Targeting liver cancer stem cells for the treatment of hepatocellular carcinoma

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Abstract: Liver cancer is one of the most common malignant tumors and prognosis remains poor. It has been increasingly recognized that liver cancer stem cells (LCSCs) are responsible for the carcinogenesis, recurrence, metastasis and chemoresistance of hepatocellular carcinoma (HCC). Targeting LCSCs is promising to be a new direction for the treatment of HCC. Herein, we summarize the potentially therapeutic targets in LCSCs at the level of genes, molecules and cells, such as knockout of oncogenes or oncoproteins, restoring the silent tumor suppressor genes; inhibition of the transcription factors and regulation of noncoding RNAs (including microRNAs and long noncoding RNAs) in LCSCs at the genetic level; inhibition of markers and blockade of the key signaling pathways of LCSCs at the molecular level; and inhibiting autophagy and application of oncolytic adenoviruses in LCSCs at the cellular level. Moreover, we analyze the potential targets in LCSCs to eliminate chemoresistance of HCC. Thereinto, the suppression of autophagy and Nanog by chloroquine and shRNA respectively may be the most promising targeting approaches. These targets may provide novel therapeutic strategies for the treatment of HCC by targeting LCSCs.

Keywords: drug resistance, HCC treatment, hepatocellular carcinoma, liver cancer stem cells, targeting LCSCs

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Introduction
Liver cancer is one of the most common human malignancies in the world, it takes the second place of mortality, only next to lung cancer. Liver cancer includes several subtypes, hepatocellular carcinoma (HCC) is by far the most common worldwide, accounting for 78%, other subtypes such as bile-duct cancer (15%), hepatoblastoma and various liver sarcomas and carcinomas (7%).

The main risk factors for HCC are definite, including hepatitis B or C virus infection, alcohol abuse, intake of the fungal metabolite aflatoxin B1 and an emerging cause that named nonalcoholic fatty liver disease or nonalcoholic steatohepatitis (NASH). In the past few decades, cancer stem cells (CSCs) have similar characteristics to normal stem cells, such as self-renewal and pluripotent activity, which have been found to be associated with the major malignant phenotypes of cancer, including recurrence, metastasis and chemoresistance. Liver cancer stem cells (LCSCs) which are identified by certain surface markers, are also known as hepatic cancer stem cells or liver tumor-initiating cells (T-ICs). Recently, the malignant behaviors of LCSCs have been increasingly recognized. They are identified to be responsible for the initiation, relapse, metastasis and chemoresistance of HCC. Thus, targeting LCSCs may be a new strategy for the treatment of HCC. This paper reviews the potentially therapeutic targets against LCSCs at the level of genes, molecules and cells, including targets of drug resistance.

Targeting LCSCs at the level of genes
Targeting LCSCs at the genetic level includes knockout of oncogenes or oncoproteins, and restoration of the silent tumor suppressor genes (Table 1).
Table 1. Targets at the level of genes.

| Targets                          | Effect | Mechanism                                                                 | Species | References                                      |
|---------------------------------|--------|---------------------------------------------------------------------------|---------|------------------------------------------------|
| **Oncogenes or oncoproteins**    |        |                                                                           |         |                                                |
| BC047440                        | +      | BC047440 increased the activation of NF-κB signaling.                     | Mouse   | You and colleagues\(^7\)                      |
| 14-3-3ζ                         | +      | Knockout of 14-3-3ζ improved IR-induced apoptosis by upregulating the expression of pro-apoptotic proteins. | Human   | Lee and colleagues\(^8\)                      |
| HSP90                           | +      | Inhibition of HSP90 induced LCSC apoptosis by downregulating the HSP90 effector proteins. | Mouse   | Yang and colleagues\(^9\)                     |
| ANXA3                           | +      | ANXA3 overexpression activated JNK pathway, leading to oncogenic events.  | Human   | Pan and colleagues\(^10\); Tong and colleagues\(^11\) |
| Hepatitis B virus PreS 1         | +      | PreS1 upregulated CSCs-related genes including Klf4, Nanog, Sox2, Oct4, and c-Myc. | Human   | Liu and colleagues\(^12\)                     |
| SIRT1                           | +      | SIRT1 regulated the transcriptional activity of Sox2, and was maintained by MEK1 signaling. | Human   | Liu and colleagues\(^13\); Cheng and colleagues\(^14\) |
| ADAM17                          | +      | ADAM17 was a key point to activate Notch signaling pathway, silencing ADAM17 reduced the activity of Notch signaling pathway. | Human   | Hong and colleagues\(^15\)                    |
| **Tumor suppressor genes**       |        |                                                                           |         |                                                |
| Shp2 and Pten                    | −      | Shp2 and Pten worked together to promoted tumorigenesis of LCSCs by the upregulation of the proto-oncogene c-jun. | Human   | Luo and colleagues\(^16\)                     |
| C8orf4                          | −      | C8orf4 impaired the self-renewal of LCSCs through inhibition of NOTCH2 signaling. | Human   | Zhu and colleagues\(^17\)                     |
| p53                             | −      | Autophagy suppressed p53 which otherwise could be activated by PINK1 to downregulate the expression of Nanog. | Human   | Liu and colleagues\(^18\)                     |
| Numb                            | −      | The phosphorylation of Numb destabilized p53 and promoted self-renewal of LCSCs in a Nanog-dependent manner. | Human   | Siddique and colleagues\(^19\)                |

\(^*\) +, promoting the stemness of LCSCs; −, suppressing the stemness of LCSCs.

ADAM17, a disintegrin and metalloproteinase-17; ANXA3, annexin A3; LCSC, liver cancer stem cell; PINK1, Pten-induced putative kinase 1; Pten, phosphatase and tensin homologue; Shp2, src-homology 2 domain-containing phosphatase 2; SIRT1, histone deacetylase sirtuin 1.
Knockout or inactivation of the oncogenes or oncoproteins in LCSCs

In recent years, increasing genes are reported to have relations with the biological behaviors of LCSCs. The activation of oncogenes or overexpression of oncoproteins increase the proliferation and tumorigenicity of LCSCs. The functions of these oncogenes and oncoproteins which may be taken as targets against LCSCs are as followed.

