Pakistan J. Zool., vol. 55(1), pp 139-144, 2023 **DOI**: https://dx.doi.org/10.17582/journal.pjz/20210914160920

SOX9 Promotes Hepatocellular Carcinoma Progression through Targeted Regulation of HSPA1B

Jiangbo Shao[1], Donglai Zhu[2], Shengqiang Zou[1], Guohong Ge[1] and Ju Huang[1]*

[1]Department of Hepatosis, The Third People's Hospital of Zhenjiang Affiliated Jiangsu University, Zhenjiang, Jiangsu 212005, China

[2]Department of Clinical Laboratory, The Third People's Hospital of Zhenjiang Affiliated Jiangsu University, Zhenjiang, Jiangsu 212005, China

Jiangbo Shao and Donglai Zhu contributed equally to this work.

ABSTRACT

Recent studies have demonstrated that SOX9 was highly expressed and played critical roles in increasing cancer stem cell expansion as well as decreasing sensitivity to the sorafenib therapy in hepatocellular carcinoma. In this study, the potential functions and mechanisms of SOX9 in promoting hepatocellular carcinoma progression were investigated through comprehensive bioinformatics analysis. We found that SOX9 mainly participated in heat stress response, protein folding as well as metabolism and biosynthesis of amino acid. SOX9 correlated with HSPA1B were negative to the progression in hepatocellular carcinoma patients. These results suggest that SOX9 participated in hepatocellular carcinoma progression through targeted regulation of HSPA1B that might provide novel insights in hepatocellular carcinoma therapy.

Article Information
Received 14 September 2021
Revised 18 October 2021
Accepted 06 November 2021
Available online 09 February 2022 (early access)
Published 22 October 2022

Authors' Contribution
JS, DZ and JH designed the study, performed the research and wrote the paper. SZ and GG did part of the analyses and interpreted the results. All authors discussed the data and revised the manuscript.

Key words
SOX9, HSPA1B, Hepatocellular carcinoma, Bioinformatic analyses, Biomarker

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fourth leading cause of cancer-related death worldwide, which seriously threats the physical and mental health of patients (Forner *et al*., 2018; El-Serag, 2020; Siegel *et al*., 2020). Early stage HCC can be treated with partial resection, liver transplantation or radiofrequency ablation (Grandhi *et al*., 2016; Liver, 2018). However, as limitations in the sensitivity and specificity of diagnosis, most patients are diagnosed at an advanced stage. At this stage, palliative therapy such as radiotherapy or chemotherapy is the only option (Balogh *et al*., 2016; Yang *et al*., 2019). Sorafenib, a small-molecule multikinase inhibitor of antiangiogenic drug, was the first-line systemic treatment of patients with advanced-stage HCC. Sorafenib has been proved for significantly suppressing tumor cell proliferation as well as effectively prolonging the survival of HCC patients (Wilhelm *et al*., 2006; Llovet *et al*., 2008). Unfortunately, most patients treated with sorafenib eventually show resistance and disease progression (Keating, 2017; Zhu *et al*., 2017).

Sex determining region Y-related high mobility group box gene 9 (SOX9), a member of the sex determining region Y box gene superfamily, is required for cartilage, formation respiratory epithelium development and melanocyte differentiation (Bi *et al*., 1999). Accumulating evidence has revealed that the up-regulated SOX9 was associated to drug resistance and recurrence in a number of tumors (Lü *et al*., 2008; Song *et al*., 2014; Ma *et al*., 2016). In HCC research, recent studies have demonstrated that SOX9 was highly expressed and played critical roles in increasing cancer stem cell expansion as well as decreasing sensitivity to the sorafenib therapy (Huang *et al*., 2017; Xiao *et al*., 2019; Wang *et al*., 2020). However, there was still little research about functions or molecular mechanisms of SOX9 in HCC.

