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Original Articles

KIAA1199 promotes sorafenib tolerance and the metastasis of hepatocellular carcinoma by activating the EGF/EGFR-dependent epithelial-mesenchymal transition program

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ABSTRACT

Patients with advanced hepatocellular carcinoma (HCC) will almost always develop acquired tolerance after sorafenib therapy, and the molecular mechanism of sorafenib tolerance remains poorly characterized. Here, using our established sorafenib-resistant HCC cell and xenograft models, we identified a novel gene, KIAA1199, which was markedly elevated among the differentially expressed genes involved in sorafenib tolerance. Moreover, elevated expression of KIAA1199 was positively correlated with a high risk of recurrence and metastasis and advanced TNM stage in HCC patients. Functionally, loss- and gain-of-function studies showed that KIAA1199 promoted the migration, invasion, and metastasis of sorafenib-resistant HCC cells. Mechanistically, KIAA1199 is required for EGF-induced epithelial-mesenchymal transition (EMT) in sorafenib-resistant HCC cells by aiding in EGFR phosphorylation. In summary, our data uncover KIAA1199 as a novel sorafenib-tolerant promoting gene that plays an indispensable role in maintaining sorafenib-resistant HCC cell metastasis.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most prevalent and aggressive neoplasms around the world and the second leading cause of cancer death in developing countries and the sixth leading cause in developed countries [1]. So far, the prognosis of patients with HCC remains dismal, and its overall 5-year survival is less than 15 % [2]. In general, a late diagnosis, high heterogeneous background and HCC cells tolerant to conventional chemotherapeutic agents are considered the principle reasons for the high mortality rates of HCC [3]. However, the molecular and genetic events underlying drug tolerance and tumor metastasis have not been elucidated.

Currently, sorafenib is the first targeted therapy for advanced HCC [4]. Sorafenib mainly blocks tumor cell proliferation by targeting Raf/MEK/ERK signaling and exerts an antiangiogenic effect by VEGFR 2/3 as well as PDGFR-β tyrosine kinases [5]. Although previous data have shown that the clinical outcome of sorafenib for advanced HCC is notable, unfortunately, long-term exposure to sorafenib often leads to tumor cells acquiring drug tolerance [6–8]. To date, the precise molecular mechanism of sorafenib tolerance remains poorly characterized. Thus, the identification of critical targets for the modulation of HCC drug tolerance and migration is valuable for the prevention of tumor cell dissemination and metastasis.

Acquired sorafenib resistance develops in the background of the tumor-host interaction. To identify novel and key targets involved in sorafenib tolerance, we established several sorafenib-resistant HCC cell and xenograft models. The HCC xenograft model more closely resembles the clinical features of HCC patients and retains the characteristics significantly associated with drug response [9].

In our current study, we found that the expression of KIAA1199 was significantly elevated in our previously established sorafenib-resistant HCC *in vitro* cell model and in an *in vivo* xenograft model. Furthermore, we found that KIAA1199 promotes HCC cell resistance to sorafenib by enhancing HCC cell migration and invasion. In addition, KIAA1199 promotes HCC cell resistance to sorafenib and cell migration through modulating the EGF-induced epithelial-mesenchymal transition (EMT)

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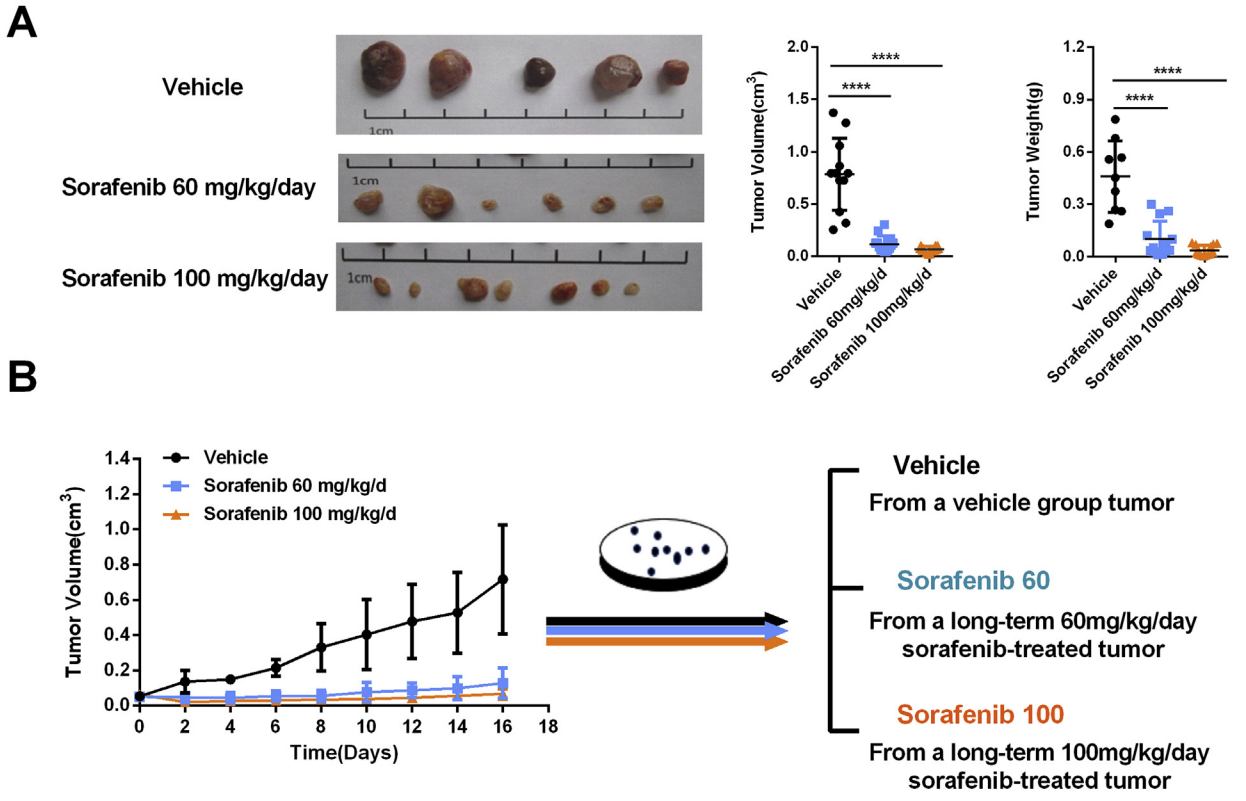