**BC047440.** BC047440 (GeneBank accession number: BC047440) is a novel HCC-related gene, the full length of BC047440 cDNA was cloned from human HCC tissues, the activation of BC047440 contributed to development of HCC. You and colleagues showed that BC047440 played an important role in maintaining stemness properties of LCSCs in the nude mouse model. The oncogenic effect of BC047440 depended on the increasing activation of nuclear factor kappa B (NF-κB), which was a critical nuclear transcription factor (TF) in tumor development. On the other hand, knockout of BC047440 resulted in tumorigenicity inhibition and hepatocyte-differentiation induction of LCSCs through enhancing the expression of hepatocyte nuclear factor 4α (HNF4α), which was important to regulate hepatocyte differentiation. Removal of the BC047440 gene may counteract the malignant behaviors of LCSCs.

**14-3-3ζ.** 14-3-3ζ belongs to the 14-3-3 family, which is a group of evolutionarily highly conserved acidic proteins. The abnormal expression of 14-3-3ζ has been detected in multiple cellular pathways in cancers. Lee and colleagues reported that 14-3-3ζ was upregulated in LCSCs after γ-irradiation (IR), it contributed to radiation resistance and survival of LCSCs. Knockout of 14-3-3ζ reduced the stemness properties of LCSCs and improved IR-induced apoptosis by upregulating the expression of pro-apoptotic proteins. The combination of radiotherapy and 14-3-3ζ silencing may be potential strategy for HCC therapy by targeting LCSCs.

**HSP90.** Heat-shock protein 90 (HSP90) can induce the heat-shock response as a chaperone protein. It facilitates cellular proteins folding and degradation, keeps protein stability under oxidative and heat stress. It also plays a critical role in the stabilization and activity of various oncogenic proteins. Yang and colleagues found that HSP90 in CD90+ LCSCs was upregulated under hyperthermic condition in nude mice, the HSP90 inhibitor could sensitize CD90+ LCSCs to hyperthermia and induce CD90+ LCSCs apoptosis by downregulating the HSP90 effector proteins, which regulated cell survival and apoptosis. Thus, blockage of HSP90 may improve the thermotherapy sensitivity and reduce thermoresistance of LCSCs. HSP90 may become a promising target against LCSCs, especially in thermotherapy of HCC.

**Annexin A3 (ANXA3).** ANXA3 is a group of Ca2+-dependent phospholipid-binding secretory proteins. It contributed to the propagation and self-renewal of LCSCs. Upregulation of ANXA3 promoted the tumorigenicity of LCSCs through aberrant regulated c-Jun NH2-terminal kinase (JNK) pathway, the JNK inhibitor could suppress the oncogenic events caused by ANXA3 overexpression. Thus, ANXA3 silencing and blockage of JNK pathway may suppress the malignant behaviors of LCSCs.

**Hepatitis B virus PreS 1.** Hepatitis B virus (HBV) infection is involved in HCC development. It is the initial step of HBV infection for the HBV envelope protein PreS1 to bind to the specific cellular surface receptor of hepatocytes. Liu and colleagues found that PreS1 acted as an oncoprotein in HCC development, it drove the expression of CSCs-related genes including Klf4, Nanog, Sox2, octamer 4 (Oct4), and c-Myc. PreS1 also upregulated CSCs-related markers, such as CD133, CD90 and CD117. Knockout of PreS1 counteracted the stemness properties of LCSCs, indicating that the suppression of Pres1 may be potential method for the therapy of HBV-related HCC through targeting LCSCs.

**SIRT1.** Histone deacetylase sirtuin 1 (SIRT1) is class III histone deacetylase, taking a role in the regulation of cellular stress responses. SIRT1 participates in the process of carcinogenesis by regulating lipid metabolism. In HCC, Liu and colleagues reported that SIRT1 promoted self-renewal and tumorigenicity of LCSCs by regulating the transcriptional activity of Sox2, which was essential TF for LCSCs. Knockdown of SIRT1 impaired the stemness capacities of LCSCs. On the other hand, the stabilization of SIRT1 was maintained by MEK1 signaling which belongs to the mitogen-activated protein kinase (MAPK) pathway. So, both SIRT1 and MEK1 signaling may be novel potential targets against LCSCs.
**ADAM17.** A disintegrin and metalloproteinase-17 (ADAM17), also known as tumor necrosis factor (TNF)-α converting enzyme (TACE), is important in processing single-spanning membrane proteins. Many proteins processed by ADAM17 including cytokines, receptors and growth factors are involved in cancer development. In HCC, it was revealed that ADAM17 contributed to radio resistance and was responsible for invasion and metastasis of CD133^+^ LCSCs. The mechanism was that ADAM17 was a key point to activate Notch signaling pathway, silencing ADAM17 reduced the radio resistance of LCSCs by suppressing Notch signaling. So, silencing ADAM17 may become novel method to improve the efficacy of radiotherapies and reduce the metastasis of HCC.

**Restoring the silent tumor suppressor genes in LCSCs**

In recent years, it has been recognized that the silencing of tumor suppressor genes caused by mutations, deletions, promoter inactivation or other epigenetic changes are relevant to malignant change in normal cells. Accordingly, it may become a potential strategy for HCC therapy to restore the expression of silent tumor suppressor genes in LCSCs.

**Ptpn11/shp2 and Pten.** Src-homology 2 domain-containing phosphatase 2 (Shp2) is an oncogenic tyrosine phosphatase, encoded by Ptpn11. Shp2 mutation was detected in several types of leukemia. The role of Shp2 on HCC development is controversial, it was demonstrated that Shp2 played a role as tumor suppressor in HCC on one hand, but on the other hand it promoted HCC development, and enhanced the invasion of LCSCs by activating β-catenin signaling.

Phosphatase and tensin homologue (Pten), deleted from chromosome 10, is a classical tumor suppressor which could block the activation of PI3K/Akt signaling pathway. Pten and Shp2 work together to exert the inhibitory effect on LCSCs. It was identified that both deficiencies of Shp2 and Pten promoted tumorigenesis in LCSCs, Pten deficiency promoted Akt over-activation and Shp2 loss induced JNK activation, resulting in the upregulation of the proto-oncogene c-jun. Both silencing of Shp2 and Pten were related to poor prognosis in patients with HCC. So, Shp2 and Pten may become novel potential targets against LCSCs, but further research is needed.

