JBUON 2021; 26(5): 1958-1963
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
Email: editorial_office@jbuon.com

ORIGINAL ARTICLE

The Akt/GSK3β/β-catenin signaling regulated by ZCCHC14 is responsible for accelerating the proliferation of hepatocellular carcinoma

Yujun Cui[1]*, Jijun Zhang[2]*, Chiyi Chen[1], Jinxuan Luo[1], Hao Yan[3], Wentao Jiang[1]

[1]Organ Transplant Center, Tianjin First Central Hospital, Tianjin, China. [2]Department of General Surgery, Tianjin Ninghe District Hospital, Tianjin, China. [3]Zhongyan Biotechnology Co., LTD., Tianjin, China.

*Yujun Cui and Jijun Zhang contributed equally to this work.

Summary

Purpose: *To clarify how ZCCHC14 affects the development of hepatocellular carcinoma (HCC).*

Methods: *Differential levels of ZCCHC14 in HCC tissues and cells were examined. Proliferative and migratory changes in HCC cells with overexpression or knockdown of ZCCHC14 were detected using 5-Ethynyl-2'- deoxyuridine (EdU) and Transwell assay, respectively. Expression changes of p-Akt/Akt, p-GSK3β/GSK3β and β-catenin in HCC cells mediated by ZCCHC14 were determined. Intervened by the p-Akt activator SC79 or overexpression of β-catenin, further validated the involvement of the Akt/GSK3β/β-catenin signaling in HCC cell phenotypes mediated by ZCCHC14.*

Results: *Upregulated ZCCHC14 in HCC accelerated in vitro proliferative potential of HCC cells. Knockdown of ZCCHC14 inactivated the Akt/GSK3β/β-catenin signaling and inhibited malignant phenotypes of HCC, which were partially reversed by SC79 induction or overexpression of β-catenin.*

Conclusions: *By activating the Akt/GSK3β/β-catenin signaling, ZCCHC14 accelerates HCC cells proliferation.*

Key words: *HCC, ZCCHC14, Akt/GSK3β/β-catenin signaling. proliferation*

Introduction

Being as a very frequent malignant tumor in China, hepatocellular carcinoma (HCC) is also commonly diagnosed around the world. It is estimated that HCC is the fifth and seventh leading cancer in men and women, respectively, which is the number two killer cancer [1]. Because HCC is highly aggressive and lowly detected in the early stage, its poor prognosis poses a huge medical burden and harms human health [2]. The pathogenesis of HCC has been extensively studied. Epidemiological studies demonstrated that HCC results from a synergistic effect of multiple factors, and among them, genetic factors play a major role [3]. At present, a combination therapy is able to prolong the survival of HCC, which is superior to a single treatment. Nevertheless, clinical treatment of HCC is a huge challenge since there is a large number of advanced HCC patients and effective treatment for them is lacking. The selection of therapeutic strategies for advanced HCC is remarkably limited owing to metastases and poor physical conditions, and their 5-year survival is only around 10% [4]. Great efforts should be made on preventing the aggravation of HCC and searching biomarkers for detecting early-stage HCC.

Corresponding author: Wentao Jiang, MD. Organ Transplant Center, Tianjin First Central Hospital, No.24, Fukang Rd, Nankai District, Tianjin 300190, China.
Tel: +86 018002009823; Email: jwt_2012@163.com
Received: 17/06/2021; Accepted: 03/08/2021

This work by JBUON is licensed under a Creative Commons Attribution 4.0 International License.

ZCCHC encodes zinc finger proteins containing a CCHC domain, which are involved in the regulation of cell differentiation, embryo development, germ cell formation and other important life activities. ZCCHC14 locates on human chromosome 16 with 86,779 bases in size. It is closely related to microcephalia [5], nicotine and alcohol addiction [6] and food addiction [7]. However, the specific mechanism of ZCCHC14 in mediating pathological conditions is unclear. It is reported that ZCCHC14 is upregulated in lung cancer samples, which stimulates proliferative and invasive potentials of cancer cells by activating the MAPK signaling [8]. This study intended to clarify the role of ZCCHC14 in mediating HCC cell phenotypes.

