# IRAK1 augments cancer stemness and drug resistance via the AP-1/AKR1B10 signaling cascade in hepatocellular carcinoma

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**Abbreviations:** Aldo-Keto Reductase Family 1, Member 10, AKR1B10; hepatocellular carcinoma, HCC; IL-1 receptor, IL-1R; Interleukin-receptor associated kinase 1, IRAK1; quantitative PCR, qPCR; Toll-like receptors, TLRs; tumor-initiating cells, T-ICs, Statistical Package for the Social Sciences, SPSS

**Significance:** IRAK4/IRAK1/AP-1/AKR1B10 signaling pathway regulates cancer stemness and drug resistance and may be a novel therapeutic target in HCC.

**Abstract**

Frequent relapse and drug resistance in patients with hepatocellular carcinoma (HCC) can be attributed to the existence of tumor-initiating cells (T-IC) within the tumor bulk. Therefore, targeting liver T-IC may improve the prognosis of these patients. From transcriptome sequencing of 16 pairs of clinical HCC samples, we report that interleukin-1 receptor-associated kinase 1 (IRAK1) in the TLR/IRAK pathway is significantly upregulated in HCC. IRAK1 overexpression in HCC was further confirmed at the mRNA and protein levels and correlated with advanced tumor stages and poor patient survival. Interestingly, IRAK4, an upstream regulator of IRAK1, was also consistently upregulated. IRAK1 regulated liver T-IC properties including self-renewal, tumorigenicity, and liver T-IC marker expression. IRAK1 inhibition sensitized HCC cells to doxorubicin and sorafenib treatment in vitro via suppression of the apoptotic cascade. Pharmacological inhibition of IRAK1 with a specific IRAK1/4 kinase inhibitor consistently suppressed liver T-IC populations. We identified aldo-keto reductase family 1 member 10 (AKR1B10) as a novel downstream target of IRAK1, which was found to be overexpressed in HCC and significantly correlated with IRAK1 expression. Knockdown of AKR1B10 negated IRAK1-induced T-IC functions via modulation of the AP-1 complex. Inhibition of IRAK1/4 inhibitor in combination with sorafenib synergistically suppressed tumor growth in an HCC xenograft model. In conclusion, targeting the IRAK4/IRAK1/AP-1/AKR1B10 signaling pathway may be a potential therapeutic strategy against HCC.

## Introduction

HCC is one of the deadliest diseases, being the fifth most commonly diagnosed cancer and the second leading cause of cancer-related mortality in the world (1). The poor prognosis of HCC patients is mainly attributed to late diagnosis, frequent relapse and the refractory nature of HCC to chemotherapy. Sorafenib is reported to increase the median survival of HCC patients by only 3 months (2), while none of the chemotherapeutic drugs developed thus far, including doxorubicin and cisplatin, can achieve a response rate higher than 25% (3,4), let alone improve patient survival. Thus, there is an urgent need to seek novel therapeutic targets that display high efficacy and specificity to HCC cells.

Recently, compelling evidence has emerged in support of a cancer stem cell (CSC) / tumor-initiating cell (T-IC) model in leukemia and a wide range of solid tumors, including HCC (5-8) Our group was the first to identify a liver T-IC population marked by the CD24 and CD47 surface phenotype (9,10). Toll-like receptors (TLRs) and IL-1 receptor (IL-1R) signaling engages IL-1R-associated kinase IRAK1, and IRAK1 phosphorylation is one of the crucial upstream regulators of NF-κB signaling in inflammatory responses, which have been implicated in tumorigenesis (11) and drug response modulation (12). Since NF-κB plays a crucial role in liver T-IC regulation and sorafenib resistance (10, 13), we hypothesize that TLRs and IL-1R signaling may play a crucial role in the regulation of liver T-ICs. By RNA sequencing analysis in 16 clinical HCC samples and their adjacent non-tumor counterparts, IRAK1 was found to be the most upregulated among other family members. Although IL-1 signaling and TLR-MyD88 have been implicated in human cancers, alterations in IRAK1 itself were not linked to human malignancy until very recently

(11,12). However, the role of IRAK1 in T-ICs remains unknown, and its role in HCC has not been clearly addressed.

In this study, we examined whether IRAK1 plays a crucial role in the regulation of liver T-IC functions. First, we found that over-expression of IRAK1 at both mRNA and protein levels was associated with advanced tumor stages. Interestingly, IRAK4, an upstream regulator of IRAK1, was also found to be consistently upregulated. IRAK1 was found to regulate the phenotypes of liver T-ICs, including tumorigenicity, self-renewal, drug resistance and liver T-IC marker expression. The results were also validated by the use of an IRAK1/4 inhibitor. Mechanistically, RNA sequencing analysis upon IRAK1 knockdown revealed Aldo-Keto Reductase Family 1 Member 10 (AKR1B10) as a potential novel downstream target of IRAK1 mediated by AP-1 activation. Pharmacological inhibition of IRAK1 by an IRAK1/4 inhibitor not only suppressed the growth of established tumors but also sensitized the tumors to sorafenib *in vivo*.