**C8orf4.** C8orf4, also named thyroid cancer 1 (TC1), was cloned from thyroid cancer. Over-expression of C8orf4 was reported being involved in tumorigenesis in several types of human cancers. It could promote clonogenicity of human lung cancer cells and also acts as an oncogene in breast cancer. But, Zhu and colleagues found an inhibitory effect of C8orf4 on HCC, it impaired the self-renewal of LCSCs through inhibition of Notch2 signaling. It counteracted the nuclear translocation of Notch2 intracellular domain. By contrast, C8orf4 silencing drove the activation of Notch2 signaling, then improved self-renewal properties of LCSCs. Anyhow, C8orf4 was found to act oncogenic role in several other types of cancer, it needs further studies about the role of C8orf4 gene in targeted therapy of HCC based on LCSCs.

**p53.** As a classical tumor suppressor gene, p53 could restrain stem cells expansion by restricting self-renewal, inhibiting symmetric division and blocking the somatic/progenitor cells reprogramming into stem cells. Liu and colleagues found that autophagy, a catabolic process of cells removing damaged organelles and protein aggregates, could positively regulate LCSCs by suppressing p53 which otherwise could be phosphorylated on mitochondria by Pten-induced putative kinase 1 (PINK1), a kinase associated with mitophagy, to downregulate the expression of CSC-related gene Nanog. So, both the restoration of p53 and blockade of autophagy may be promising methods to control LCSCs.

**Numb.** Numb is originally discovered in drosohila embryos as a cell fate determinant during sensory organ formation. Accumulating evidences support that Numb acts as tumor suppressor in several types of cancer by inhibiting Notch signaling and epithelial-mesenchymal transition (EMT). In pancreatic cancer, Numb was down-regulated by Musashi2, a RNA-binding protein which was required for tumorigenesis as a translational repressor. In HCC, Siddique and colleagues reported that Numb could conjunct with p53 forming the Numb-p53 complex to prevent the degradation of p53, the phosphorylation of Numb destabilized p53 and promoted self-renewal of LCSCs in a Nanog-dependent manner. Nanog phosphorylated Numb by enhancing...
the kinase activities of both Aurora A kinase (AURKA) and atypical protein kinase C zeta (aPKCζ) through the Nanog-AURKA-aPKCζ pathway. Thus, the Nanog-Numb-p53 signaling axis is important in the self-renewal and tumorigenesis of LCSCs, it may provide novel strategies for the treatment of HCC.

**Targeting LCSCs at the level of molecules**

Targeting LCSCs at the molecular level includes inhibition of the TFs, regulation of noncoding RNA (including microRNA and long noncoding RNA), decrease of markers and interruption of the essential signaling pathways of LCSCs (Table 2).

**Inhibition of the key TFs in LCSCs**

**Twist.** The Twist proteins belong to the highly conserved basic heli-loop-helix TF family; the Twist genes include Twist1 and Twist2. It is reported that Twist is associated with EMT and self-renewal of LCSCs by regulating the CSCs marker CD24, promoting the development of HCC. Some phytochemicals show inhibitory effect on Twist signaling in LCSCs, such as casticin and 8-bromo-7-methoxychrysin (BrMC). Casticin is derived from Fructus Viticis (Chinese name, Manjingzi). BrMC is a novel synthetic analogue of chrysin (5,7-dihydroxyflavone). Both casticin and BrMC could inhibit EMT and the stemness of LCSCs by negatively regulating Twist. Thus, Twist inhibitors might be therapeutic agents by targeting LCSCs.

**HIF-1α and ELK3.** Hypoxia-inducible factor 1 (HIF-1) is a basic-helix-loop-helix-PAS heterodimeric TF which mediates transcriptional responses in hypoxic cells. HIF-1 is composed of two subunits, the HIF-1α and HIF-1β. HIF-1α is identified an important role in tumor development, including the regulation of oncogenes expression, cellular metabolism and proliferation, tumor metastasis and invasion. Moreover, HIF-1α inhibited the biological characteristics of LCSCs, such as self-renewal, migration, invasion.

The TF ELK3, also named Net/SAP-2/Erp, is part of the large family of ETS-domain TFs, belonging to the ternary complex factor subfamily. ELK3 is associated with wound healing, angiogenesis, and tumor growth as a downstream of the RAS/ERK signaling pathway. Lee and colleagues showed that ELK3 promoted the migration and invasion of LCSCs by targeting HIF-1α. So, both HIF-1α and ELK3 might act as the therapeutic targets for the treatment of HCC.

**KLF8.** Krüppel-like factor 8 (KLF8) belongs to the KLF family of TFs as DNA-binding transcriptional regulators in many cellular processes. KLF8 was highly expressed not only in LCSCs, but also in HCC tumors and distant migrated tissues. Overexpression of KLF8 promoted the maintenance and chemoresistance of LCSCs, accordingly, played a role in HCC tumorigenesis. Mechanistically, KLF8 facilitated the activation of the Wnt/β-catenin signaling pathway. The knockout of KLF8 gene showed markedly apoptosis of LCSCs. So, KLF8 could be taken as a novel target to suppress LCSCs.

**Targeting noncoding RNAs in LCSCs**

Noncoding RNAs include microRNAs (miRNAs) and long noncoding RNAs (lncRNAs). MiRNAs are a class of small noncoding RNAs which can post-transcriptionally regulate the expression of target genes and play roles as tumor suppressors or oncogenes in different tumorigenic progression. Long noncoding RNAs (lncRNAs) are >200 bases long and have low or no protein-coding potential. They are known to regulate splicing, recruit TFs, and regulate mRNA stability. Increasing studies demonstrate that both miRNAs and lncRNAs play important roles in LCSCs, they may be taken as therapeutic targets for the treatment of HCC.