Methods

Cancer samples

Thirty-five HCC cases diagnosed at our hospital were recruited. Their cancer tissues and non-tumor ones (pathological examination confirmed the absence of residual cancer cells) were surgically resected, fixed in 4% formalin (pH=7) and embedded for section preparation. Postoperative diagnosis of primary HCC was completed by two experienced pathologists. Clinical data and their follow-up information were thoroughly recorded. Inclusion criteria: (1) Primary HCC cases; (2) Follow-up period lasted at least 5 years; (3) No residues of cancer cells at the margin; (4) Clinical data could be traced. Exclusion criteria: (1) Pathological diagnosis was unclear; (2) Combined with other critical illness or other tumors; (3) Therapeutic efficacy could not be assessed. This study was approved by the Ethics Committee of Tianjin First Central Hospital. Signed written informed consents were obtained from all participants before the study entry.

Cell culture and transfection

Human HCC cell lines (Bel-7404, HepG2 and SNU-423) and hepatocyte epithelial cell line (THLE-3) provided by American Type Culture Collection (ATCC) (Manassas, VA, USA) were cultured in Roswell Park Memorial Institute 1640 (RPMI 1640) (HyClone, South Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), 1000 U/mL penicillin and 100 mg/mL streptomycin in an incubator containing 5% CO2 at 37°C. Monolayer cells at 80% confluence were digested for cell passage.

Transfection plasmids were diluted in serum-free OptiMEM and gently mixed with diluted Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) for 20 min. After 48 h of cell transfection using the mixture, fresh medium was replaced and transfection efficacy was tested.

Transwell assay

300 μL of serum-free cell suspension was seeded in an insert (1×108 cells/L) placed in each well of 24-well plates, where 500 μL medium with 10% FBS per well was contained. After 24-h cell culture, penetrating cells were fixed in 4% paraformaldehyde. Twenty min later, cells were dyed in 0.4% trypan blue for 20 min and observed under a microscope (40×).

5-Ethynyl-2'- deoxyuridine (EdU)

Cells were seeded in 96-well plates with 1×103 cells per well, and stained with EdU as recommended by the commercial kit (Beyotime, Shanghai, China). EdU-positive cells were captured for calculating using Image J.