## Materials and Methods

### Cell lines and cell culture

The human HCC cell lines MHCC97L (Liver Cancer Institute, Fudan University, China), Huh7 (Japan Cancer Research Bank, Tokyo, Japan), Hep3B (ATCC) , PLC/PRF/5 (Japanese Cancer Research Bank), Bel7402, and SMMC7721 (Shanghai Institute of Cell Biology, Chinese Academy of Sciences) were maintained in DMEM containing high glucose (Gibco BRL, Grand Island, NY), 10% heat-inactivated fetal bovine serum (Gibco BRL), 100 mg/mL penicillin G, and 50 μg/mL streptomycin (Gibco BRL) at 37°C in a humidified atmosphere containing 5% $CO_2$. All cell lines used in this study were obtained between 2013 and 2016, regularly authenticated by morphologic observation and AuthentiFiler STR (Invitrogen) and tested for absence of mycoplasma contamination (MycoAlert, Lonza). Cells were used within 20 passages after thawing.

### RNA-sequencing

Total RNA of PLC/PRF/5 of non-target control (NTC) and IRAK1 knockdown (shIRAK1_H3) groups were extracted using TRIzol Reagent (Life Technologies) according to manufacturer's protocol. The quality of total RNA was checked by Agilent 2100 bioanalyzer (Agilent Technologies Inc.) to have OD260/280 ratio of between 1.8-2.0 and RNA integrity number (RIN) value higher than 8.0. The RNA samples which met the quality assessment were then subjected to Illumina Solexa sequencing using Hiseq 1500 sequencer (Illumina) for performing HiSeq sequencing run (pair-end sequencing of 101bp). Each sample had an average throughput of 10.8Gb and a total throughput of 21.5Gb. An average of 94% of the bases achieved a quality score of Q30 where Q30 denotes the accuracy of a base call to be 99.9%. Expression estimation and tests for differential expression were processed by Cufflinks v2.1.1 (Trapnell et al., 2013). All data was expressed as fragments per kilobase of exon per million fragments mapped (FPKM) values and

fold changes in transcript levels relative to the NTC group from PLC/PRF/5. RNA-sequencing data is available publicly at GSE94025.

### *In vivo* drug treatment assay

1 x $10^6$ PLC/PRF/5 and PDTX#1 cells were prepared according to the above mentioned protocol and were injected into the flank of BALB/c nude mice. Once the tumors established and reached a size of approximately 0.4cm in diameter, the mice were randomly divided into four groups, each consisting of 6 and 4 mice for PLC/PRF/5 and PDTX#1: the vehicle control group, the IRAK1/4-Inh receiving group, the sorafenib receiving group and the combined treatment group. Sorafenib (LC Laboratories, Woborn, MA) was dissolved in DMSO and further diluted in 100μl $H_2O$ for oral gavage; IRAK1/4-Inh (Sigma-Aldrich, Inc, Saint Louis, MO) was dissolved in DMSO then diluted in 100μl of solution containing 90% $H_2O$, 5% Tween80 (Anatrace, Maumee, OH) and 5% PEG400 (Sigma-Aldrich, Inc, Saint Louis, MO). The tumor volume and body weight were measured every three days. The tumor volume was calculated by height x width x depth / 2. The mice were treated for 31 and 20 days before sacrificed and tumors harvested for documentation. The study protocol was approved by and performed in accordance with the Committee of the Use of Live Animals in Teaching and Research at The University of Hong Kong and Hong Kong polytechnic University.

### Statistical analysis

The statistical significance of the results obtained from qPCR, spheroid formation assays, flow cytometry analysis, invasion and migration assays was determined by Student's t-test using Microsoft Office Excel software (Microsoft Corporation, Redmond, WA, USA). The results are shown as the means and standard deviations, and p-values less than 0.05 were considered statistically

significant (* $p<0.05$, ** $p<0.01$, *** $p<0.001$). Chi-square test was used to assess the correlations between clinicopathological parameters and IRAK1 expression. Kaplan-Meier survival analysis was used to analyze disease-free survival and overall survival, and the statistical significance was calculated by log-rank test; these analyses were carried out using SPSS 20.

Additional experimental procedures are provided in the supplementary information.