**Regulation of microRNAs in LCSCs**

a. **MicroRNA 122.** MicroRNA 122 (miR-122) is a liver-specific miRNA, accounting for 70% of the total miRNAs in human liver. MiR-122 was identified as a tumor suppressor in HCC. Upregulation of miR-122 inhibited EMT, proliferation and invasion of hepatoma cells by suppressing the Wnt/β-catenin signaling pathway. Moreover, miR-122 inhibited glycolysis by targeting glycolytic genes PDK4, accordingly, suppressed the stemness properties and growth of CD133+ LCSCs.

b. **MicroRNA 152.** MicroRNA-152 (miR-152) is involved in diverse biological functions and induces apoptosis in HCC as a tumor suppressor. In HBV-related HCC, the
### Table 2. Targets at the level of molecules.

| Targets          | Effect* | Mechanism                                                                 | Species | References                          |
|------------------|---------|---------------------------------------------------------------------------|---------|-------------------------------------|
| **TFs**          |         |                                                                           |         |                                     |
| Twist            | +       | Twist is associated with EMT and self-renewal of LCSCs by regulating the CSCs marker CD24. | Human   | Liu and colleagues\(^42\); He and colleagues\(^43\); Ren and colleagues\(^44\) |
| HIF-1 and ELK3   | +       | ELK3 promoted the migration and invasion of LCSCs by targeting HIF-1\(\alpha\). | Human   | Lee and colleagues\(^45\)          |
| KLF8             | +       | KLF8 facilitated the activation of the Wnt/\(\beta\)-catenin signaling pathway. | Human   | Shen and colleagues\(^44\)         |

| Noncoding RNAs   |         |                                                                           |         |                                     |
| MicroRNAs (miRNAs) |       |                                                                           |         |                                     |
| MiR-122          | –       | MiR-122 suppressed Wnt/\(\beta\)-catenin signaling pathway and inhibited glycolysis by targeting glycolytic genes PDK4. | Human   | Song and colleagues\(^47\)         |
| MiR-152          | –       | MiR-152 directly binding to 3 untranslated region and downregulating the expression of KIT which is a proto-oncogene. | Human   | Huang and colleagues\(^48\)       |
| MiR-21           | –       | MiR-21 positively regulated the expression of LCSC markers CD13, EpCAM, CD90 and Oct4. | Human   | Jiang and colleagues\(^49\)       |
| MiR-155          | +       | MiR-155 mediated TP53INP1 to regulate CSC phenotype, and enhanced the LCSC markers CD90, CD133 and Oct4. | Human   | Liu and colleagues\(^50\)         |
| MiR-25           | +       | Knockdown of miR-25 increased apoptosis of LCSCs induced by TRAIL, via PI3K/Akt/Bad signaling pathway. | Human   | Feng and colleagues\(^51\)       |
| MiR-200 family   | +/-     | MiR-429 decreased RBBP4 expression and resulted in the activation of Oct4. But, miR-200a suppressed the EMT phenotype of LCSCs. | Human   | Li and colleagues\(^52\); Wang and colleagues\(^53\) |
| MicroRNA let-7    | –       | Let-7a negatively regulating EMT and Wnt signaling pathway. Let-7c targeted PBX3 and suppressed the transcriptional activity of CSCs-related genes including CACNA2D1, EpCAM, Sox2 and Notch3. | Human   | Jin and colleagues\(^54\); Han and colleagues\(^55\) |
| MiR-1246         | +       | MiR-1246 activated the Wnt/\(\beta\)-catenin pathway through inhibiting the expression of Axin2 and GSK3\(\beta\). | Human   | Chai and colleagues\(^56\)       |

(Continued)
| Targets | Effect | Mechanism                                                                 | Species  | References       |
|---------|--------|---------------------------------------------------------------------------|----------|------------------|
| HULC and MALAT1 | +      | They cooperated to regulate the TRF2.                                      | Human    | Wu and colleagues57 |
| LncDILC | -      | LncDILC inhibited the autocrine IL-6/STAT3 signaling, and mediated the crosstalk between TNF-α/NF-κB signaling and IL-6/JAK2/STAT3 cascade. | Human    | Wang and colleagues58 |
| CUDR and H19 | +      | Pten depletion promoted the binding of CUDR to the oncogene CyclinD1, the CUDR-cyclinD1 complex then enhanced the H19 expression. | Human    | Pu and colleagues59 |
| HOTAIR  | +      | HOTAIR accelerated LCSC malignant proliferation through downregulating SETD2.      | Human    | Li and colleagues60 |
| LncTCF7 | +      | LncTCF7 recruited the SWI/SNF complex to activation of Wnt signaling.       | Human    | Wang and colleagues61 |
| LncSox4 | +      | LncSox4 recruited the TF Stat3 to the Sox4 promoter to trigger the expression of Sox4. | Human    | Chen and colleagues62 |
| LncBRM  | +      | LncBRM associated with BRM to trigger the BRG1/BRM switch and BAF, leading to activation of the transcriptional cofactors YAP1. | Human    | Zhu and colleagues63 |
| Lncβ-Catm | +      | Lncβ-Catm associated with β-catenin and the methyltransferase EZH2, promoting the methylation and stability of β-catenin. | Human    | Zhu and colleagues64 |
| LncCAMTA1 | +      | LncCAMTA1 associated with CAMTA1 promoter to inhibit its transcription.   | Human    | Ding and colleagues65 |
| CD133   | +      | The downregulation of CD133 decreased the level of NF-κB.                  | Human    | Liu and colleagues66 |
| ICAM-1  | +      | ICAM-1 is upregulated by Nanog, promoting the stemness of LCSCs.          | Human    | Liu and colleagues67 |
| Wnt/β-catenin pathway | +      | The Wnt/β-catenin pathway promoted the self-renewal and unlimited cell proliferation of CSCs. | Human    | Chen and colleagues68; Kim and colleagues69; Seto and colleagues70 |

(Continued)
HBV X protein facilitates AFP expression, which activates PI3K/Akt signal pathways.

Inhibition of the protein kinase Akt reduced the self-renewal of LCSCs.

IL-6, activating STAT3 and elevating the cellular glucose uptake. TLR4 cooperated with STAT3 via Nanog to activate Twist1.

Depleting MEK or reducing ERK1/2 phosphorylation suppressed the proliferation, invasion and migration of LCSCs. MEK maintained the stabilization of SIRT1 protein.

ANXA3 could enhance the activity of JNK pathway in CD133+ LCSCs by upregulating the expression of c-MYC.

The Notch signaling cascade associated with Wnt, MAPK and NF-κB signaling.