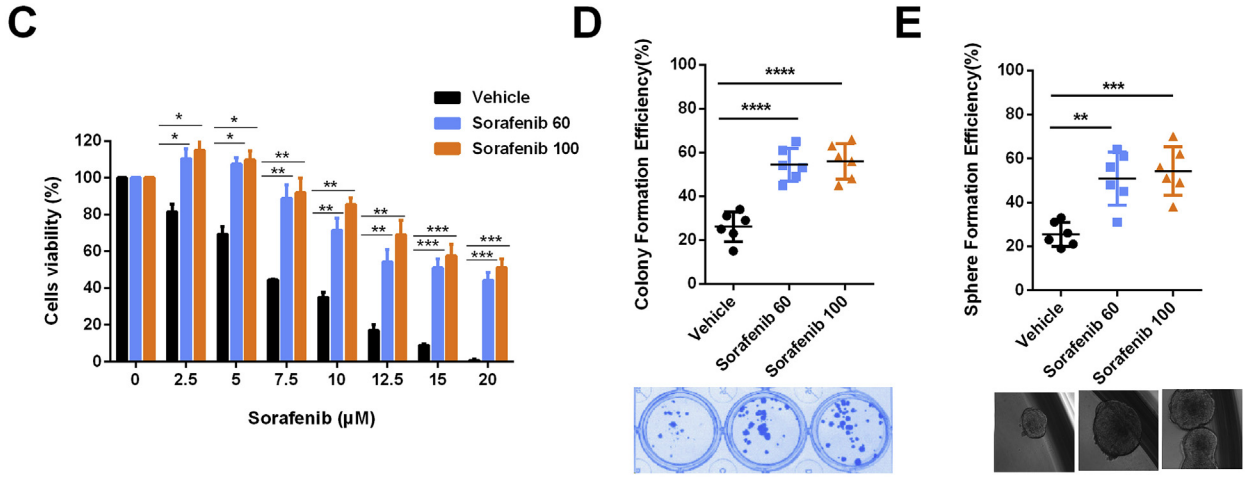

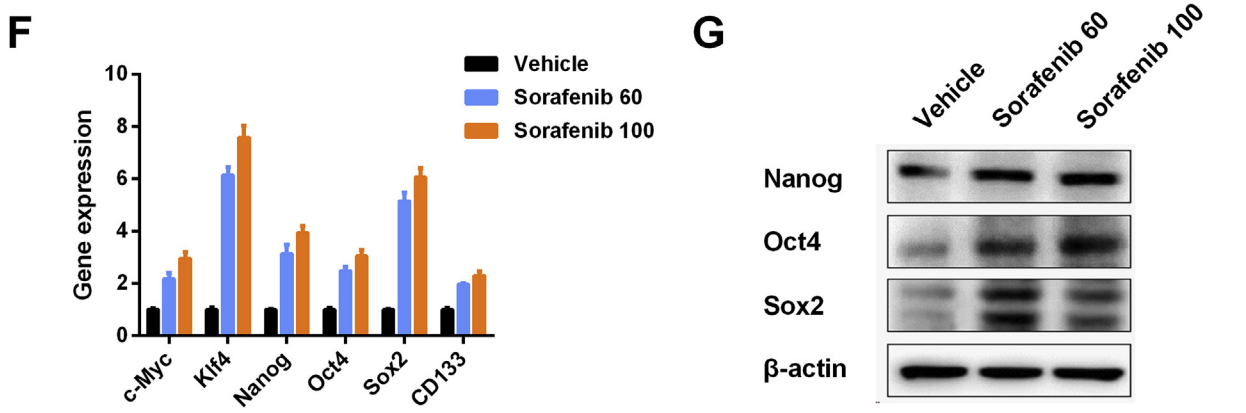

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Fig. 1. Establishment and confirmation of sorafenib-resistant HCC xenografts *in vivo*. (A). NOD/SCID mice were randomly divided into three groups: (i) vehicle; (ii) 60 mg/kg/day sorafenib; and (iii) 100 mg/kg/day sorafenib. HCC xenografts were orally gavaged with 60 mg/kg or 100 mg/kg of sorafenib daily, and tumor morphology, tumor volume and tumor weight were compared by one-way ANOVA (****, $P < 0.0001$). (B). Cells were isolated from the vehicle, 60 mg/kg/day sorafenib and 100 mg/kg/day sorafenib groups and treated with sorafenib at the indicated doses for 48 h. (C). Cell viability was determined by the CCK-8 assay. (D, E). The self-renewal ability of sorafenib-resistant HCC cells was examined by colony formation efficiency (D) and sphere formation efficiency (E) assays. One-way ANOVA was used to analyze the differences among at least three groups (**, $P < 0.01$; ***, $P < 0.001$, ****, $P < 0.0001$). (F, G). The expression of HCC CSC markers was detected by RT-PCR (F) and western blotting (G).

process. Our study suggests that KIAA1199 is a novel candidate target for sorafenib tolerance and metastasis in HCC.

2. Materials and methods

2.1. Cell culture and reagents

Human HCC cell lines PLC/PRF/5 and HepG2 were purchased from the ATCC (https://www.atcc.org/). Huh7 and HLE cells were obtained from the cell bank at the Chinese Academy of Sciences (Shanghai, China, http://www.cellbank.org.cn/). 293T cells were purchased from the ATCC for lentivirus production. T421, T1115 and T1224 primary HCC cells were previously established in our laboratory. Cells were cultured according to the providers' instructions for less than 2 months and confirmed by mycoplasma detection before experimental application.

Sorafenib (Nexavar) was purchased from Cell Signaling Technology (Sorafenib, #8705). Recombinant human EGF was purchased from R&D Systems (Cat. # AB-236-NA). The EGFR inhibitor erlotinib was purchased from Selleckchem.com (Houston, TX, USA, Cat. #S7786).