## RESULTS

### *Upregulation of IRAK1 and its upstream regulator, IRAK4, in clinical HCC specimens*

By transcriptome sequencing analysis in tumorous (T) and non-tumorous (NT) liver tissues in 16 cases of HCC patients, we analyzed the expression levels of 10 TLR and 4 IRAK family members. Among the TLR family members, TLR5 and TLR9 were the only overexpressed TLRs in HCC, which showed significant upregulation of 2.78-fold and 1.90-fold, respectively (Fig. S1). Among the IRAK family members, IRAK1 was the only protein kinase that was significantly overexpressed in HCC (Fig. S1). To further validate this result, we assessed IRAK1 mRNA expression in a cohort of 69 patients by qPCR analysis (Fig. 1A). In concordance with the transcriptome sequencing data, the IRAK mRNA level was 2.28-fold more abundant in tumor, and overexpression of IRAK1 mRNA (>2-fold) was found in 49.28% (34/69) of HCC patients (Fig. 1B). Statistical analysis using Statistical Package for the Social Sciences (SPSS) software revealed upregulation of IRAK1 was significantly associated with advanced tumor stages ($p$=0.04) (Table S1). By TCGA data analysis of 372 HCC patients, we have stratified HCC patients into two groups based on their IRAK1 expression. We found that patients with IRAK1 expression higher than median level have a poorer overall survival when compared with those lower than median (p=0.0033, log rank test) (Fig. 1C). To further confirm if IRAK1 is also overexpressed at the

protein level in HCC, Western blot analysis was performed on protein lysates extracted from T and adjacent NT liver tissues in a cohort of 15 patients (Fig. 1D). IRAK1 overexpression was defined as a fold change>1.5. Among the 15 pairs of HCC samples, 13 (86.67%) displayed IRAK1 overexpression in T compared to the NT liver tissues. Consistently, IRAK1 overexpression in HCC was confirmed by immunohistochemical staining for IRAK1 in a pair of clinical HCC tissue sections (Fig. 1E). Both IRAK1 and IRAK4 play important roles in the transduction of TLR/IRAK signaling; therefore, we were surprised to find that IRAK4 was downregulated at the mRNA level. We also evaluated the expression of IRAK4 at the protein level in 16 pairs of clinical HCC samples by Western blotting and found that IRAK4 was highly expressed at the protein level in HCC (Fig. 1F). By comparing the mRNA and protein levels of IRAK4 in a series of clinical samples, it was noted that the correlation between IRAK4 mRNA and protein levels was very poor (Fig. S2). Our data may suggest that IRAK4 is upregulated in HCC, possibly through post-translational modification. By analyzing the Oncomine database, we found that overexpression of IRAK1 was also observed in a variety of other cancers, including brain, colon, breast, bladder, lung and ovarian cancers (Fig. S3).

***IRAK1 regulates self-renewal, tumorigenicity, and liver T-IC marker expression in HCC cells***

To further examine whether IRAK1 functionally contributes to the traits of liver T-ICs, we performed IRAK1-knockdown and IRAK1-overexpression experiments using a lentiviral-based approach. By Western blotting, we found high expression of IRAK1 in a panel of HCC cell lines, including Bel7402 and PLC/PRF/5, among others (Fig. 2A); therefore, these two cell lines were chosen for the knockdown experiment, while Huh7 cells were chosen for the overexpression experiment. Western blotting was used to confirm the successful establishment of the knockdown and overexpression clones (Fig. 2B). Knockdown of IRAK1 dramatically reduced the spheroid

forming ability of HCC cells in both PLC/PRF/5 and Bel7402 cells. Accordingly, IRAK1 overexpression was shown to enhance the spheroid forming ability of Huh7 (Fig. 2C). In an *in vivo* tumorigenicity assay, rate-limiting dilution analysis was performed to determine how IRAK1 may affect the tumorigenicity of HCC cells. In both PLC/PRF/5 and Bel7402 cells, knockdown of IRAK1 dramatically reduced the size and number of tumors formed (Fig. 2D, Table S2). In line with our findings, IRAK1 overexpression in Huh7 cells significantly enhanced tumorigenicity, with an increase in the estimated T-IC frequency (Fig. 2D, Table S2). Finally, flow cytometry was used to assess the expression level of two known liver T-IC markers, including CD24 and CD47. We found that the shIRAK1-transfected cells exhibited a decrease in the percentages of these markers (Fig. 2E). By immunofluorescence staining, we found that spheroids derived from shIRAK1 knockdown showed decreased expression of CD24 as compared to control cells (Fig. 2F).