In this study, we deeply analyzed the raw data of GSE143477 from GEO database and comprehensively explored the potential functions and mechanisms of SOX9 in promoting hepatocellular carcinoma progression, which will provide novel insights in HCC therapy.

MATERIALS AND METHODS

Microarray, Metascape, JASPAR, TIMER and Kaplan Meier plotter were used for that present study.

* Corresponding author: fwzzyyx@126.com
0030-9923/2023/0001-139 $ 9.00/0

The gene expression profiles chip (GSE143477) was downloaded from GEO database and was checked by an Agilent Bioanalyzer 2100 (Agilent technologies, Santa Clara, CA, US) (Barrett *et al*., 2005). This microarray included three SOX9 knockdown and three scrambled control HepG2 cell samples (Wang *et al*., 2020). Genes with adjusted |log2FC|≥1 and p value < 0.05 were obtained as the differentially expressed genes.

Metascape (https://metascape.org) is a comprehensive online analysis tool for inputted genes (Zhou *et al*., 2019). In this study, Gene ontology (GO) analysis, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, PPI analysis and mCODE enrichment analysis of differentially expressed genes were performed by Metascape.

JASPAR (http://jaspar.genereg.net/) is a database for derivation of eukaryotic potential transcription factor binding sites (Sandelin *et al*., 2004). In this study, we explored the possible target gene and binding sequence to SOX9 in HCC with JASPAR.

TIMER (https://cistrome.shinyapps.io/timer/) is an online web server for providing information for differential gene expression in tumor/normal tissue and correlations between genes (Li *et al*., 2017).

Kaplan Meier plotter (http://kmplot.com/analysis/) is a reliable tool for a meta-analysis based discovery and validation of survival biomarkers (Nagy *et al*., 2018). In this study, prognostic analysis was performed using a Kaplan-Meier curve.

RESULTS

Differentially expressed genes of GSE143477

We firstly performed analysis of SOX9 and its correlated genes in HCC. The gene expression profiles of GSE143477 included three SOX9 knockdown and three scrambled control HepG2 cell samples. Genes with adjusted |log2FC|≥1 and p value < 0.05 were obtained. Heat map Figure 1 shows a total of 676 differentially expressed genes were identified, including 212 up-regulated and 464 down-regulated genes.

Functions of SOX9 and HCC

To further explore the potential functions of SOX9 and its correlated genes in HCC, Metascape was utilized for analysis of the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway. Table I shows the top 10 enrichment functions of the 212 up-regulated and 464 down-regulated genes, respectively. As presented in the table, the up-regulated genes were significantly enriched in heat stress response and protein folding. While the down-regulated genes were significantly enriched in metabolism and biosynthesis of amino acid.

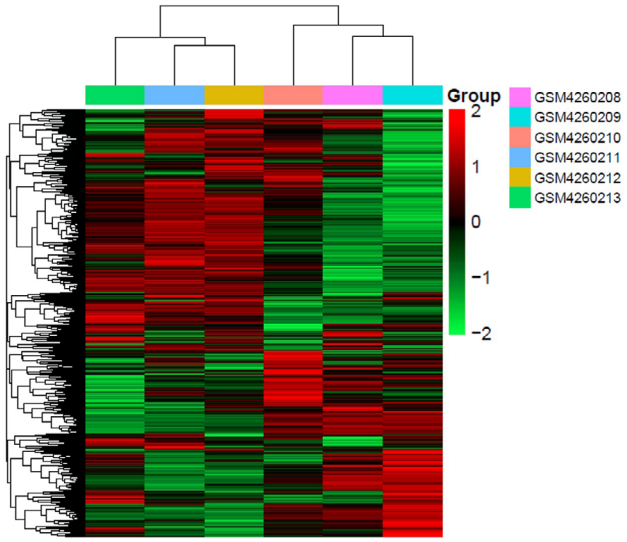

Fig. 1. Identification differentially expressed genes correlated with SOX9 in HCC cell samples. Analysis differentially expressed genes correlated SOX9 from SOX9 knockdown (GSM4260211, GSM42602012, GSM4260213) and scrambled control (GSM4260208, GSM42602089, GSM4260210) HepG2 cell samples.