2.2. HCC patient specimens

HCC tumor tissues and matched peritumoral tissue specimens were collected from the Department of Hepatobiliary Surgery, Southwest Hospital of Amy Medical University from 2009 to 2012. Fresh HCC tumor tissues were obtained from patients with informed consent and complied with the protocols of the Institutional Review Board of Southwest Hospital, Amy Medical University. Furthermore, a panel of 45 fresh frozen HCC tissues and the corresponding peritumoral tissues were examined in the current study. Moreover, sorafenib-resistant HCC tissues (n = 10) and sorafenib-sensitive HCC tissues (n = 4) were also included in our study. Other clinical parameters, including age, gender, tumor size, tumor differentiation and location, were also collected. A portion of patients received sorafenib therapy prior to surgery.

2.3. Sorafenib-resistant HCC in vitro cell model

The establishment of sorafenib-resistant HCC cell models was conducted according to our previous report [10].

2.4. Sorafenib-resistant HCC in vivo xenograft model

Twenty female NOD/SCID mice (4 – 6 weeks old) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. All animals received humane care that complied with the Guide for the Care and Use of Laboratory Animals prepared by Amy Medical University. Huh7 cells were counted and then resuspended in serum-free medium, and 1×10^6 cells were subcutaneously injected into female NOD/SCID mice. Tumor formation efficiency was measured regularly by palpation of injection sites, and tumor volume was calculated. When the tumor reached a size of approximately 0.5 cm in diameter, twenty NOD/SCID mice were randomly divided into three groups: (i) Control (vehicle group, n = 6); (ii) 60 mg/kg/day sorafenib (n = 7); and (iii) 100 mg/kg/day sorafenib (n = 7). For *in vivo* gavage experiments, sorafenib was dissolved in Cremophor EL/95 % ethanol (1:1) solution, and solutions of 60 mg/kg or 100 mg/kg sorafenib were prepared for oral gavage. All animals were administered sorafenib via gavage for at least 2 weeks, and body weight and tumor volume were measured. Tumor volume was calculated by the following formula: length × width^2/2.

2.5. mRNA microarray analysis

Total RNAs were isolated from sorafenib-resistant HCC cells, xenograft tissues and the corresponding parental counterparts. RNA concentration and integrity were qualified by a NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Foster City, CA), respectively. The Affymetrix Human Genome U133 Plus 2.0 array platform was applied to explore the gene expression profiles of ten samples (CapitalBio Corporation, Beijing, China). Data were analyzed by Quant Array R software and SAM2.0 software. Genes that were up- or downregulated 1.5-fold were considered differentially expressed.

2.6. In vitro migration and invasion assays

In vitro migration and invasion assays were performed as described in Supplementary Materials and Methods.

2.7. Cell proliferation assay and in vitro cytotoxicity assay

Cell proliferation and cytotoxicity were examined by the CCK-8 assay, and detailed information is described in Supplementary Materials and Methods.

2.8. Quantitative RT-PCR analysis for mRNA expression

Total RNA was extracted from HCC cells and/or fresh frozen tissues using Trizol reagent, and the mRNA expression of genes was determined by qRT-PCR. Detailed information is described in Supplementary Materials and Methods.

2.9. Western blot analysis

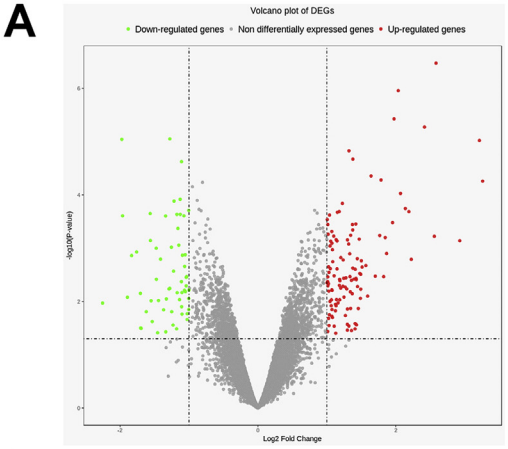
Whole-cell lysates and tissue specimens were extracted, and the expression of KIAA1199 and the indicated markers was examined by western blotting. Detailed information is described in Supplementary Materials and Methods.

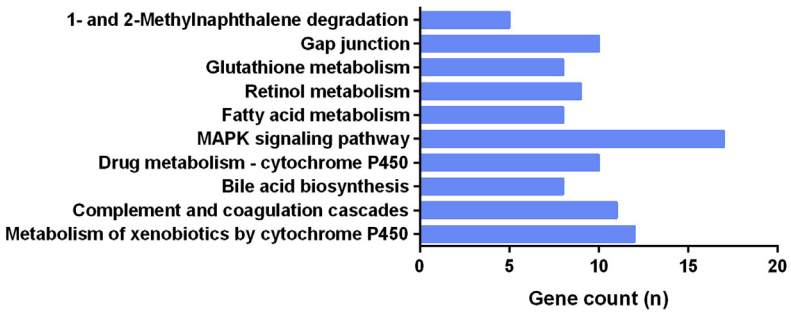
2.10. Sphere formation efficiency and cloning formation efficiency assays

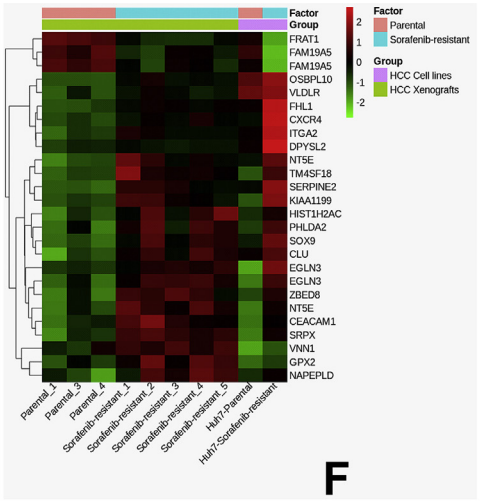
Cell sphere formation efficiency (SFE) and cloning formation efficiency (CFE) assays were performed as described in Supplementary Materials and Methods.