***IRAK1 regulates the drug resistance of HCC cells through the regulation of the apoptotic cascade***

Next, the effect of IRAK1 on drug resistance was evaluated by Annexin V assay. Upon treatment with doxorubicin, the shIRAK1-transfected clones (H2 and H3) derived from both PLC/PRF/5 and Bel7402 cells exhibited enhanced sensitivity towards doxorubicin (Fig. 3A). In addition, the shIRAK1-transfected clones showed enhanced sensitivity towards sorafenib (Fig. 3A). In contrast, IRAK1 overexpression reduced cell death in Huh7 cells when they were treated with either doxorubicin or sorafenib (Fig. 3B). To further confirm the role of IRAK1 and its upstream regulator, IRAK4, in sorafenib resistance, cells were treated with sorafenib, and then the levels of IRAK1 and IRAK4 mRNA were evaluated. Upon sorafenib treatment, IRAK1 and

IRAK4 mRNA expression in PLC/PRF/5 and Bel7402 cells was increased (Fig. 3C). Consistently, IRAK1, p-IRAK1 and IRAK4 protein expression was elevated in response to sorafenib treatment (Fig. 3D). Interestingly, IRAK1, p-IRAK1 and IRAK4 expression was elevated in sorafenib-resistant Bel7402 cells (13) (Fig. 3E). In addition, the effect of sorafenib on the apoptosis of shIRAK1-transfected clones and NTC-transfected cells was validated by the differential expression of apoptosis cascade-related proteins, including Bcl2, Bax, cleaved caspase-9, caspase-3 and PARP. Compared with the NTC control cells, increased expression levels of key proteins in apoptosis, including BAX, cleaved caspase 9, cleaved caspase 3, and cleaved PARP, were observed in the shIRAK1-transfected clones derived from PLC/PRF/5 cells (Fig. 3F).

***Pharmacological inhibition of IRAK1 by IRAK1/4 inhibitor suppresses the traits of liver T-ICs***

To further explore the potential of therapeutic targeting of IRAK1 in HCC, we wanted to examine whether the pharmacological inhibition of IRAK1 by IRAK1/4-Inh (Sigma) exerts an effect similar to that of IRAK1 knockdown using a lentiviral-based approach. The chemical structure and purity of IRAK1/4-Inh was shown in Fig. S4A. By western blot analysis, we found that IRAK1/4-Inh suppressed the phosphorylation level of IRAK1 in dose dependent manner (Fig. S4B). Then, we determined the dose of IRAK1/4-Inh at which cell proliferation is minimally affected. By direct cell counting up to day 5, we found that the doses of IRAK1/4-Inh ranging from 1 μM to 5 μM exerted minimal effects on cell growth in both Bel7402 and PLC/PRF/5 cells expressing high levels of IRAK1 (Fig. S4C). Based on these results, we then determined whether IRAK1/4-Inh at doses ranging from 1 μM to 10 μM could exert a suppressive effect on the self-renewal ability of the cells. By sphere formation assay, we found that IRAK1/4-Inh at 5 μM significantly suppressed the number and size of spheres formed (Fig. 4A) and their secondary

passages (Fig. S4D). Pretreatment with IRAK1/4-Inh significantly inhibited the *in vivo* tumorigenicity of both PLC/PRF/5 and Bel7402 cells, as indicated by the reduced number and size of the tumors formed post-treatment (Fig. 4B). As shown by migration and invasion assays, 5 μM IRAK1/4-Inh significantly suppressed both the migration and invasion of Bel7402 and PLC/PRF/5 cells (Fig. 4C). Consistently, treatment with IRAK1/4-Inh decreased the T-IC population, as indicated by the reduced expression of T-IC markers, including CD24 and CD47 (Fig. 4D). Last, to confirm whether IRAK1 suppression could sensitize the cells to doxorubicin and sorafenib, Bel7402 and PLC/PRF/5 cells were treated with IRAK1/4-Inh alone and in combination with the two drugs. By Annexin V staining, we found that the suppression of IRAK1 by IRAK1/4-Inh in both Bel7402 and PLC/PRF/5 cells resulted in a reduction in survival ability and lower resistance to both doxorubicin and sorafenib (Fig. S4E&F).