MicroRNA 21. MicroRNA 21 (miR-21) was identified as a pro-metastatic miRNA, which regulated the expression of metastasis-related proteins in HCC.49 MiR-21 was upregulated in LCSCs.95 Overexpression of miR-21 promoted the tumorigenesis, invasion and migration of LCSCs and positively regulated the expression of Oct4 and LCSC markers CD13, EpCAM and CD90.49 MiR-21 played an oncogenic role in LCSCs which might be taken as a target.

decrease of miR-152 contributed to the epigenetic inactivation of RIZ1, a candidate HCC suppressor gene which could be repressed by HBV X protein.93 In LCSCs, miR-152 inhibited clonogenicity and growth of CD133+ LCSCs by directly binding to 3' untranslated region and downregulating the expression of KIT which is a proto-oncogene.48

c. MicroRNA 21. MicroRNA 21 (miR-21) was identified as a pro-metastatic miRNA, which regulated the expression of metastasis-related proteins in HCC.49 MiR-21 was upregulated in LCSCs.95 Overexpression of miR-21 promoted the tumorigenesis, invasion and migration of LCSCs and positively regulated the expression of Oct4 and LCSC markers CD13, EpCAM and CD90.49 MiR-21 played an oncogenic role in LCSCs which might be taken as a target.

d. MicroRNA 155. MicroRNA 155 (miR-155) could either be an oncogene or a tumor suppressor in several type of cancers.96 In HCC, miR-155 was demonstrated to contribute to tumorigenesis.97 MiR-155 facilitated the formation and self-renewal of LCSCs by mediating TP53INP1 (tumor protein 55-induced nuclear protein 1) to regulate CSC phenotype.50 Moreover, the overexpression of miR-155 enhanced the levels of Oct4 and LCSC markers CD90 and CD133, knockdown of miR-155 led to a decrease of LCSCs populations.50
Abnormal expression of MicroRNA 25 (miR-25) mediates tumorigenesis in many human malignant tumors. In HCC, miR-25 played a role in promoting proliferation, invasion and migration of hepatoma cells. It was overexpressed in LCSCs compared with non-LCSCs, knockdown of it increased apoptosis of LCSCs induced by TNF-related apoptosis inducing ligand (TRAIL), via the Pten/P3K/Akt/Bad signaling pathway. It indicates that both miR-25 and TRAIL may become novel anticancer agents by targeting LCSCs.

MicroRNA 200 family. MicroRNA 200 family (miR-200 family) consist of five evolutionarily conserved members in the human genome with miR-200b, miR-200a and miR-429 located in 1p36 cluster, and miR-200c, miR-141 located in 12p13 cluster. MiR-429 was demonstrated as oncogene in HCC, its upregulation promoted the self-renewal, tumorigenicity and chemoresistance of EpCAM+LCSCs, it led to the activated transcription of Oct4 by E2F transcription factor 1 (E2F1) via reducing Rb binding protein 4 (RBBP4) expression, which otherwise could inhibit E2F1 transcriptional activity. Whereas, miR-200a acted as tumor suppressor, its upregulation suppressed the EMT phenotype of LCSCs by increasing the epithelial marker E-cadherin and decreasing the mesenchymal markers, fibronectin, vimentin, and N-cadherin. Moreover, miR-200b/miR-200c/miR-429 subfamily was found to suppress HCC metastasis by inhibiting Rho/ROCK signaling pathway. However, the miR-200 family play indispensable roles in maintenance of LCSCs which still need further studies.

MicroRNA let-7. The let-7 family consists of 12 members: let-7a-1, -2, -3; let-7b; let-7c; let-7d; let-7e; let-7f-1, -2; let-7g; let-7i; MIR98, most of them are found to be downregulated in several cancers, restoration of normal expression prevents tumorigenesis. In HCC, let-7a and let-7c were identified as tumor suppressors by targeting LCSCs. Let-7a suppressed the self-renewal of LCSCs by negatively regulating EMT and Wnt signaling pathway. Let-7c targeted PBX3 which was essential for the maintenance of LCSCs and suppressed the transcriptional activity of CSCs-related genes including CACNA2D1, EpCAM, Sox2 and Notch3.

MicroRNA 1246. The upregulation of microRNA 1246 (miR-1246) was observed in several types of cancer, which was identified to play an oncogenic role. In HCC, miR-1246 was demonstrated to promote migration and invasion of hepatoma cells. Circulating miR-1246 has been shown to be a predictor of survival and tumor recurrence in HCC patients after liver transplantation. MiR-1246 promoted stemness of LCSCs by activating the Wnt/β-catenin pathway through inhibiting the expression of Axin2 and glycogen synthase kinase 3β (GSK3β), which could induce the degradation of β-catenin. Moreover, Oct4 acted as the direct upstream regulator of miR-1246 by direct promoter binding which drove β-catenin activation in LCSCs.
CUDR could induce upregulation of HULC and β-catenin, and control human liver stem cells malignant differentiation, which was identified the possible origin of LCSCs. CUDR and lncRNA H19 were found to have a combined action on LCSCs. H19 is encoded by a highly conserved imprinted gene and is essential for human tumor growth, its high expression was associated with poor clinical outcomes of patients with solid tumors. In human HCC, H19 was reported to play a role in tumorigenesis of HCC. It stimulated angiogenesis and promoted the adhesion of CD90+ hepatoma cells to endothelial cell monolayer. It was demonstrated that Pten depletion promoted the binding of CUDR to the oncogene CyclinD1, the CUDR-CyclinD1 complex then enhanced the H19 expression by loading onto the promoter region of H19, which improved the cell telomerase activity and extended the telomere length in LCSCs, finally promoting LCSCs growth.

LncRNA HOX transcript antisense RNA. LncRNA HOX transcript antisense RNA (HOTAIR) acts as a transcriptional modulator in various fundamental biological activities, its over-expression is also related with poor prognosis of patients with HCC. Excessive HOTAIR promoted the malignant transformation of normal liver stem cells to LCSCs via inducing EMT. HOTAIR also governed the oncogenic action of inflammatory related gene IKKα, IKKβ, and IKKγ in LCSCs. Most of all, it accelerated human LCSCs malignant proliferation through downregulating SETD2, which is an essential enzyme in DNA double-strand breaks.

LncRNA TCF7. LncTCF7 is a novel lncRNA which is highly expressed both in HCC and LCSCs, the protein-coding gene TCF7 acts as an upstream trigger to activate the Wnt signaling cascade. LncTCF7 recruited the SWI/SNF complex, a group of evolutionally conserved multi-subunits, to the promoter of TCF7 to initiate its expression, leading to activation of Wnt signaling and promoting self-renewal and tumorigenic capacity of LCSCs.

LncRNA Sox4. LncSox4 is also a novel lncRNA which is recently discovered to be highly expressed in HCC and LCSCs. LncSox4 facilitated the self-renewal of LCSCs, mechanistically by targeting STAT3-Sox4 pathway, it recruited the TF STAT3 to the Sox4 promoter to trigger the expression of Sox4, which was required for the self-renewal of LCSCs.