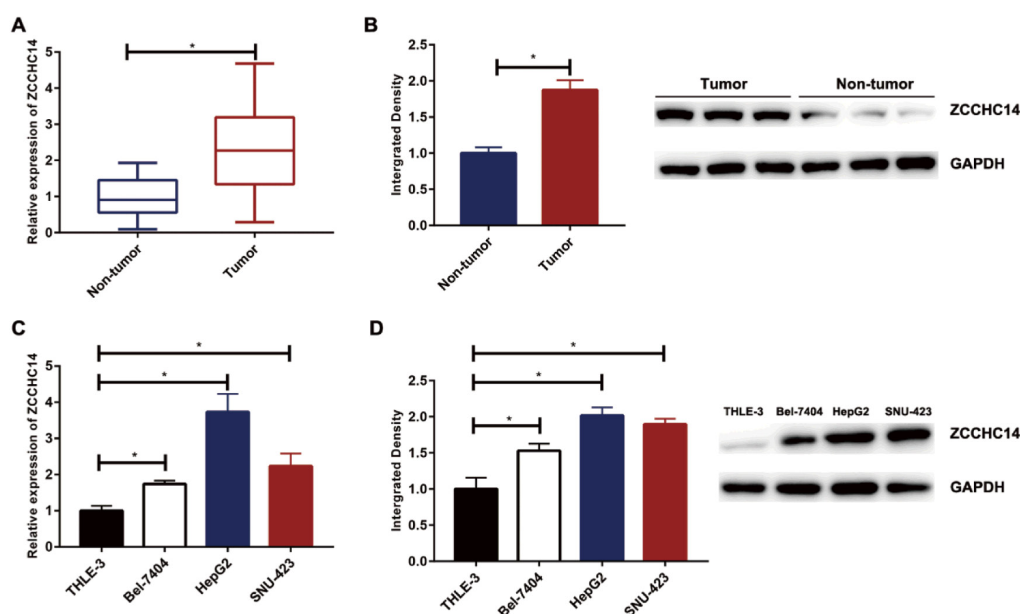

Figure 1. Differential level of ZCCHC14 in HCC. **A:** Differential level of ZCCHC14 in HCC (n=35) and non-tumor tissues (n=35) detected by qRT-PCR. **B:** Differential level of ZCCHC14 in HCC (n=35) and non-tumor tissues (n=35) detected by Western blot. **C:** Differential level of ZCCHC14 in HCC cell lines and hepatic epithelial cell line detected by qRT-PCR. **D:** Differential level of ZCCHC14 in HCC cell lines and hepatic epithelial cell line detected by Western blot. *p<0.05.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNAs were extracted using TRIzol (Invitrogen, Carlsbad, CA, USA), and the concentrations were measured using NanoDrop 2000 (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Using the PrimeScript™RT Master Mix, reversely transcribed complementary DNAs (cDNAs) were further subjected to qPCR. Relative level was calculated using ABI Step One (Applied Biosystems, Foster City, CA, USA). Sequences of primers were ZCCHC14: Forward, 5'- CGGGAGTCACGCCACTATC-3' and reverse, 5'-CCTGCTACGAGACCTCTCTCT-3'; glyceraldheyde 3-phosphate dehydrogenase (GAPDH): Forward, 5'-GATACAGTTCCCGTGTTGTTGAC-3' and reverse, 5'-CATAAAGACACATAACACCACACTC-3'.

Western blot

Cells or tissues were lysed on ice for 30 min and the mixture was centrifuged at 4°C, 12,000 rpm for 15 min. Protein samples were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred on polyvinylidene fluoride (PVDF) membranes (Roche, Basel, Switzerland). After blocking non-specific antigens on membranes, they were induced with primary and secondary antibodies under indicated

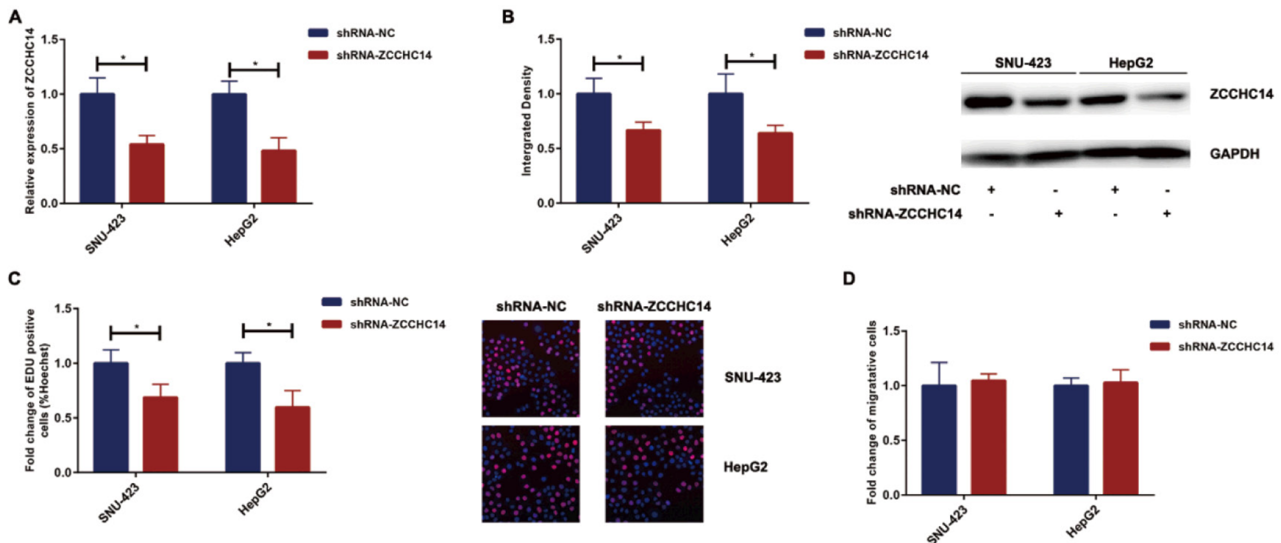

Figure 2. Silence of ZCCHC14 suppressed proliferative potential of HCC. **A:** Transfection efficacy of ZCCHC14 shRNA in SNU-423 and HepG2 cells detected by qRT-PCR. **B:** Transfection efficacy of ZCCHC14 shRNA in SNU-423 and HepG2 cells detected by Western blot. **C:** EdU-positive cell rate in SNU-423 and HepG2 cells transfected with ZCCHC14 shRNA or shRNA-NC. **D:** Migratory cell rate in SNU-423 and HepG2 cells transfected with ZCCHC14 shRNA or shRNA-NC. *p<0.05.