***IRAK1 regulates the traits of liver T-ICs via the AP-1/AKR1B10 signaling cascade***

We employed RNA sequencing analysis to compare the gene expression profiles of shIRAK1-transfected (H3) and non-target control cells derived from PLC/PRF/5 cell line. In total, 1171 genes were found to be significantly different in terms of their expression level in the shIRAK1_H3 cells relative to the NTC cells. Among these 1171 genes, 646 genes were significantly upregulated, and 525 genes were significantly downregulated (Fig. 5A). The list of the most deregulated genes was further narrowed down through literature review to evaluate their relevance to cancer development (Table S3). Then, the consistency of expression of these genes relative to IRAK1 expression level was confirmed at the mRNA level in both shIRAK1_H2 and shIRAK1_H3 clones in PLC/PRF/5 and Bel7402 cells, as well as in IRAK1-overexpressing Huh7 cells. Among these candidates, Aldo-Keto Reductase Family 1 Member B10 (AKR1B10) was the

only gene we analyzed that showed a strong correlation to IRAK1 expression. As shown in Fig. 5B, AKR1B10 was consistently downregulated upon IRAK1 knockdown in shIRAK1 clones in PLC/PRF/5 and Bel7402 cells and consistently upregulated in IRAK1-overexpressing Huh7 clone. The downregulation of AKR1B10 at the protein level upon IRAK knockdown was further confirmed in PLC/PRF/5 cells (Fig. 5B). The overexpression of AKR1B10 in HCC was further confirmed in a cohort of 79 patients, and AKR1B10 was found to be upregulated by 3.75-fold in tumor tissues (Fig. S5A&B). Patients with overexpression of AKR1B10 had a tendency of poorer disease-free survival (Fig. S5C). Most importantly, IRAK1 and AKR1B10 were significantly correlated in this cohort of HCC samples ($r=0.318$, $p=0.001$) (Fig. S5D). Similar correlation was also observed in a publicly available dataset (GSE145200) (Fig. S5E). Upon knockdown of AKR1B10 in Bel7402 and PLC/PRF/5 cells, shAKR1B10 showed decreased expression of liver T-IC markers and abilities in self-renewal and sorafenib resistance (Fig. S6A-D). Through *in silico* analysis, we discovered a putative AP-1 binding site located at -218 to -213 bp upstream of the AKR1B10 transcription start site, suggesting that AKR1B10 could be regulated by AP-1 signaling (Fig. S7A). Furthermore, through dual-luciferase reporter assay, it was shown that IRAK1 knockdown in PLC/PRF/5 cells resulted in dose-dependent downregulation of AP-1 promoter activity, with the shIRAK1_H3 clone exhibiting a 0.76-fold difference in downregulation (Figure S7B). To further confirm the role of AKR1B10 as the downstream effector of IRAK1-mediating T-IC functions, we repressed the expression of AKR1B10 in IRAK1-overexpressing cells to investigate whether the effects of IRAK1 overexpression could be eliminated upon suppression of AKR1B10. Using a lentiviral-based knockdown approach, the expression of AKR1B10 was repressed in IRAK1-overexpressing Huh7 cells (Fig. 5C). After successful confirmation of AKR1B10 repression in IRAK1-overexpressing Huh7 cells, we compared the sphere forming

ability, sorafenib sensitivity and T-IC marker expression of IRAK1-overexpressing Huh7 with or without AKR1B10 repression. We found that the suppression of AKR1B10 negated the effect of IRAK-mediated sphere formation, sorafenib resistance and T-IC marker expression (Fig. 5D-F).

***IRAK1/4-Inh, in combination with sorafenib, results in maximal tumor suppression in HCC xenograft models***

Given the crucial role of IRAK1 in regulating sorafenib resistance, we examined the therapeutic role of targeting IRAK1 alone and its combined effect with sorafenib *in vivo* using PLC/PRF/5 cells and PDTX#1. In these two *in vivo* models, mice with established tumors were given either vehicle control, IRAK1/4-Inh, sorafenib or a combination of both for 31 and 20 days for PLC/PRF/5 cells and PDTX#1 respectively. Compared to the vehicle control group, the IRAK1/4-Inh, sorafenib and combined treatment groups all could reduce the tumor volume in a time dependent manner (Fig. 6A). For both HCC xenograft models, IRAK1/4-Inh suppressed the tumor volumes in a manner similar to the effect of sorafenib. In addition, IRAK1/4-Inh in combination with sorafenib exerted maximal suppression of the tumors, compared with the control group (Fig. 6B&C). The body weight of mice was measured as an indication of the toxicity of the drugs. Although there was a consistent decrease in body weight throughout the treatment period, there was no significant difference among the four treatment groups; therefore, the reduction in body weight was likely due to the tumor burden and not the treatment received by mice (Fig. 6D).

## DISCUSSION

Through transcriptome analysis of 16 pairs of clinical HCC samples, TLR5 and TLR9 were both significantly upregulated in HCC, while TLR4, TLR7 and TLR8 were both downregulated. A previous study had reported the upregulation of TLR9 in human HCC through tissue microarray analysis (14). Inhibition of TLR9 with IRS-954 or chloroquine reduced Huh7 cell proliferation *in vitro* and inhibited tumor growth *in vivo* (14). Compared to studies on TLRs, there exists only a limited number of studies on the expression of IRAK family members in HCC. From our data, we observed significant upregulation of IRAK1 and downregulation of IRAK3 and IRAK4 in HCC tissues. This result is consistent to the previous report showing upregulation of IRAK in HCC (15,16). IRAK1 overexpression has also been reported in lung cancer and is associated with clinical TNM stage, lymph node metastasis and tumor size (17). More recently, IRAK1 has been reported as a therapeutic target in cancer treatment (18,19).