LncRNA Brahma. LncBRM (gene symbol LINCR-0003) is recently identified to be highly expressed in LCSCs and is required for the oncogenicity and self-renewal of LCSCs. The mechanism was that lncBRM associated with Brahma (BRM) to trigger the BRG1/BRM switch and the BRG1-associated factor (BAF) complex, leading to activation of the transcriptional cofactors YAP1 which was required for the self-renewal of LCSCs.

LncRNA β-Catm. Lncβ-Catm (gene symbol LINC00184) is a novel lncRNA which is recently found to be highly expressed both in LCSCs and HCC. Lncβ-Catm contributed to LCSCs self-renewal by activating Wnt/β-catenin signaling. Mechanistically, it associated with β-catenin and the methyltransferase EZH2, promoting the methylation and stability of β-catenin, which resulted in the activation of Wnt/β-catenin signaling.

LncRNA CAMTA1. LncCAMTA1 (gene symbol RP11-312B8.1) is a novel lncRNA which is identified to be highly expressed both in LCSCs and HCC, promoting the stemness and tumorigenesis of LCSCs. Mechanistically, lncCAMTA1 associated with the calmodulin binding transcription activator 1 (CAMTA1) promoter to inhibit the transcription of CAMTA1 which served as a tumor suppressor in HCC.

Decreasing LCSCs biomarkers
CD133. CD133 is an essential LCSCs biomarker which participates in the regulation of EMT, tumorigenicity and invasion of LCSCs. The downregulation of CD133 decreased the level of nuclear factor-κB (NF-κB), leading to inhibition of EMT and the stemness of LCSCs. The expression of CD133 in HCC could be negatively regulated by Ikaros, a member of the Krüppel family, which interacted with the transcription repressor CtBP to form a complex directly binding to the CD133 promoter.

ICAM-1. Intercellular adhesion molecule 1 (ICAM-1) is a transmembrane molecule which is involved in many important cellular processes and also in the development of various human cancers, such as HCC, breast cancer and renal cancer. ICAM-1 is considered as a stem cell marker which is upregulated by Nanog, it is also
high expressed in LCSCs, promoting the tumorigenicity and stemness of LCSCs.\textsuperscript{67}

**Interruption of the essential signaling pathways in LCSCs**

Several signaling pathways are essential in the development of LCSCs and HCC, which provide some promising targets against LCSCs for the treatment of HCC.

**Wnt/β-catenin pathway.** The Wnt/β-catenin pathway is responsible for the self-renewal and unlimited cell proliferation of CSCs. It has also been widely confirmed in maintenance of LCSCs. Chen and colleagues\textsuperscript{68} revealed that constitutive expression of Wnt/β-catenin was detected in LCSCs, knockdown of it suppressed the cell phenotype of LCSCs.\textsuperscript{69,70,119} Moreover, some phytochemicals, such as casticin, triptolide and BrMC, also have been demonstrated to restrain the self-renewal and proliferation of LCSCs by suppressing Wnt/β-catenin signaling.\textsuperscript{120,121}

**PI3K/Akt/mTOR pathway.** The PI3K/Akt signal pathway plays a key role in tumorigenesis.\textsuperscript{122} In HCC, The PI3K/Akt/mTOR pathway promoted formation of LCSCs, leading to hepatocarcinogenesis.\textsuperscript{123,124} The inhibitor of PI3K or mTOR could suppress the proliferation of LCSCs. Moreover,
HBV contributes to the activation of the pathways, HBV X protein facilitates alpha fetoprotein (AFP) expression, which promotes the proliferation of LCSCs, by activating PI3K/Akt signal pathways.\textsuperscript{71}

Akt/GSK-3β/β-catenin pathway. In HCC, the Akt/GSK-3β/β-catenin pathway promotes the proliferation and invasion of LCSCs.\textsuperscript{72,73} Inhibition of Akt which is a protein kinase in multiple cellular processes, reduced the self-renewal and propagation of LCSCs.\textsuperscript{73,122} However, increasing evidence shows that adverse effects including liver injury and inflammation, hyperglycemia and hyperinsulinemia accompany the complete deletion of Akt.\textsuperscript{122} Thus, the definite mechanisms of systemic inhibition of Akt or the pathways need further study.
**STAT3 signaling pathway.** Signal transducer and activator of transcription 3 (STAT3) plays an oncogenic role in cancer progression involving in the regulation of EMT and CSCs, it could be activated by Toll-like receptors, microRNA or cytokine receptors such as the IL-6 family of cytokines. It was identified that IL-6 activated STAT3 and elevated the cellular glucose uptake, finally promoting the stemness of LCSCs. On the other hand, TLR4 could also trigger the expression of Nanog, the latter directly interacted with STAT3 which could also phosphorylated by the leptin receptor (OB-R) pathways to promote the generation and invasion of LCSCs by upregulating Twist1. Moreover, STAT3 mediated Sox4 expression to favor the activity of LnxSox4 as mentioned above.

**RAS/RAF/MEK/ERK signaling pathway.** Aberrant regulation of the RAS/RAF/MEK/ERK pathway has been detected in several malignancies including HCC. Experimental research demonstrated that blockade of the RAS/RAF/ERK pathway suppressed the proliferation, invasion and migration of LCSCs by depleting MEK or reducing ERK1/2 phosphorylation. Moreover, MEK signaling was identified to promote the self-renewal of LCSCs by maintaining the stabilization of SIRT1 protein which was involved in carcinogenesis.

**JNK signaling pathway.** The JNK signaling pathway is involved in cancer development by regulating the tumor-initiating capacity of CSCs. In HCC, increased JNK activity is associated with tumor proliferation. The CSCs-related gene ANXA3 could enhance the activity of the JNK pathway in CD133+ LCSCs by upregulating the expression of the proto-oncogene c-MYC, finally improving the self-renewal of LCSCs.

**Notch signaling pathway.** The Notch receptors consist of four members (Notch1-4) in mammals, which can combine with various ligands and be activated. The Notch signaling cascade participates in the progress of various cancers associating with Wnt, MAPK and NF-κB signaling. In HCC, the activated Notch pathway was detected in LCSCs promoting the expression of CSC-related genes and maintaining the stemness of LCSCs. Notch1 in LCSCs was identified to be a downstream of Wnt/β-catenin. Notch2 which was activated in LCSCs could be suppressed by C8orf4.