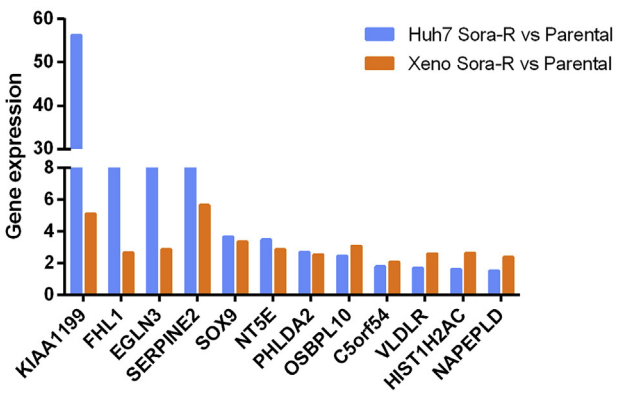
2.11. In vivo metastatic assay

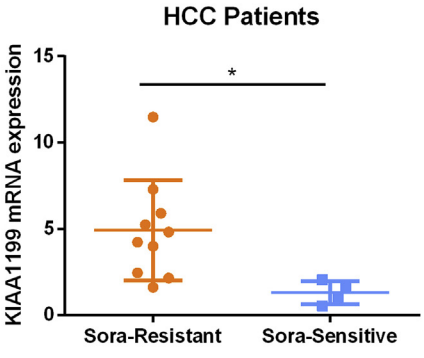
4- to 6-week-old female NOD/SCID mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. Thirty-six female NOD/SCID mice were randomly divided into four groups: (i) parental; (ii) sorafenib-resistant; (iii) sorafenib-resistant HCC cells infected with Scramble; and (iv) sorafenib-resistant HCC cells with depleted KIAA1199 expression. NOD/SCID mice were anesthetized with

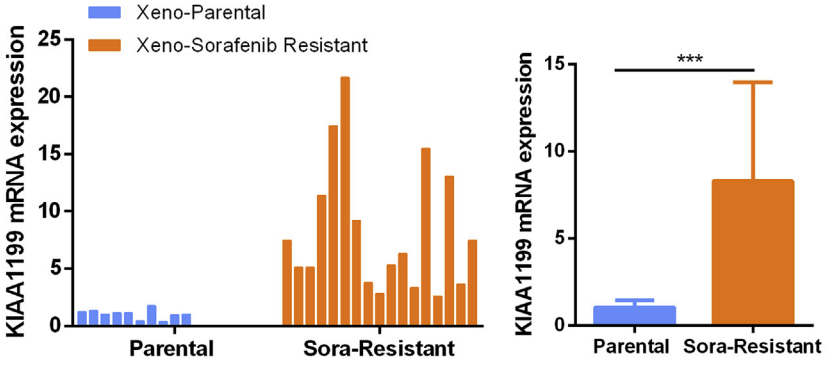


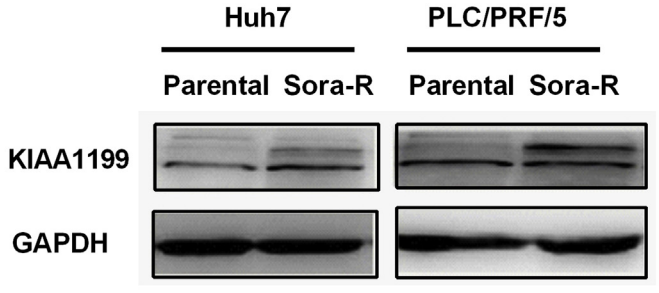












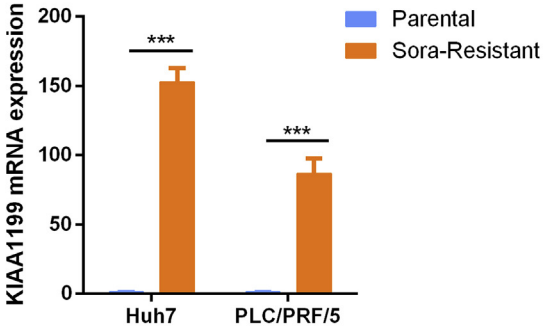


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Fig. 2. KIAA1199 expression is elevated in sorafenib-resistant HCC cells and xenografts. (A). Volcano plot of differentially expressed genes (DEGs) between sorafenib-resistant HCC cells and the parental counterparts. (B). KEGG pathway analysis of DEGs. (C). Hierarchical clustering of quantitative gene expression profiling for sorafenib-resistant HCC cells and xenografts versus the corresponding parental cells. (D). Top 12 upregulated genes in sorafenib-resistant HCC cell and xenograft models. (E). KIAA1199 expression was upregulated in sorafenib-resistant HCC patients compared with sorafenib-sensitive HCC patients (*, $P < 0.05$). (F, G and H). KIAA1199 expression was confirmed by RT-PCR and western blotting in sorafenib-resistant HCC xenografts (F) and sorafenib-resistant HCC cells (G, H) (***, $P < 0.001$).

isoflurane, and then cells (1×10^6 cells/0.05 ml/site) suspended in 50 % Matrigel (Corning, 356237) of saline were injected into the spleen. Metastatic lesions were monitored after the injection of D-luciferin (GoldBio, 6707.072817A) by IVIS Spectrum and analyzed with living image software (Lumina III, PerkinElmer, Waltham, MA). Liver metastases were counted and enumerated after 2 – 3 weeks under a fluorescence stereoscope. All animal experiments were performed after approval by the Institute Animal Care and Use Committee of Amy Medical University.

2.12. Hematoxylin & eosin (H&E) staining

Hematoxylin & eosin (H&E) staining was performed as described in Supplementary Materials and Methods.

2.13. Statistical analysis

The statistical analysis of all data was performed using SPSS 25.0 statistic software (SPSS Inc., Chicago, USA). The association between clinicopathologic parameters and KIAA1199 expression was determined with χ^2 tests. Survival curves were calculated by the Kaplan-Meier method. Student's t-test and/or one-way ANOVA was used to determine statistical significance between groups. The correlation between the expression of KIAA1199 and the migration potential of HCC cells was analyzed by the Pearson correlation coefficient. For all tests, data are presented as the means ± SD. $P < 0.05$ was regarded as statistically significant and is indicated by *; **$P < 0.01$; and ***$P < 0.001$.