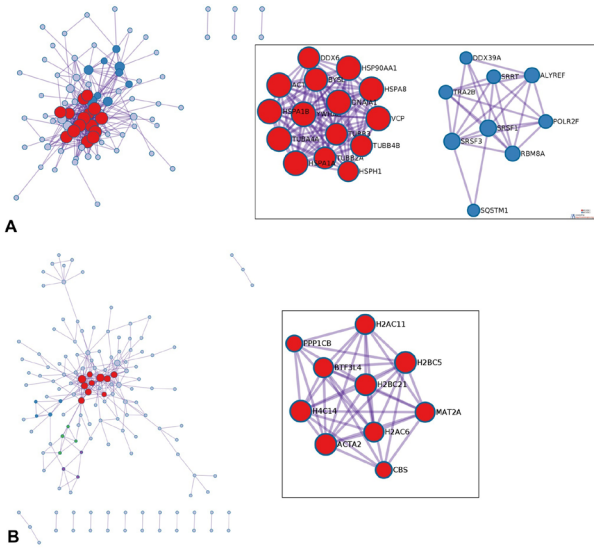

Fig. 2. The PPI network and mCODE components of differently expressed genes. (A) PPI network and mCODE of up-regulated genes. (B) PPI network and mCODE of down-regulated genes.

To deeply understand the correlation between SOX9 and HCC, the protein–protein interaction (PPI) network was analyzed. Two mCODE components were identified in the up-regulated genes list and mainly associated with HSP90 chaperone cycle for steroid hormone receptors

(SHR), selective autophagy and metabolism of RNA (Fig. 2A). In addition, one mCODE component was identified in the down-regulated genes list and related to alcoholism, HDACs deacetylate histones and RHO GTPases activate PKNs (Fig. 2B).

Table I. Enrichment analysis of 676 differentially expressed genes in different samples.

Category	Gene function	-Log P
Up-regulated genes		
GO	response to heat	8.8
GO	cellular response to heat	7.8
GO	regulation of cellular response to heat	7.4
GO	response to temperature stimulus	7.3
GO	protein folding	6.6
GO	chaperone-mediated protein folding	5.7
GO	response to unfolded protein	5.6
GO	response to topologically incorrect protein	5.2
KEGG	protein processing in endoplasmic reticulum	5.0
GO	regulation of protein complex assembly	4.8
Down-regulated genes		
GO	alpha-amino acid metabolic process	9.1
GO	cellular amino acid metabolic process	8.7
KEGG	biosynthesis of amino acids	7.4
GO	serine family amino acid biosynthetic process	7.3
GO	cellular amino acid biosynthetic process	6.9
GO	alpha-amino acid biosynthetic process	6.8
GO	L-serine metabolic process	6.6
GO	serine family amino acid metabolic process	5.7
GO	cysteine biosynthetic process via cystathionine	5.7
KEGG	cysteine biosynthesis	5.7

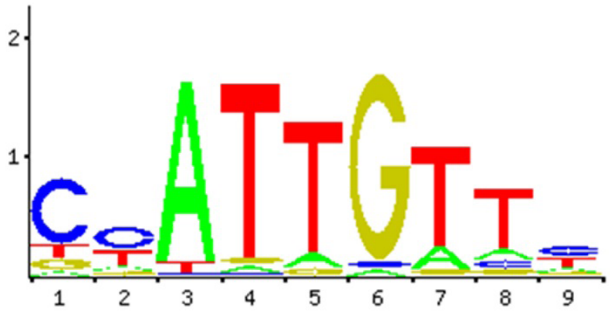

Fig. 3. Identification the binding sequence according to JASPAR. ATTGTT could be the core binding sequence for the SOX9 motif.