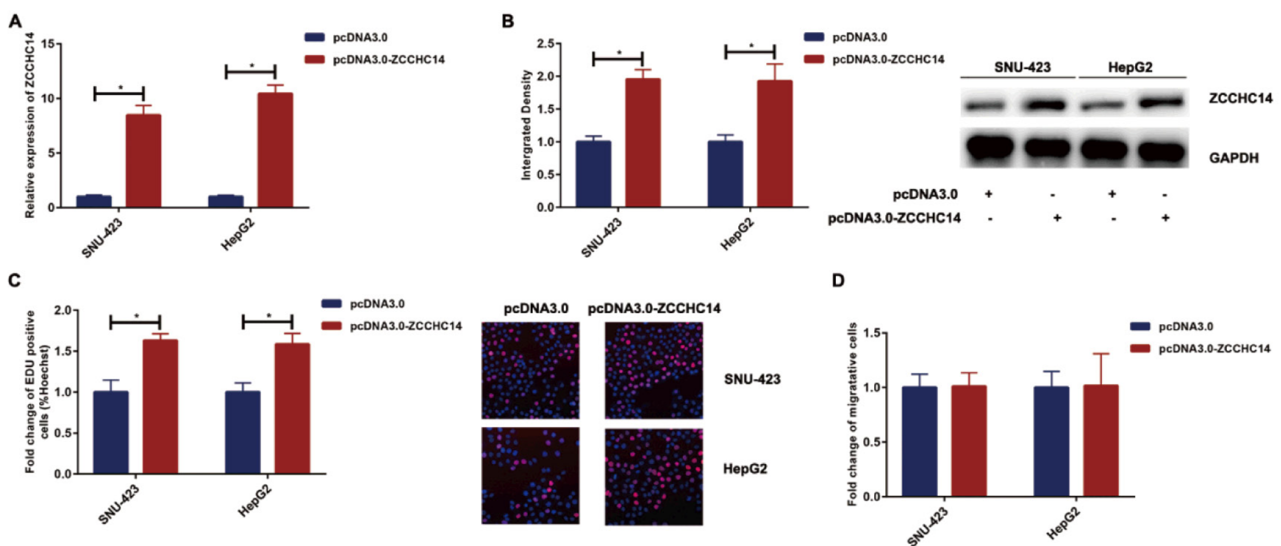

Figure 3. Overexpression of ZCCHC14 accelerated proliferative potential of HCC. **A:** Transfection efficacy of pcDNA3.0-ZCCHC14 in SNU-423 and HepG2 cells detected by qRT-PCR. **B:** Transfection efficacy of pcDNA3.0-ZCCHC14 in SNU-423 and HepG2 cells detected by Western blot. **C:** EdU-positive cell rate in SNU-423 and HepG2 cells transfected with pcDNA3.0-ZCCHC14 or pcDNA3.0. **D:** Migratory cell rate in SNU-423 and HepG2 cells transfected with pcDNA3.0-ZCCHC14 or pcDNA3.0. *p<0.05.

conditions. Protein signals were detected using Luminol substrate solution.

Statistics

SPSS 23.0 (IBM, Armonk, NY, USA) was used for statistical processing. Measurement data were expressed as mean±SD, and differences between groups were compared using the independent t-test. All experiments were performed in triplicate. Significant difference was set at $p<0.05$.

Results

Differential level of ZCCHC14 in HCC

Compared with non-tumor tissues, ZCCHC14 was upregulated in HCC (Figure 1A). Its protein level was identically upregulated in HCC tissues (Figure 1B). Meanwhile, ZCCHC14 was highly expressed in HCC cell lines than that of hepatic epithelial cell line (Figure 1C, 1D). HepG2 and SNU-423 cells were abundant with ZCCHC14, and they were used in in vitro ZCCHC14 knockdown models.

Silence of ZCCHC14 suppressed the proliferative potential of HCC

In vitro ZCCHC14 knockdown models were established by transfection of ZCCHC14 shRNA in SNU-423 and HepG2 cells (Figure 2A,2B). As EdU data showed, silence of ZCCHC14 declined the rate of EdU-positive cells, indicating attenuated proliferative potential (Figure 2C). Nevertheless, silence of ZCCHC14 did not influence the rate of migratory cells (Figure 2D).