3. Results

3.1. In vivo establishment of sorafenib-resistant HCC xenograft models

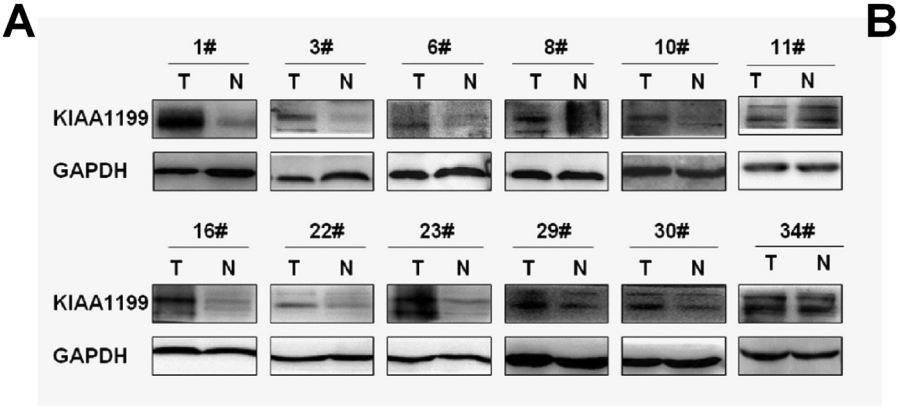
First, we developed several sorafenib-resistant *in vivo* xenograft models of HCC based on our previously established sorafenib-resistant HCC *in vitro* cell models [10]. Of note, we systematically assessed sorafenib-resistant HCC cells *in vitro* before use in *in vivo* studies. Expectedly, sorafenib-resistant HCC cells possess slow growth characteristics (Supplementary Fig. S1A) and require higher doses of sorafenib for partial inhibition *in vitro* (Supplementary Fig. S1B). More importantly, in sorafenib-resistant HCC xenograft models, our results indicated that tumor volume was significantly smaller in the sorafenib-resistant group than the control counterparts during the initial days. Furthermore, xenograft tumor volume did not further decrease following long-term exposure to sorafenib, suggesting that the HCC xenograft models are resistant to sorafenib (Fig. 1A and B). To further validate the successful establishment of sorafenib-resistant HCC xenograft models, the effect of sorafenib on HCC cell viability was evaluated. Our data showed that sorafenib 60 and sorafenib 100 tumor cells were more resistant to sorafenib than vehicle tumors (Fig. 1B and C). More importantly, we also found that sorafenib 60 and sorafenib 100 tumor cells possessed higher self-renewal ability (Fig. 1D and E). To obtain further evidence for the self-renewal ability of sorafenib-resistant HCC xenograft models, the expression of several known HCC CSC markers was examined (Fig. 1F and G). Altogether, our data demonstrated the successful establishment of sorafenib-resistant HCC xenograft models suitable for further investigation of sorafenib resistance.

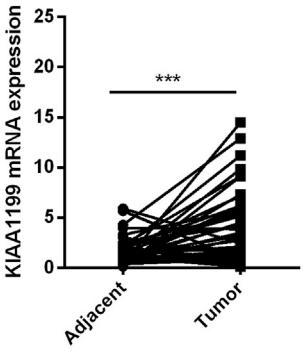
3.2. KIAA1199 is highly expressed in sorafenib-resistant HCC cell and xenograft models

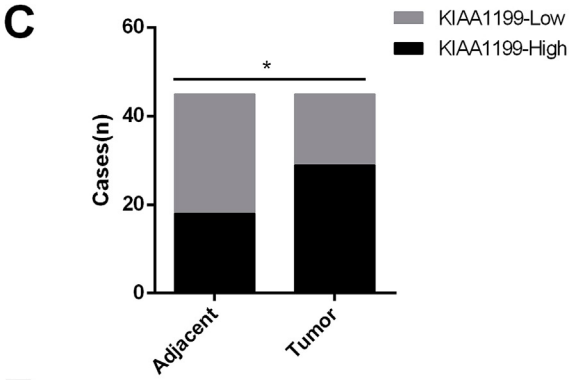
To better identify the key genes involved in sorafenib tolerance and to uncover potential targets for HCC therapy, whole human genome microarray analysis was used to screen differentially expressed genes in sorafenib-resistant HCC cells, xenograft models and the corresponding counterparts. The results revealed that the pattern of gene expression in sorafenib-resistant HCC cells was significantly different from that in the parental counterparts. Our data revealed 2776 and 153 differentially expressed genes in sorafenib-resistant HCC cells and xenografts versus their corresponding counterparts, respectively (Fig. 2A). Moreover, a volcano plot and KEGG pathway analysis were used to further analyze the differentially expressed genes (DEGs) involved in the signaling pathway (Fig. 2A and B and Supplementary Table S1). Fig. 2C displays 25 DEGs with 1.5-fold or more differential expression in sorafenib-resistant HCC cells, xenografts and the corresponding counterparts. The top 12 genes with the highest expression are shown in Fig. 2D. Among these differentially expressed genes, the most intensively induced target was KIAA1199 (56.32-fold change in sorafenib-resistant HCC cells vs parental cells; 5.12-fold change in sorafenib-resistant HCC xenografts vs the corresponding counterparts, ***$P < 0.001$) (Fig. 2D). On the basis of the microarray analysis, we selected KIAA1199 for further investigation. First, we evaluated KIAA1199 expression in sorafenib-resistant (n = 10) and sorafenib-sensitive (n = 4) HCC patients. Our data revealed that the average expression of KIAA1199 was 4.92 ± 2.90 in Sora-resistant HCC patients, which was significantly higher than that of Sora-sensitive HCC patients (1.75 ± 0.95, $P = 0.0226$) (Fig. 2E). Subsequently, we confirmed KIAA1199 expression in sorafenib-resistant HCC xenograft (Fig. 2F) and cell (Fig. 2G and H) models. More specifically, for the first time, we systematically noticed that KIAA1199 expression was obviously upregulated in sorafenib-resistant HCC cells, xenografts and patients with HCC, implying that KIAA1199 might be a novel potential sorafenib tolerant promoting gene in HCC.