Among the 4 IRAK family members, IRAK1 and IRAK4 are the only two kinases that exhibit serine/threonine kinase activity and are therefore targetable by small molecule inhibitors. Therefore, we were surprised to find that the expression of IRAK1 and IRAK4 was not correlated. In view of this finding, we evaluated the expression of IRAK4 at the protein level by Western blot analysis of 16 pairs of clinical HCC samples and found that IRAK4 is indeed highly upregulated in HCC tissues at the protein level. By comparing the mRNA and protein levels of IRAK4 in a series of clinical samples, it was noted that the correlation between IRAK4 mRNA and protein levels was very poor. Our preliminary analysis demonstrated that IRAK4 was indeed upregulated in HCC possibly through post-translational modification. In addition, We observed downregulation of IRAK3 in HCC. As IRAK3 plays a negative regulatory role in the IRAK

signaling cascade, the downregulation of IRAK3 comes in line with the rest of the observations. In concordance with our observation, a very recent study demonstrated that IRAK3 expression in colorectal cancer can be induced by TLR activation and that IRAK3 deficiency is associated with enhanced inflammation (20). Furthermore, by analysis of the Oncomine database, IRAK1 was found to be overexpressed in a wide range of other cancers, strongly suggesting its significant and universal role in promoting cancer.

IRAK1 expression levels were positively correlated with the expression levels of liver T-IC markers, including CD24 (9) and CD47 (10). Using both overexpression and knockdown approaches, we found that suppression of IRAK1 not only reduced the sphere forming ability but also inhibited the tumor forming ability of HCC cells. Consistently, IRAK1 was found to play a regulatory role in mammosphere and tumor formation in a breast cancer model (18). Some of the earlier studies linked IRAK1 and chemoresistance. In T-ALL, knockdown of either IRAK1 or IRAK4 with shRNA could significantly enhance apoptosis induced by the chemotherapeutic agent ABT-737 (12). In melanoma, knockdown of either IRAK1 or IRKA4 with siRNA augmented the toxic effects of vinblastine, and the effect was more prominent when both IRAK1 and IRAK4 were simultaneously knocked down (21). Consistent with previous findings, we found that IRAK1 regulates the sensitivity of HCC cells towards doxorubicin and sorafenib. Next, we wanted to confirm the role of IRAK1 and its upstream regulator, IRAK4, in drug resistance. Upon sorafenib treatment, both IRAK1 and IRAK4 were elevated at the mRNA and protein levels, and the phosphorylation of IRAK1 was increased upon sorafenib treatment. This finding indicates that the activation of IRAK4/IRAK1 contributes to acquisition of resistance to sorafenib.

Through transcriptome sequencing analysis, we identified AKR1B10 as a potential novel downstream target of IRAK1. Further, AKR1B10 and IRAK1 were also positively correlated in an expanded cohort of clinical HCC samples, strongly indicating that AKR1B10 is likely to be regulated by IRAK1. The expression level of AKR1B10 is significantly upregulated in HCC (22). Upon the discovery of its relevance to HCC, AKR1B10 was also discovered to be upregulated in breast cancer (23), gastric cancer (24), and lung cancer (25). Functionally, AKR1B10 silencing was shown to inhibit anchorage-independent cell growth in colorectal cancer (26). Recently, AKR1B10 was reported to be associated with chemoresistance in a wide range of cancers, including doxorubicin resistance in gastric cancer (27). In this study, we confirmed AKR1B10 as a downstream effector of IRAK1, and overexpression of AKR1B10 was employed to rescue the T-IC phenotypes in shIRAK1-transfected cells. Interestingly, overexpression of AKR1B10 reversed the suppressive effect of IRAK1 knockdown on self-renewal, sorafenib resistance and T-IC marker expression in HCC cells. To identify the possible link between IRAK1 and AKR1B10, we found a potential binding site of AP-1 in the promoter of AKR1B10. Consistent with this finding, we found a reduction in AP-1 promoter activity in the two shIRAK1 clones. Our study suggested a novel regulatory mechanism for AKR1B10 expression that may function through the IRAK1/AP-1/AKR1B10 signaling axis, thus mediating T-IC phenotypes.