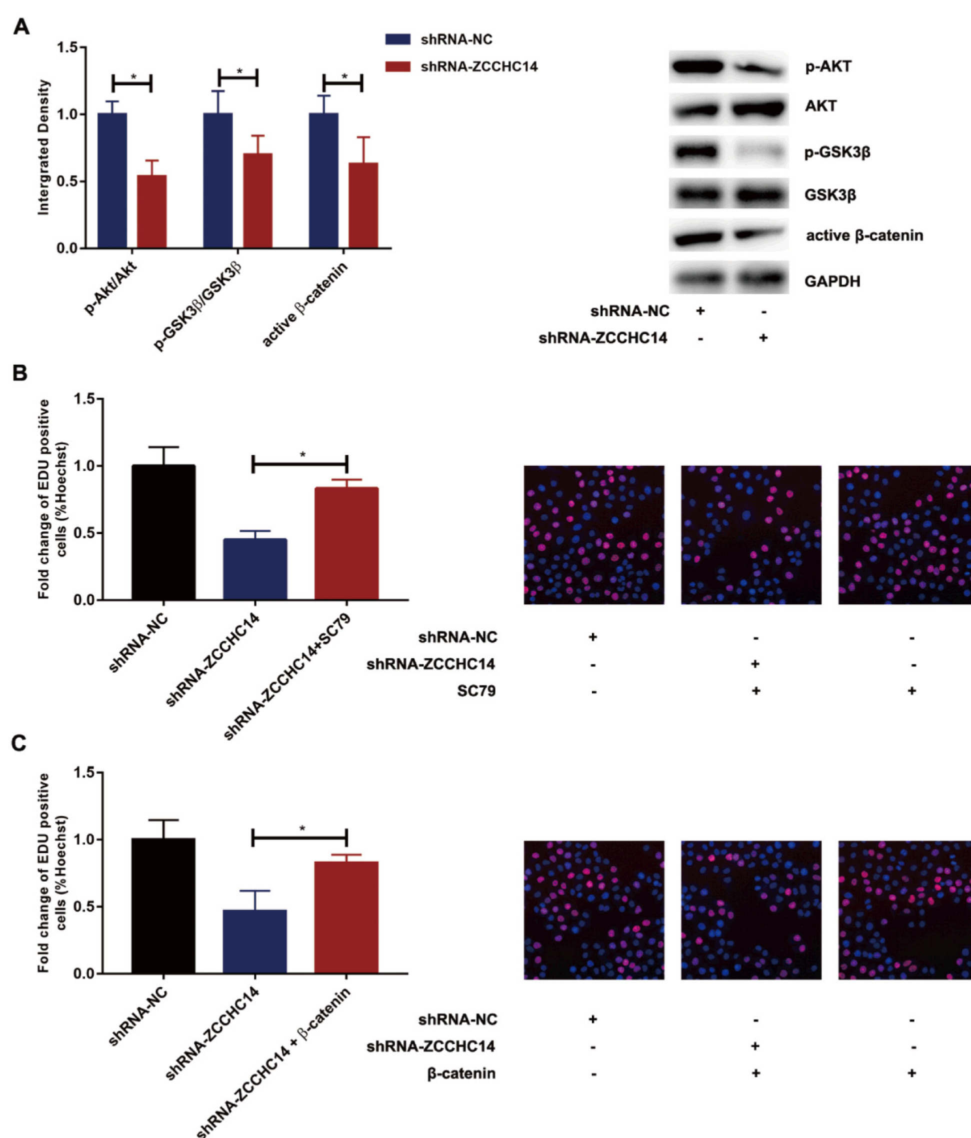

Figure 4. Silence of ZCCHC14 inactivated the Akt/GSK3β/β-catenin signaling. **A:** Protein levels of p-Akt, Akt, p-GSK3β, GSK3β and active β-catenin in HepG2 cells transfected with ZCCHC14 shRNA or shRNA-NC. **B:** EdU-positive cell rate in HepG2 cells regulated by ZCCHC14 or SC79. **C:** EdU-positive cell rate in HepG2 cells regulated by ZCCHC14 or β-catenin. *$p<0.05$.