To find the core gene of the mCODE components, we explored the possible target gene and binding sequence of SOX9 in HCC using JASPAR. We found that ATTGTT could be the core binding sequence to SOX9 transcriptional factor and there were twelve potential binding sites in HSPA1B promoter region (Fig. 3, Table II).

Table II. Binding site sequences to SOX9 transcriptional factor in HSPA1B promoter region.

Model ID	Model name	Score	Start	End	Predicted site sequence
MA0077.1	SOX9	7.650	19	27	CTATAGTTG
MA0077.1	SOX9	6.315	702	710	ATATTGTTA
MA0077.1	SOX9	7.874	859	867	ATATTGTTA
MA0077.1	SOX9	7.105	909	917	CATTTGTTT
MA0077.1	SOX9	6.222	994	1002	CAATTGAGC
MA0077.1	SOX9	6.944	1087	1095	CTATTTTTT
MA0077.1	SOX9	7.222	1140	1148	CTATTGCTT
MA0077.1	SOX9	8.391	1179	1187	CCTTTGTTA
MA0077.1	SOX9	9.086	1219	1227	CCTTTGTTT
MA0077.1	SOX9	8.316	1425	1433	GAATTGTTT
MA0077.1	SOX9	6.113	1524	1532	TTTTTGTTT
MA0077.1	SOX9	6.964	1530	1538	CTGTTGTTT

We finally assessed the expression levels and association of SOX9 and HSPA1B in HCC tumor and normal tissues with TIMER (Fig. 4A, B). As expected, the expression levels of SOX9 and HSPA1B in HCC were significantly elevated and there was a positive correlation between them (p=0.0241). Subsequently, correlation between differently expressed SOX9/HSPA1B and the overall survival of HCC patients were assessed (Fig. 4C). As a result, HCC patients with low transcriptional levels of SOX9 or HSPA1B were associated with longer survival. These data suggest that SOX9 might play critical roles in the progression and prognosis of HCC via targeted regulation of HSPA1B.

DISCUSSION

The SOX family consists of more than 20 members and have regulatory functions in specific biological processes. Over-expression of SOX9 in HCC clinical samples significantly associates with tumor progression and poor prognosis of HCC (Guo *et al*., 2012). Inhibiting SOX9 could effectively suppress HCC tumorigenicity, proliferation, invasion and sorafenib resistance, which suggested that SOX9 was involved in multiple biological

functional regulations (Liu *et al*., 2016; Xiao *et al*., 2019; Wang *et al*., 2020). In this study, we explored the gene expression profiles chip of SOX9 knockdown HCC cell line and performed the differential gene enrichment analysis. The data suggested that SOX9 was mainly participated in heat stress response, protein folding as well as metabolism and biosynthesis of amino acid in HCC. Recently, SOX9 was found to be involved in sorafenib resistance in HCC. Wang *et al*. (2020) reported that sorafenib treatment increased the proportion of $SOX9^{+}$ HCC cell. Overexpression of exogenous SOX9 in HCC increased sorafenib resistance both in vitro and in vivo, whereas down-regulation led to inhibition of sorafenib resistance, which indicated that SOX9 enhanced sorafenib resistance (Wang *et al*., 2020). However, the mechanism needs to be further explored.