IRAK1/4-Inh suppressed the self-renewal, tumorigenicity, migration and invasion of HCC cells. These results are in line with the findings in breast cancer (18). In addition, when IRAK1/4-Inh was combined with sorafenib or doxorubicin, cell apoptosis was significantly enhanced. To investigate whether the application of IRAK1/4-Inh augments the efficacy of sorafenib *in vivo*, IRAK1/4-Inh was tested alone or in combination with sorafenib in tumor-bearing nude mice. We

found that IRAK1/4-Inh at the dose of 5 mg/kg suppressed tumor growth but did not cause significant weight loss in the tumor-bearing nude mice during treatment. Next, we examined the therapeutic efficacy of IRAK1/4-Inh in combination with sorafenib by oral gavage. After 21 and 31 days, the effects IRAK1/4-Inh and sorafenib treatment alone were relatively modest compared to that in the co-treatment group. Interestingly, the co-treatment group exhibited a significant reduction in tumor volume, suggesting that IRAK1/4-Inh may be used as a novel inhibitor to augment the efficacy of sorafenib and target HCC and liver T-ICs. In a study on MDS, inoculation of MDSL into mice followed by IRAK1/4-Inh treatment also improved the survival of mice compared to the control (19). Similarly, mice with established melanoma were divided into 4 treatment groups receiving vehicle control, vinblastine, IRAK1/4-Inh or combined treatment, and the combined treatment group displayed much greater suppression in tumor size and longer survival (21).

In conclusion, we have demonstrated that IRAK1 regulates liver T-ICs and sorafenib resistance in HCC cells through the regulation of the AP-1/AKR1B10 signaling cascade. Targeting T-ICs with an IRAK1/4 inhibitor in combination with sorafenib may be a novel therapeutic strategy for the treatment of HCC patients.

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**Figure 1. Overexpression of IRAK1 and IRAK4 in HCC clinical specimen**. (A) qPCR analysis showed the IRAK1 mRNA expression of T and their corresponding NT tissues in 69 cases of HCC patients. Values are normalized with house-keeping gene HPRT. (B) Waterfall plot ascendingly showed the relative IRAK1 mRNA expression of tumorous tissues in 69 cases of HCC patients. The values were normalized with their NT counterpart. Overexpression was defined as 2-fold upregulation. (***P<0.001, *t* test). (C) The overall survival rate of HCC patients with high IRAK1 over-expression was significantly lower than those patients with low IRAK1 expression (*P*=0.0033, log-rank test). (D) Western blotting analysis showed IRAK1 protein expression of HCC and their corresponding NT tissue in clinical samples. Actin was used as internal reference marker. (E) Representative immunohistochemistry staining on IRAK1 in HCC clinical specimen (#262). Scale bar represents 100μm. (F) Western blotting analysis demonstrated up-regulation of IRAK4 expression in clinical HCC at protein level. Chart representation of the percentage of cases with various IRAK4 expression level.

**Figure 2**. **IRAK1 regulates stem/progenitor characteristics of HCC cells.** (A) By western blot analysis, IRAK1 was found to be highly expressed in Bel7402 and PLC/PRF/5 among other cell lines; while Huh7 and SMMC7721 showed lowest expression. (B) Two different shIRAK1 sequences were used (H2 and H3). Western blotting showed the successful knockdown of IRAK1 in PLC/PRF/5 and Bel7402 cells. Successful overexpression of IRAK1 was also evidenced in Huh7 cells (C) Knockdown of IRAK1 also reduced the size and number of hepatospheres formed by PLC/PRF/5 and Bel7402 cells (*$P$<0.05 and **$P$<0.01, respectively, *t* test), while

overexpression of IRAK1 increased the size and number of hepatospheres formed by Huh7 cells (*$P$<0.05, $t$ test). Scale bar represents 100μm. (D) Knockdown of IRAK1 in Bel7402 and PLC/PRF/5 cell lines suppressed the tumorigenicity when compared with NTC cells. Representative photos showing injection of 5000 and 1000 cells derived from PLC/PRF/5 and Bel7402 were shown. IRAK1 OE showed increased tumorigenicity of Huh7 cells. Representative photo showing injection of 10000 cells were shown. Scale bar represents 1cm. (E) Knockdown of IRAK1 decreased expression of CD24 and CD47 in both PLC/PRF/5 and Bel7402 cells, when compared with NTC cells (*p<0.05, ** p<0.01, $t$ test). (F) Spheroids from shIRAK1 and control cells were stained with CD24 antibody (green) and DAPI (blue) and analyzed by confocal microscopy. Scale bar represent 25μm. Error bars represent the standard deviation (SD) from at least three independent experiments.