3.3. KIAA1199 is upregulated in clinical HCC samples, and elevated KIAA1199 is positively correlated with a high risk of recurrence and metastasis in HCC patients

To further unravel the role of KIAA1199 in HCC, we investigated KIAA1199 expression in primary HCC tumor tissues and the corresponding adjacent noncancerous tissues. Of note, we demonstrated that KIAA1199 protein expression was obviously higher in HCC tumor tissues than in matched tumor-adjacent tissues ($n = 12$) (Fig. 3A). Furthermore, our data also indicated that KIAA1199 mRNA expression was 2.41-fold higher on average in primary HCC tumor tissues than in matched adjacent tissues ($n = 45$) (Fig. 3B, $P = 0.0002$). Accordingly, these data show that KIAA1199 expression is significantly increased in HCC tumor tissues compared with paired adjacent tissues (Fig. 3C and D, $P = 0.020$). To further investigate the clinical significance of KIAA1199 expression in HCC patients, three publicly available GEO databases (GSE52018, GSE14520 and GSE17856) were analyzed for validation. Specimens were classified into two groups based on KIAA1199 expression, in which high KIAA1199 expression was positively correlated with patient recurrence (Fig. 3E) and inversely associated with patient recurrence-free survival (Fig. 3F). Furthermore, we next showed that elevated KIAA1199 was an indicator for predicting the high metastatic potential of HCC (Fig. 3G and H, $P = 0.0015$). In



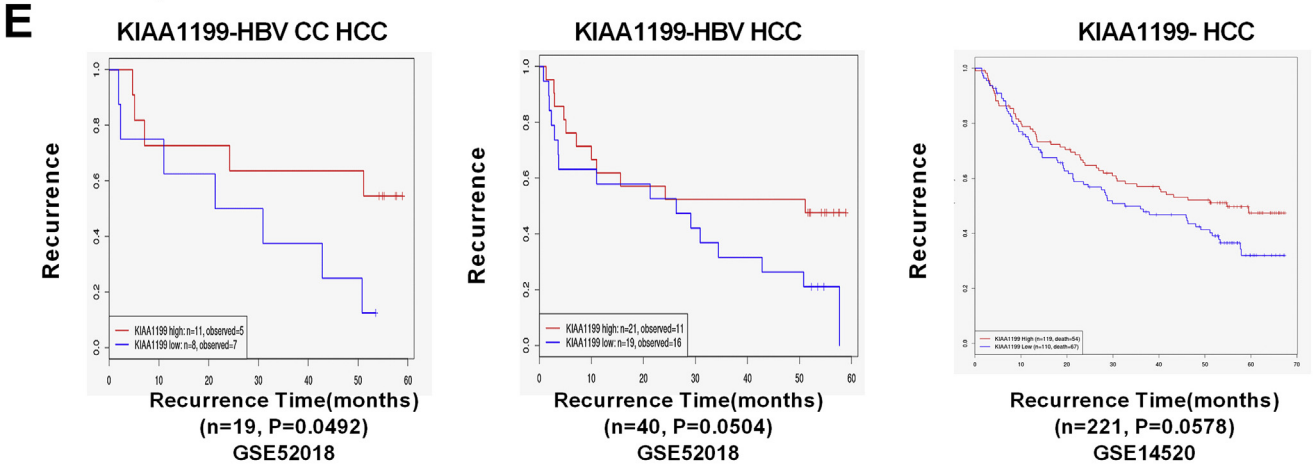


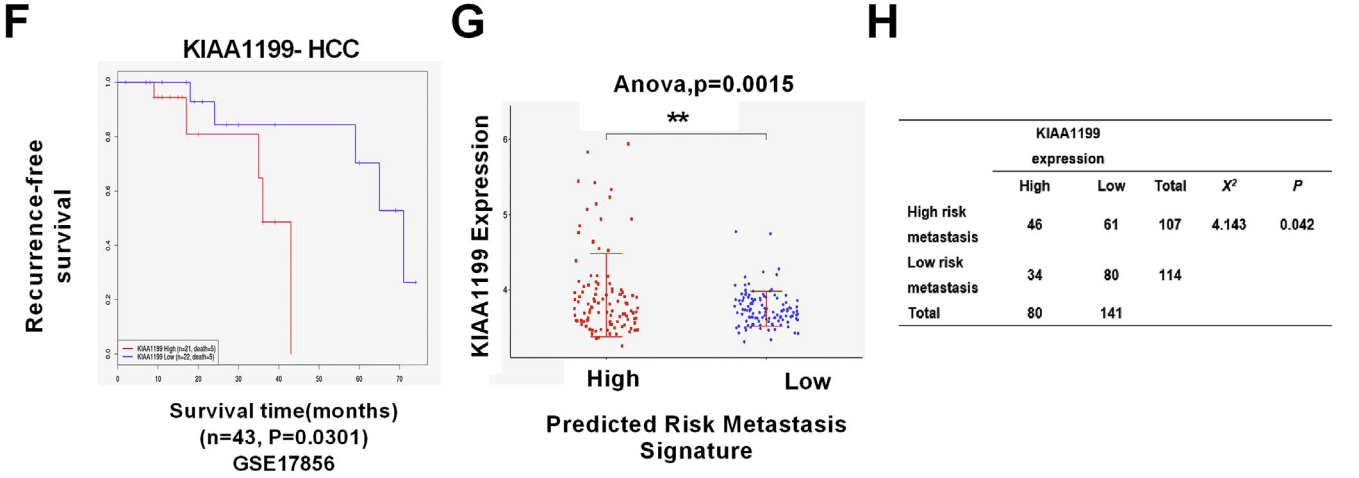


D

Index	KIAA1199 expression		Total	X^2	*P*
	High	Low			
Adjacent Tissues	18	27	45	5.389	0.020
HCC Tissues	29	16	45		
Total	47	43			

P: Chi-square test. HCC: Hepatocellular carcinoma





H

	KIAA1199 expression				
	High	Low	Total	X^2	*P*
High risk metastasis	46	61	107	4.143	0.042
Low risk metastasis	34	80	114		
Total	80	141			