Overexpression of ZCCHC14 accelerated the proliferative potential of HCC

Moreover, proliferative and migratory abilities of HCC cells overexpressing ZCCHC14 were examined. By transfection of pcDNA3.0-ZCCHC14, both mRNA and protein levels of ZCCHC14 markedly increased in SNU-423 and HepG2 cells (Figure 3A,3B). The rate of EdU-positive cell was much higher in HCC cells overexpressing ZCCHC14 in comparison to those transfected with negative control (Figure 3C). The migratory potential of HCC, however, was not affected by overexpression of ZCCHC14 (Figure 3D).

Silence of ZCCHC14 inactivated the Akt/GSK3β/β-catenin signaling

Previous evidence has proven the major role of the Akt/GSK3β/β-catenin signaling in HCC development. Western blot analyses uncovered that protein levels of p-Akt, p-GSK3β and active β-catenin were downregulated by transfection of ZCCHC14 shRNA (Figure 4A). To validate the involvement of such a critical signaling in HCC development, an intervention of SC79 or overexpression of β-catenin was carried out. Interestingly, the decreased rate of EdU-positive cells was partially reversed by either SC79 induction (Figure 4B) or overexpression of β-catenin (Figure 4C).

Discussion

HCC has become one of the main reasons of cancer-related death globally [9,10]. Cirrhosis and portal hypertension during the deterioration of HCC increase the difficulties in its management [11]. Our findings demonstrated that ZCCHC14 accelerates HCC cells to proliferate by activating the Akt/GSK3β/β-catenin signaling. We believe that suppression of ZCCHC14 may be a promising approach in HCC treatment.

The Akt signaling is useful in preventing cancer cell apoptosis [12,13]. Dysregulated Akt is frequently detected in multiple types of cancers [14-16]. Akt is the main downstream effector of PI3K, and the phosphorylation of Akt requires Thr-308 and Ser-473. Phosphorylated Akt (p-Akt) inhibits apoptosis of cancer cells by regulating GSK3β and other apoptotic factors, and it is also involved in the development of drug resistance [17,18]. The activity of GSK3β differs from phosphorylation sites [19]. Specific inhibitors of GSK3β contribute to alter the polarization of HCC cells, therefore attenuating their migratory potential [20]. Our results clarified that the Akt/GSK3β signaling was positively regulated by ZCCHC14 and participated in the regulation of HCC cell functions.

Protein complexes containing GSK3 are mediators of cancer cell fate. The classic Wnt signaling is well-known for its cancer cell regulation. GSK3 binding protein in this signaling controls the binding to the substrate β-catenin, thereafter leading to high specificity in the regulation of GSK3 activity [21,22]. In the absence of stimuli, the skelemin axin binds to GSK3, alongside casein kinase I and β-catenin. As a result, casein kinase phosphorylates Ser-45 of β-catenin to produce sites for pretreatment of GSK3 phosphorylation and Thr-41. Subsequently, phosphorylated modifications of Ser-37 and Ser-33 induce degradation of β-catenin [23]. The activated Wnt signaling reduces the phosphorylation of β-catenin, and then the accumulated β-catenin activates cyclin D1, Myc and c-jun [24]. Here, ZCCHC14 was able to downregulate β-catenin, thus suppressing the proliferative ability of HCC cells. How β-catenin mediates the proliferative potential requires further explorations. In addition, our findings should be validated in an in vivo HCC model, and the reason why ZCCHC14 is upregulated needs to be discussed.

Conclusions

Through activating the Akt/GSK3β/β-catenin signaling, ZCCHC14 makes HCC cells to proliferate.

Conflict of interests

The authors declare no conflict of interests.

References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2020. CA Cancer J Clin 2020;70:7-30.
2. Trevisan FDLL, Broszczak D, Zhang X, Bridle K, Crawford D, Punyadeera C. The use of minimally invasive biomarkers for the diagnosis and prognosis of hepatocellular carcinoma. Biochim Biophys Acta Rev Cancer 2020;1874:188451.
3. Villanueva A. Hepatocellular Carcinoma. N Engl J Med 2019;380:1450-62.
4. Bekir HM, Kostek O, Karabulut S et al. Efficacy of

regorafenib in the second-and third-line setting for patients with advanced hepatocellular carcinoma: A real life data of multicenter study from Turkey. JBUON 2020;25:1897-903.