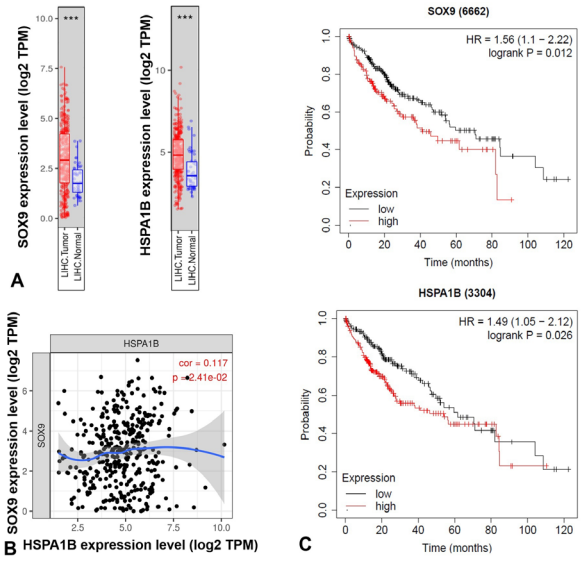

Fig. 4. Expression and prognostic value of SOX9 and HSPA1B in HCC patients. (A) The transcriptional levels of SOX9 and HSPA1B in HCC and normal tissues. (B) The correlation between SOX9 and HSPA1B in HCC. (C) The overall survival of SOX9 and HSPA1B in HCC. The p value cut-off was set at 0.05. ***, p<0.001.

Heat shock proteins (HSPs) 70, as one of the molecular markers of HCC, is significantly over-expressed in advanced stage (Chuma *et al*., 2003). HSPA1B is the member of the HSP70 family, but the role and its prognostic implication in HCC are unknown. Recently, a case-control study elucidated that single nucleotide polymorphism (SNP) in 1267 allele of HSPA1B increased the risk and threated prognosis of HCC (Jeng *et al*., 2008), which indicated that HSPA1B played important roles in the development of HCC. In our study, HSPA1B was predicted as the possible target gene and binding sites of SOX9. Besides, we investigated the high-expression and prognostic value of SOX9 and its target HSPA1B in clinical HCC patients from TCGA datasets. All these results also suggested that HSPA1B might participate in the development of HCC.

Limitations of this research should not be ignored. The different expressed genes correlated with SOX9 in HCC were investigated by constructing SOX9 knockdown cell line. Whether these genes expressed in clinical samples remains to be further confirmed. In addition, this study only evaluated the potential functions and mechanisms of SOX9 through bioinformatics analysis. Future studies should further verify these results both *in vitro* and *in vivo*.

In conclusion, our research indicated that SOX9 was high-expressed in HCC and mainly participated in heat stress response, protein folding as well as metabolism and biosynthesis of amino acid through targeted regulation of HSPA1B, which were associated with poor prognosis in HCC patients. These will provide novel insights in SOX9 research and HCC therapy.

ACKNOWLEDGEMENT

This work was supported by grant from Science and Technological Development Foundation of Zhenjiang (SH2020035).

Statement of conflict interest

The authors have declared no conflict of interest.

REFERENCES

Balogh, J., Victor, D., Asham, E.H., Burroughs, S.G., Boktour, M., Saharia, A., Li, X., Ghobrial, R.M. and Monsour Jr, H.P., 2016. Hepatocellular carcinoma: A review. *J. Hepatocell. Carcinoma*, **3**: 41. https://doi.org/10.2147/JHC.S61146

Barrett, T., Suzek, T.O., Troup, D.B., Wilhite, S.E., Ngau, W.-C., Ledoux, P., Rudnev, D., Lash, A.E., Fujibuchi, W. and Edgar, R., 2005. Ncbi geo: Mining millions of expression profiles—database and tools. *Nucl. Acids Res*., **33(suppl_1)**: D562-D566.

Bi, W., Deng, J.M., Zhang, Z., Behringer, R.R. and De Crombrugghe, B., 1999. Sox9 is required for cartilage formation. *Nat. Genet*., **22**: 85-89. https://doi.org/10.1038/8792

Chuma, M., Sakamoto, M., Yamazaki, K., Ohta, T., Ohki, M., Asaka, M. and Hirohashi, S., 2003. Expression profiling in multistage hepatocarcinogenesis: Identification of hsp70 as a molecular marker of early hepatocellular carcinoma. *Hepatology*, **37**:

198-207.