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Fig. 3. KIAA1199 is upregulated in clinical HCC samples, and high expression of KIAA1199 is positively correlated with a high risk of recurrence and metastasis in HCC patients. (A). Western blot analysis of the expression of KIAA1199 in HCC tumor tissues (T) and the corresponding non-tumor (N) tissues. GAPDH served as the internal reference (n = 12). (B). KIAA1199 mRNA expression is upregulated in HCC tumor tissues (n = 45) compared with the corresponding adjacent tissues (n = 45). GAPDH served as an internal reference (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). (C, D). Statistical analysis showed elevated KIAA1199 expression in HCC tumor tissues compared with adjacent tissues. (E). Association between patient recurrence probability and KIAA1199 expression in three databases of patients with HCC. (F). KIAA1199 expression was negatively correlated with the recurrence-free survival of patients with HCC. (G, H). KIAA1199 expression was positively associated with the metastatic potential of patients with HCC (**, $P < 0.01$).

Table 1
Correlation between the expression of KIAA1199 and clinicopathological characteristics in hepatocellular carcinoma.

Characteristic	Cases (n)	High KIAA1199 expression	Low KIAA1199 expression	X^2	*P* value
Gender					
Male	191	72	119	1.366	0.243
Female	30	8	22		
Age (years)					
< 60	178	69	109	2.606	0.106
≥60	43	11	32		
Tumor size					
≤5	141	53	88	0.326	0.568
> 5	80	27	53		
TNM Stage					
I	95	37	58	12.173	0.002*
II	77	25	52		
III	49	18	31		
Cirrhosis					
Yes	203	73	130	0.061	0.804
No	18	7	11		
Metastasis					
Low risk	114	34	80	4.413	0.042*
High risk	107	46	61		
Recurrence months					
< 50	131	45	86	0.476	0.490
≥50	90	35	55		
Survival months					
< 36	85	34	51	0.864	0.353
≥36	136	46	90		
ALT (U/L)					
> 50	91	31	60	0.305	0.581
≤50	130	49	81		
Multinodular					
Yes	45	15	30	0.201	0.654
No	176	65	111		
AFP (ng/mL)					
> 300	99	39	60	0.620	0.431
≤300	121	41	80		

addition, we further analyzed the GSE14520 database to investigate the correlation between KIAA1199 expression and the clinicopathological features of HCC. The data showed that enhanced KIAA1199 expression was positively correlated with metastatic probability ($P = 0.042$) and TNM stage ($P = 0.002$) (Table 1). Accordingly, our data suggested that KIAA1199 was obviously enhanced in HCC patients and might promote progression and metastasis. More importantly, our study further demonstrated a novel association between KIAA1199 overexpression and human HCC metastatic probability.

3.4. KIAA1199 is elevated in sorafenib-resistant HCC cells, which exhibit epithelial-mesenchymal transition and enhanced migration and invasion, and promotes tumor metastasis in vivo

The correlation between KIAA1199 expression and cancer metastasis in HCC clinical specimens prompted us to further investigate whether KIAA1199 is a crucial indicator or functional participator in HCC drug tolerance and metastasis. To confirm this speculation, we first examined KIAA1199 expression in three primary HCC cells and four HCC cell lines (Fig. 4A and B). Intriguingly, KIAA1199 was dramatically elevated in T1224 primary cells, which were isolated from HCC patient with vein tumor thrombus and exhibit stronger metastatic potential. More importantly, our data also revealed that endogenous KIAA1199 expression in HCC cells was positively correlated with migration ability (Fig. 4C, $P < 0.0001$). Furthermore, we observed that sorafenib-resistant HCC cells were remarkably more migratory and invasive than the corresponding parental counterparts (Fig. 4D). To explore whether the acquisition of sorafenib resistance could induce specific molecular changes consistent with EMT, we evaluated the expression changes in certain classical genes in sorafenib-resistant HCC cells following sorafenib stimulation. Of note, our data demonstrated that the expression of KIAA1199 and the mesenchymal marker Vimentin was markedly upregulated, and the epithelial markers E-cadherin, ZO-1 and Occludin were significantly downregulated in sorafenib-resistant HCC cells compared with the corresponding counterparts (Fig. 4E and F). Previous studies have revealed that enhanced cell invasion could promote metastasis [11]. To further investigate the association between our *in vitro* findings and *in vivo* settings, a spleen-liver metastasis model was used. Our data indicated that the number of metastatic tumor nodules was increased in NOD/SCID mice injected with PLC/PRF/5 Sora-R cells compared to the parental counterparts, implying that forced expression of KIAA1199 in sorafenib-resistant HCC cells promotes the formation of metastatic foci (Fig. 4G and H). Moreover, we further determined that the numbers of liver metastasis foci were much higher in PLC/PRF/5 Sora-R mouse livers than in the parental counterparts (Fig. 4I and J). Altogether, these results suggest that elevated KIAA1199 expression promotes sorafenib-resistant HCC cell metastasis *in vivo*.

3.5. Depletion of KIAA1199 expression attenuates sorafenib-tolerant HCC cell migration, invasion and cancer metastasis

To explore the function of KIAA1199 in the development of sorafenib resistance, we detected phenotypic changes in sorafenib-resistant HCC cells with depleted KIAA1199 expression. Our data indicated that KIAA1199 expression was significantly dampened in the KIAA1199 depletion group compared with the control and Scramble groups (Fig. 5A and B). Intriguingly, the depletion of KIAA1199 notably repressed parental cell growth *in vitro* and had no effect on sorafenib-resistant cells (Supplementary Figs. S2A and B). Moreover, we also noted that the inhibition of KIAA1199 expression had no obvious effect on cell viability (Supplementary Figs. S3A and B), cell cycle (Supplementary Figs. S4A and B) or cell apoptosis ratio (Supplementary Figs. S5A and B) in sorafenib-resistant HCC cells. However, the migratory and invasive abilities of sorafenib-resistant HCC cells were evidently impaired upon KIAA1199 depletion (Fig. 5C). More importantly, the repressed migratory and invasive potentials of sorafenib-resistant cells correlated with EMT phenotypic changes, as indicated by increased epithelial markers and decreased mesenchymal markers (Fig. 5D and E). In addition, migration potential was restored by rescuing KIAA1199 expression via the exp-KIAA1199 lentivirus in silenced sorafenib-resistant HCC cells (Fig. 5F, G and H). Subsequently, we disrupted KIAA1199 expression and investigated the changes in metastatic potential in sorafenib-resistant HCC cells. Our data indicated that the mean fluorescence intensity (MFI) was remarkably reduced in NOD/SCID mice bearing KIAA1199-depleted cells compared with Scramble mice (Fig. 5I). Furthermore, the spleen orthotopic tumor did not differ between the sh-KIAA1199 and Scramble groups (Fig. 5J). However, the percentage of tumor micrometastases was higher in the livers of seven mice from the Scramble group (100 %) than in two of