**Targeting LCSCs at the level of cells**
Targeting LCSCs at the cellular level includes inhibiting autophagy and application of oncolytic adenoviruses in LCSCs.

**Blocking the process of autophagy in LCSCs**
Autophagy is a conserved lysosomal degradation process during which the cytoplasmic components including macromolecules and organelles are degraded by the lysosome, accordingly maintaining the cellular homeostasis including anti-stress, immunity and antiaging. The effect of autophagy varies in different phases of tumorigenesis, it suppresses malignant transformation in healthy cells, but reduces intracellular and extracellular stress of cancer cells to promote tumor development. In healthy human liver, basal rates of autophagy maintain hepatocyte homeostasis by degrading misfolded proteins, protein aggregates and damaged mitochondria. Autophagy increased during the development of cirrhosis. Increased autophagy triggered the expression of hepatocyte growth factor (HGF) which bind with its receptor Met, leading to activation of JNK and STAT3 signaling to induce the formation of the Axin2+CD90+ LCSCs in liver cirrhosis. In another way, mitophagy which selectively removes the mitochondria by autophagy, positively regulated LCSCs by suppressing the tumor suppressor p53 which otherwise could be activated by PINK1 on mitochondria to downregulate the expression of Nanog (Figure 1). Moreover, autophagy contributed to the initiation and invasion of HCC. In the tumor microenvironment, autophagy copes with hypoxia and nutritional deprivation, favoring the survival of LCSCs and the chemoresistance of hepatoma cells. Chloroquine, the inhibitor of autophagy, showed inhibitory effect on the stemness of LCSCs. It inhibited autophagic flux by decreasing autophagosome-lysosome fusion which formed autolysosomes. Chloroquine may inhibit the formation of LCSCs by suppressing pSTAT3 and Nanog, and keep the p53 activity from being suppressed by autophagy. Thus, the application of chloroquine or other autophagy inhibitors in liver cirrhosis and HCC may be potential targeting approaches against LCSCs.
**Application of oncolytic adenovirus in LCSCs**

In recent years, the antitumor use of viruses has been studied in depth. Oncolytic adenoviruses which are genetically modified, could selectively enter and spread inside tumors, show their cytotoxicity and suppression of tumors.\textsuperscript{136} In HCC, it was reported that a new oncolytic adenovirus GD55 demonstrated a stronger killing effect on human LCSCs.\textsuperscript{137} Moreover, Zhang and colleagues designed a novel oncolytic adenovirus which carried the tumor suppressor gene TSLC1 and targeted Wnt signaling pathway, the adenovirus inhibited growth and metastasis of LCSCs \textit{in vivo} by mediating TSLC1 and suppressing Wnt signaling.\textsuperscript{138} Therefore, oncolytic adenovirus may serve as a potentially therapeutic application by targeting LCSCs.

**Eliminating the chemoresistance of LCSCs**

Targeting LCSCs by depleting or inactivating the drug resistance genes or proteins and blocking the drug resistance-associated signaling pathways in LCSCs may be effective to eliminate its chemoresistance (Table 3).

**Depletion or inactivating the drug resistance genes or proteins in LCSCs**

\textbf{ABCG2.} The ATP-binding cassette (ABC)G2 is a member of the superfamily of ABC transporters drug efflux pumps, which lead to multidrug resistance in liver cancer.\textsuperscript{139} ABCG2 is mediated by Oct4 for the chemotherapeutic resistance in hepatoma cells \textit{via} a potential Oct4-Akt-ABCG2 pathway.\textsuperscript{149} Both Oct4 and Nanog, another upstream of ABCG2, have a positive association with ABCG2, cooperating to favor the chemoresistance of LCSCs.\textsuperscript{139,140} Thus, ABCG2 may become one of the most important targets for drug resistance of LCSCs.

\textbf{ZIC-2–Octamer-binding transcription factor 4 (Oct4) axis and promyelocytic leukemia–Oct4 axis.} Octamer-binding transcription factor 4 (Oct4) which is encoded by the Pou5f1 gene belongs to the POU (Pit, Oct and Unc) family of DNA-binding proteins.\textsuperscript{150} As mentioned above, the overexpression of Oct4 favored the stemness of LCSCs by upregulating miR-1246 to drive \(\beta\)-catenin activation.\textsuperscript{56} Its expression in LCSCs can be upregulated by PreS1, miR-21, miR-155 and miR-429/RBBP4/E2F1/Oct4 pathway.\textsuperscript{12,49,50,52} It was also found that the zinc finger TF ZIC2 which was highly expressed in LCSCs acted upstream of Oct4, ZIC2 recruited the nuclear remodeling factor (NURF) complex binding to the promoter of Oct4, thereby initiating Oct4 activation.\textsuperscript{141} Another protein upstream of Oct4 is the promyelocytic leukemia (PML) protein which was originally identified in acute promyelocytic leukemia.\textsuperscript{151,142} PML took a role as tumor suppressor in multiple pathways, but it was found to preserve the activity of Oct4 gene in stem cells.\textsuperscript{151,152} In LCSCs, suppression of PML decreased the level of Oct4, indicating that the PML favored the stemness of LCSCs \textit{via} the PML/Oct4 axis.\textsuperscript{142} However, Oct4 overexpression is also involved in the acquisition of the drug-resistant phenotype of cancer cells.\textsuperscript{153} It has a positive association between ABCG2, both of them contributed to drug resistance of CD90\textsuperscript{+}CD133\textsuperscript{+}LCSCs.\textsuperscript{139} Oct4 is one of the most promising therapeutic targets for HCC treatment by targeting LCSCs.