El-Serag, H.B., 2020. *Epidemiology of hepatocellular carcinoma*. *The liver: Biology and pathobiology*. pp. 758-772. https://doi.org/10.1002/9781119436812.ch59

Forner, A., Reig, M. and Bruix, J., 2018. Hepatocellular carcinoma. *Lancet*, **391**: 1301-1314. https://doi.org/10.1016/S0140-6736(18)30010-2

Grandhi, M.S., Kim, A.K., Ronnekleiv-Kelly, S.M., Kamel, I.R., Ghasebeh, M.A. and Pawlik, T.M., 2016. Hepatocellular carcinoma: From diagnosis to treatment. *Surg. Oncol*., **25**: 74-85. https://doi.org/10.1016/j.suronc.2016.03.002

Guo, X., Xiong, L., Sun, T., Peng, R., Zou, L., Zhu, H., Zhang, J., Li, H. and Zhao, J., 2012. Expression features of sox9 associate with tumor progression and poor prognosis of hepatocellular carcinoma. *Diagn. Pathol*., **7**: 44. https://doi.org/10.1186/1746-1596-7-44

Huang, M., Chen, C., Geng, J., Han, D., Wang, T., Xie, T., Wang, L., Wang, Y., Wang, C. and Lei, Z., 2017. Targeting kdm1a attenuates wnt/β-catenin signaling pathway to eliminate sorafenib-resistant stem-like cells in hepatocellular carcinoma. *Cancer Lett.*, **398**: 12-21. https://doi.org/10.1016/j.canlet.2017.03.038

Jeng, J.E., Tsai, J.F., Chuang, L.Y., Ho, M.S., Lin, Z.Y., Hsieh, M.Y., Chen, S.C., Chuang, W.L., Wang, L.Y. and Yu, M.L., 2008. Heat shock protein a1b 1267 polymorphism is highly associated with risk and prognosis of hepatocellular carcinoma: A case-control study. *Medicine*, **87**: 87-98. https://doi.org/10.1097/MD.0b013e31816be95c

Keating, G.M., 2017. Sorafenib: A review in hepatocellular carcinoma. *Target. Oncol*., **12**: 243-253. https://doi.org/10.1007/s11523-017-0484-7

Li, T., Fan, J., Wang, B., Traugh, N., Chen, Q., Liu, J.S., Li, B. and Liu, X.S., 2017. Timer: A web server for comprehensive analysis of tumor-infiltrating immune cells. *Cancer Res*., **77**: e108-e110. https://doi.org/10.1158/0008-5472.CAN-17-0307

Liu, C., Liu, L., Chen, X., Cheng, J., Zhang, H., Shen, J., Shan, J., Xu, Y., Yang, Z. and Lai, M., 2016. Sox9 regulates self-renewal and tumorigenicity by promoting symmetrical cell division of cancer stem cells in hepatocellular carcinoma. *Hepatology*, **64**: 117-129. https://doi.org/10.1002/hep.28509

Liu, Y., Zhang, W., Liu, K., Liu, S., Ji, B. and Wang, Y., 2016. Mir-138 suppresses cell proliferation and invasion by inhibiting sox9 in hepatocellular carcinoma. *Am. J. Transl. Res*., **8**: 2159.

Liver, E.A.F.T.S.O.T., 2018. Easl clinical practice guidelines: Management of hepatocellular carcinoma. *J. Hepatol*., **69**: 182-236. https://doi.org/10.1136/vr.k883

Llovet, J.M., Ricci, S., Mazzaferro, V., Hilgard, P., Gane, E., Blanc, J.F., De Oliveira, A.C., Santoro, A., Raoul, J.L. and Forner, A., 2008. Sorafenib in advanced hepatocellular carcinoma. *N. Engl. J. Med*., **359**: 378-390. https://doi.org/10.1056/NEJMoa0708857

Lü, B., Fang, Y., Xu, J., Wang, L., Xu, F., Xu, E., Huang, Q. and Lai, M., 2008. Huang and M. Lai, 2008. Analysis of sox9 expression in colorectal cancer. *Am. J. clin. Pathol*., **130**: 897-904. https://doi.org/10.1309/AJCPW1W8GJBQGCNI