**Figure 3. IRAK1 regulates drug resistance of HCC cells through regulation of apoptotic cascade.** (A) shIRAK1 cells derived from PLC/PRF/5 and Bel7402 showed a higher percentage of Annexin V-positive cells in response to doxorubicin and sorafenib treatment at 0.5μg/mL for 24 hours and 8 μM for 48 hrs respectively, when compared with the NTC control cells (*$P$<0.05 and **$P$<0.01, respectively, $t$ test). (B) IRAK1 OE cells showed lower percentage of Annexin V-positive cells in response to doxorubicin and treatment at 1μg/mL for 24 hours and 8 μM for 48 hrs respectively, when compared with the EV control cells (*$P$<0.05, respectively, $t$ test). (C) The mRNA expression level of IRAK1 and IRAK4 was increased upon 8μM sorafenib treatment in PLC/PRF/5 and Bel7402 for 48 hrs respectively. (D) Protein level of IRAK1, phosphorylated IRAK1 and IRAK4 in PLC/PRF/5 was increased upon 8μM sorafenib treatment for 48 hours. (E) Protein level of IRAK1, phosphorylated IRAK1 and IRAK4 in

was upregulated in sorafenib-resistant Bel7402, when compared with the mock cells. (F) Western blotting analysis showed the protein expression level of key members in the apoptotic cascade upon IRAK1 knock-down in PLC/PRF/5 post sorafenib treatment.

**Figure 4. Pharmacological inhibition of IRAK1 by IRAK1/4-Inh suppresses T-IC properties**. (A) Inhibition of IRAK1 by IRAK1/4-Inh significantly suppressed sphere formation of PLC/PRF/5 and Bel7402 cells at dose of 5μM and 10μM (**p<0.01, *** p<0.001, *t* test). Scale bar represents 100μm. (B) Pretreatment with IRAK1/4-Inh inhibited *in vivo* tumor growth in terms of number and size in PLC/PRF/5 and Bel7402 (*P<0.05, *t* test). Scale bar represents 1cm. (C) Pretreatment with IRAK1/4-Inh at 5μM inhibited migration and invasion of PLC/PRF/5 and Bel7402 (*P<0.05, **P<0.01, ***P<0.001, *t* test). (D) Flow cytometry analysis showed the expression of T-IC markers CD24 and CD47 reduced upon IRAK1/4-Inh pretreatment in PLC/PRF/5 and Bel7402 (*P<0.05, **P<0.01, ***P<0.001, *t* test). Error bars represent the standard deviation (SD) from at least three independent experiments.

**Figure 5. IRAK1 regulates liver T-IC properties via regulation of AKR1B10.** (A) Transcriptome sequencing analysis of PLC/PRF/5 NTC and shIRAK1_H3 clones. After excluding genes that did not satisfy parameters including TPM >0.1, Fold change -1< or >1 and P value <0.05, a total of 1171 genes were significantly different in terms of expression level. Among which 646 genes were significantly up-regulated while 525 genes were significantly downregulated. (B) AKR1B10 mRNA expression level upon IRAK1

knock-down in PLC/PRF/5 and Bel7402; and upon IRAK1 overexpression in Huh7. AKR1B10 protein expression level upon IRAK1 knock-down in PLC/PRF/5. (C) The IRAK1 *ORF* was successfully transfected into a AKR1B10 knockdown clone of Huh7 cells. (D) IRAK1 over-expression increased the sizes and number of hepatospheres formed in Huh7, while this effect was abolished upon AKR1B10 suppression (**$P$<0.001, *t* test). Scale bar represents 100μm. (E) Suppression of AKR1B10 induced sorafenib sensitization in IRAK OE transfected Huh7 cells (*$P$<0.05, **$P$<0.01 respectively, *t* test). (F) Suppression of AKR1B10 decreased expression of liver T-IC markers induced in IRAK OE transfectants (*P<0.05, **P<0.01, ***P<0.001, *t* test).

**Figure 6. IRAK1/4-Inh in combination with sorafenib for HCC treatment *in vivo*.** (A) Mice with established subcutaneous HCC tumors of similar size were randomly divided into four groups and were given vehicle control, 5mg/kg IRAK1/4-Inh via intraperitoneal injection, 10mg/kg sorafenib via oral gavage, or combined treatment daily. (Left panel: PLC/PRF/5; right panel: PDTX#1). The change in tumor volume during the treatment period transfectants (*P<0.05, **P<0.01, *t* test). (B) The tumor at the end of the treatment, scale bar represents 1cm. (C) Graph showing the weight of tumors at the end of the treatment, each dot represents one single tumor (*P<0.05, **P<0.01, ***P<0.001, *t* test) (N=6 for PLC/PRF/5 and N=4 for PDTX#1). (D) The change in the body weight of mice during the treatment period.