\textbf{Nanog.} Nanog is a homeodomain-containing TF which plays a key role in the regulation of stemness acquisition and cancer development.\textsuperscript{154} As mentioned above, Nanog is critical to the stemness of LCSCs, and can be activated by PreS1 and TLR4.\textsuperscript{12,76} Its abnormal expression upregulated the level of ICAM-1.\textsuperscript{67} The activated Nanog directly interacted with pSTAT3 to drive the expression of Twist1 which activated CD24.\textsuperscript{42,76} Autophagy is associated with Nanog. It favors the activity of Nanog not only by activating the HGF/Met/STAT3 pathway, but also by suppressing p53, which otherwise downregulates the expression of Nanog.\textsuperscript{18,133} On the other hand, Nanog suppresses p53 by phosphorylation of Numb and destabilization of the Numb-p53 complex \textit{via} the Nanog-AURKA-aPKC\textsubscript{ζ} pathway.\textsuperscript{19} Moreover, Nanog was found to be involved in chemoresistance of LCSCs; knockdown of it led to increased chemosensitivity to antitumor agents by decreasing the expression of ABCB1 (ABC subfamily B member 1) and ABCG2 in LCSCs which belong to the ABC drug efflux pumps (Figure 1).\textsuperscript{140} It was found that Nanog silence by short hairpin RNA (shRNA) or small interfering RNA (siRNA) impaired both the self-renewal and the chemoresistance of LCSCs.\textsuperscript{19,67,140} The Nanog shRNA \textit{via} lentiviral particles could efficiently block the expression of Nanog at both mRNA and protein levels by the cleavage of Nanog mRNA. It showed a long-term depletion of the targeted gene. Thus, the suppression of Nanog \textit{via} shRNA lentiviral particles or other gene therapy vectors may be potential LCSCs-targeting approaches.
Laminin-332. Laminin-332 belongs to the laminin family which is a kind of extracellular matrix proteins.\(^{143}\) Laminin acts as part of hepatic progenitor cell (HPC) niches to maintain the phenotype of HPCs during human liver damage.\(^{155}\) Govaere and colleagues\(^{143}\) demonstrated that laminin-332 also acted as part of the human LCSCs niches, it supported stemness and chemoresistance of LCSCs under doxorubicin and sorafenib treatment by upregulating keratin(K)19 expression, downregulating phospho-histone H3 expression and inducing phosphorylation of mTOR. However, the effect of laminin-332 was decreased upon the inhibition of mTORC1 and mTORC2, and enhanced when mTORC1 was inhibited alone,\(^{143}\) which need further studies.

CHD4. The chromodomain-helicase-DNA-binding proteins (CHDs) is part of the histone deacetylase (NuRD) complex protein, which was essential in transcriptional events of oncogenesis.\(^{156}\) CHD4 was found to contribute to the repair of DNA damage in a poly (ADP-ribose) polymerase (PARP)-dependent manner in EpCAM\(^+\) LCSCs as a chromatin remodeling enzyme.\(^{144}\) The overexpression of CHD4 enhanced the chemoresistance of LCSCs to anticancer drugs, while inhibition of CHD4 by doubly suppressing PARP and histone deacetylase (HDAC) suppressed LCSC proliferation.\(^{144}\) Thus, knockout of CHD4 may enhance the chemosensitivity of LCSCs which deserves further investigation.

Musashi 2. The Musashi family is a kind of evolutionarily conserved RNA-binding protein which comprises Musashi 1 and Musashi 2 (MSI2).\(^{40}\) MSI2 is required in the tumorigenesis of several human cancers, and participates in inhibiting the tumor suppressor Numb and wildtype p53.\(^{19,40}\) Fang and colleagues\(^{145}\) found that MSI2 also contributed to the chemoresistance of LCSCs by upregulation of Lin28A, which is critical CSC-related RNA-binding proteins. Knockdown of MSI2 or Lin28A impaired the chemoresistance of LCSCs, moreover, MSI2 knockdown also reduce the level of Nanog, Oct4 and Sox2.\(^{149}\) Thus, MSI2 may become new target to improve the chemosensitivity of LCSCs.

GRAM domain-containing protein 1A. GRAM domain-containing protein 1A (GRAMD1A) is a novel protein, the function of which has not been explored.\(^{146}\) It is highly expressed in HCC and sustains the self-renewal and chemotherapy resistance of LCSCs by regulating the transcriptional activity of STAT5 (signal transducer and activator of transcription 5). However, other evidence showed that STAT5 could prevent the formation of aggressive HCC.\(^{157}\) So, both GRAMD1A and STAT5 need more research.

Dysadherin. Dysadherin is a cell membrane glycoprotein which upregulates the production of chemokine and downregulates cell adhesion mediated by E-cadherin, accordingly, creating survival conditions for many types of human cancer.\(^{158}\) In HCC, high expression of dysadherin sustained high resistance to chemotherapeutic drugs in LCSCs which might depend on upregulation of drug efflux pumps.\(^{147}\) Knockdown of dysadherin showed increased sensitivity to chemotherapy agents and apoptotic cell death, as well as remarkable decreasing expression of stemness-related proteins.\(^{147}\)

Blockade of the drug resistance-associated signaling pathways in LCSCs

JNK signaling pathway. As mentioned above, the JNK signaling pathway is associated with the self-renewal and tumorigenicity of LCSCs.\(^{11}\) JNK signaling was also involved in multidrug resistance of HCC. It was found that the number of side population (SP) of cells and phosphorylation of phospho-stress-activated protein kinase (SAPK)/JNK increased upon anticancer treatment, the increase of SP cells which was considered as LCSCs could be blocked by JNK signaling inhibition, indicating that the activation of JNK may be responsible for drug resistance in HCC.\(^{148}\)

Akt signaling pathway. As mentioned above, the Akt signaling pathway is involved in the proliferation of LCSCs. Also, Akt signaling contributed to the chemotherapeutic resistance of LCSCs, the inhibition of Akt signaling enhanced the sensitivity of LCSCs to sorafenib by upregulating ERK signaling, which was a primary target of sorafenib.\(^{72}\)

Conclusion

In recent years, the important role of LCSCs in the initiation, relapse, metastasis and drug resistance of HCC has been identified. Targeting LCSCs is expected to be a promising approach for the treatment of HCC. In this review, we summarize potentially therapeutic targets which are key points
of LCSCs at the genetic, molecular and cellular level. Moreover, we analyze the promising approaches to eliminate chemoresistance of LCSCs. These targets provide new opportunities for HCC treatment. However, it has been recognized that these key elements in the differentiation and self-renewal of LCSCs interacted with each other through a complex crosstalk. Thus, combined therapies by targeting LCSCs may enhance the therapeutic efficacy of HCC treatment. Moreover, although many of the targets mentioned above are highly expressed in LCSCs compared with normal liver cells or normal liver stem cells, but the systemic inhibition of these targets may result in injury of normal cells, how to save normal cells from damage in the clinical application is certainly a concern, which needs further research. In summary, targeting LCSCs could be a promising therapeutic strategy for the treatment of HCC.

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Conflict of interest statement
The authors declare that there is no conflict of interest.

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