5. Butler MG, Dagenais SL, Garcia-Perez JL et al. Microcephaly, intellectual impairment, bilateral vesicoureteral reflux, distichiasis, and glomuvenous malformations associated with a 16q24.3 contiguous gene deletion and a Glomulin mutation. Am J Med Genet 2012;158A:839-49.
6. Wang KS, Liu X, Zhang Q, Zeng M. ANAPC1 and SLCO3A1 are associated with nicotine dependence: meta-analysis of genome-wide association studies. Drug Alcohol Depend 2012;124:325-32.
7. Pedram P, Zhai G, Gulliver W, Zhang H, Sun G. Two novel candidate genes identified in adults from the Newfoundland population with addictive tendencies towards food. Appetite 2017;115:71-9.
8. Shi X, Han X, Cao Y, Li C, Cao Y. ZCCHC14 regulates proliferation and invasion of non-small cell lung cancer through the MAPK-P38 signalling pathway. J Cell Mol Med 2021;25:1406-14.
9. Zhong F, Cheng X, Sun S, Zhou J. Transcriptional activation of PD-L1 by Sox2 contributes to the proliferation of hepatocellular carcinoma cells. Oncol Rep 2017;37:3061-7.
10. Wang L, Xian Y, Yang Z. Comparison of 10 prognostic staging systems in patients with advanced hepatocellular carcinoma. JBUON 2019;24:150-7.
11. Adhoute X, Penaranda G, Raoul JL et al. Usefulness of staging systems and prognostic scores for hepatocellular carcinoma treatments. World J Hepatol 2016;8:703-15.
12. Porta C, Paglino C, Mosca A. Targeting PI3K/Akt/mTOR Signaling in Cancer. Front Oncol 2014;4:64.
13. Papadimitrakopoulou V. Development of PI3K/AKT/mTOR pathway inhibitors and their application in personalized therapy for non-small-cell lung cancer. J Thorac Oncol 2012;7:1315-26.
14. Xia P, Xu XY. PI3K/Akt/mTOR signaling pathway in cancer stem cells: from basic research to clinical application. Am J Cancer Res 2015;5:1602-9.
15. Huang J, Zhang L, Greshock J et al. Frequent genetic abnormalities of the PI3K/AKT pathway in primary ovarian cancer predict patient outcome. Genes Chromosomes Cancer 2011;50:606-18.
16. Lin CC, Zhou JP, Liu YP et al. The silencing of Pokemon attenuates the proliferation of hepatocellular carcinoma cells in vitro and in vivo by inhibiting the PI3K/Akt pathway. PLoS One 2012;7:e51916.
17. Simon D, Herva ME, Benitez MJ et al. Dysfunction of the PI3K-Akt-GSK-3 pathway is a common feature in cell culture and in vivo models of prion disease. Neuropathol Appl Neurobiol 2014;40:311-26.
18. Fu Y, Wang X, Cheng X, Ye F, Xie X, Lu W. Clinicopathological and biological significance of aberrant activation of glycogen synthase kinase-3 in ovarian cancer. Onco Targets Ther 2014;7:1159-68.
19. Jope RS, Johnson GV. The glamour and gloom of glycogen synthase kinase-3. Trends Biochem Sci 2004;29:95-102.
20. Beurel E, Grieco SF, Jope RS. Glycogen synthase kinase-3 (GSK3): regulation, actions, and diseases. Pharmacol Ther 2015;148:114-31.
21. Mukai F, Ishiguro K, Sano Y, Fujita SC. Alternative splicing isoform of tau protein kinase I/glycogen synthase kinase 3beta. J Neurochem 2002;81:1073-83.
22. Bijur GN, Jope RS. Rapid accumulation of Akt in mitochondria following phosphatidylinositol 3-kinase activation. J Neurochem 2003;87:1427-35.
23. Manoukian AS, Woodgett JR. Role of glycogen synthase kinase-3 in cancer: regulation by Wnts and other signaling pathways. Adv Cancer Res 2002;84:203-29.
24. Ciani L, Salinas PC. WNTs in the vertebrate nervous system: from patterning to neuronal connectivity. Nat Rev Neurosci 2005;6:351-62.