Ma, F., Ye, H., He, H.H., Gerrin, S.J., Chen, S., Tanenbaum, B.A., Cai, C., Sowalsky, A.G., He, L. and Wang, H., 2016. Sox9 drives wnt pathway activation in prostate cancer. *J. clin. Invest*., **126**: 1745-1758. https://doi.org/10.1172/JCI78815

Nagy, Á., Lánczky, A., Menyhárt, O. and Győrffy, B., 2018. Validation of mirna prognostic power in hepatocellular carcinoma using expression data of independent datasets. *Sci. Rep*., **8**: 1-9. https://doi.org/10.1038/s41598-018-27521-y

Sandelin, A., Alkema, W., Engström, P., Wasserman, W.W. and Lenhard, B., 2004. Jaspar: An open-access database for eukaryotic transcription factor binding profiles. *Nucl. Acids Res*., **32**(**suppl_1**): D91-D94. https://doi.org/10.1093/nar/gkh372

Siegel, R.L., Miller, K.D. and Jemal, A., 2020. Cancer statistics, 2020. *Cancer J. Clin*., **70**: 7-30. https://doi.org/10.3322/caac.21590

Song, S., Ajani, J.A., Honjo, S., Maru, D.M., Chen, Q., Scott, A.W., Heallen, T.R., Xiao, L., Hofstetter, W.L. and Weston, B., 2014. Hippo coactivator yap1 upregulates sox9 and endows esophageal cancer cells with stem-like properties. *Cancer Res*., **74**: 4170-4182. https://doi.org/10.1158/0008-5472.CAN-13-3569

Wang, M., Wang, Z., Zhi, X., Ding, W., Xiong, J., Tao, T., Yang, Y., Zhang, H., Zi, X. and Zhou, W., 2020. Sox9 enhances sorafenib resistance through upregulating abcg2 expression in hepatocellular carcinoma. *Biomed. Pharmacother*., 129: 110315. https://doi.org/10.1016/j.biopha.2020.110315

Wilhelm, S., Carter, C., Lynch, M., Lowinger, T., Dumas, J., Smith, R.A., Schwartz, B., Simantov, R. and Kelley, S., 2006. Discovery and development of sorafenib: A multikinase inhibitor for treating cancer. *Nat. Rev. Drug Discov*., **5**: 835-844. https://doi.org/10.1038/nrd2130

Xiao, Y., Sun, Y., Liu, G., Zhao, J., Gao, Y., Yeh, S.,

Gong, L. and Chang, C., 2019. Androgen receptor (ar)/mir-520f-3p/sox9 signaling is involved in altering hepatocellular carcinoma (hcc) cell sensitivity to the sorafenib therapy under hypoxia via increasing cancer stem cells phenotype. *Cancer Lett*., **444**: 175-187. https://doi.org/10.1016/j.canlet.2018.11.004

Yang, J.D., Hainaut, P., Gores, G.J., Amadou, A., Plymoth, A. and Roberts, L.R., 2019. A global view of hepatocellular carcinoma: Trends, risk, prevention and management. *Nat. Rev. Gastroenterol. Hepatol*., **16**: 589-604. https://doi.org/10.1038/s41575-019-0186-y

Zhou, Y., Zhou, B., Pache, L., Chang, M., Khodabakhshi, A.H., Tanaseichuk, O., Benner, C. and Chanda, S.K., 2019. Metascape provides a biologist-oriented resource for the analysis of systems-level datasets. *Nat. Commun*., **10**: Article number 1523. https://doi.org/10.1038/s41467-019-09234-6

Zhu, Y.J., Zheng, B., Wang, H.Y. and Chen, L., 2017. New knowledge of the mechanisms of sorafenib resistance in liver cancer. *Acta Pharmacol. Sin*., **38**: 614-622. https://doi.org/10.1038/aps.2017.5