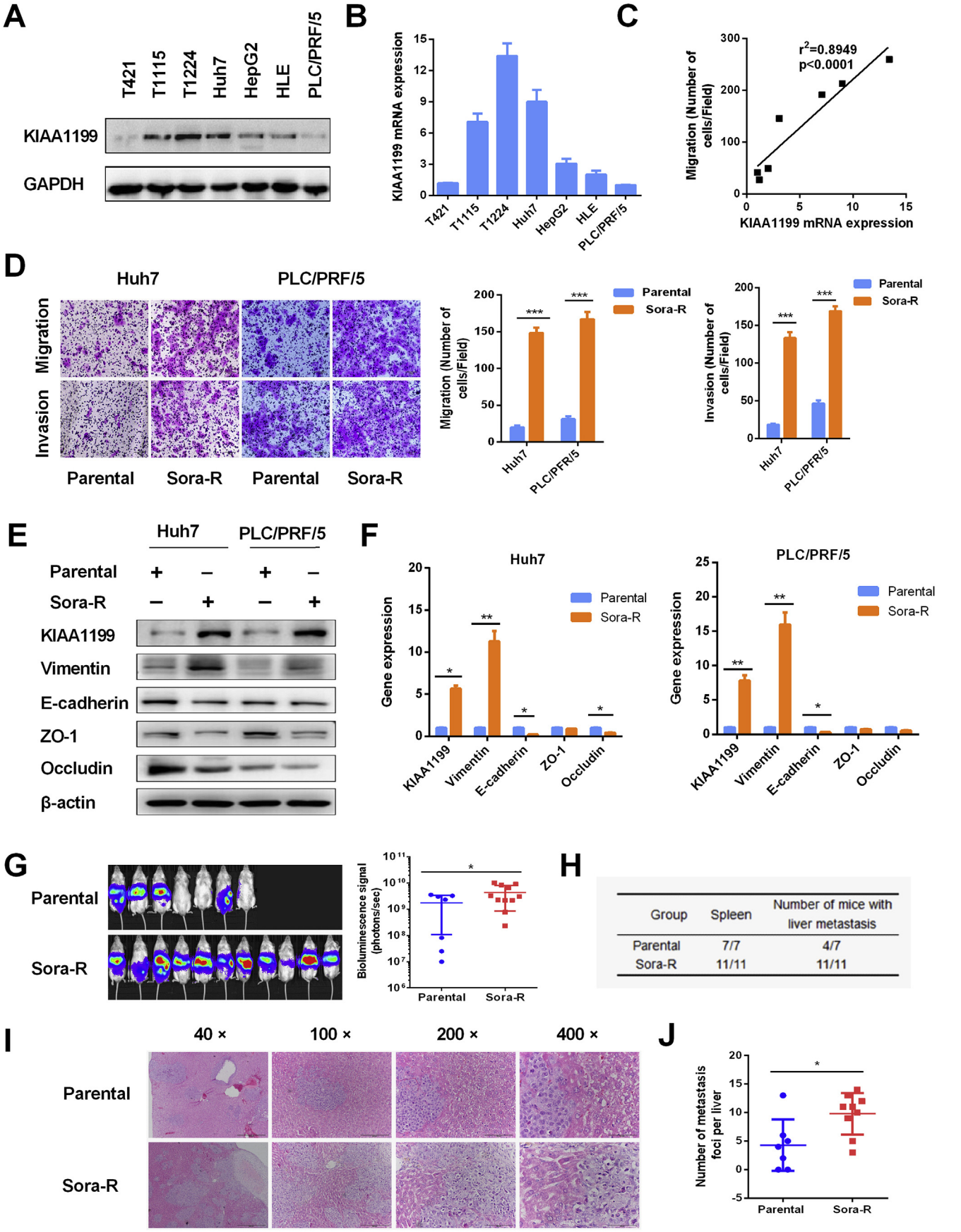

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Fig. 4. KIAA1199 promotes sorafenib-resistant HCC cell migration and invasion *in vitro* and metastasis *in vivo*. (A, B). KIAA1199 expression in HCC cell lines (Huh7, HepG2, HLE and PLC/PRF/5) and HCC primary cells (T421, T1115 and T1224) was examined by western blotting (A) and RT-PCR (B). (C). The correlation between endogenous KIAA1199 expression and the migration abilities of HCC cells was analyzed by the Pearson correlation assay. (D). Transwell assays indicated the migration and invasion abilities of sorafenib-resistant cells compared with the parental counterparts. Scale bar = 100 μm (***, $P < 0.001$). (E, F). The expression of EMT markers in sorafenib-resistant PLC/PRF/5 and Huh7 cells was detected by western blotting (E) and RT-PCR (F) (*, $P < 0.05$; **, $P < 0.01$). (G). A total of 1×10^6 PLC/PRF/5 Sora-R cells and PLC/PRF/5 cells were implanted into the spleens of NOD/SCID mice. Tumors were monitored every week using an *in vivo* imaging system. The imaging and quantification of mean fluorescence intensity are shown (*, $P < 0.05$). (H). Statistical analysis of the number of mice with liver metastasis. (I). Liver metastatic foci were detected by HE staining, and images were taken at the indicated magnifications and times. (J). Statistical analysis of the number of metastatic foci per liver (*, $P < 0.05$).

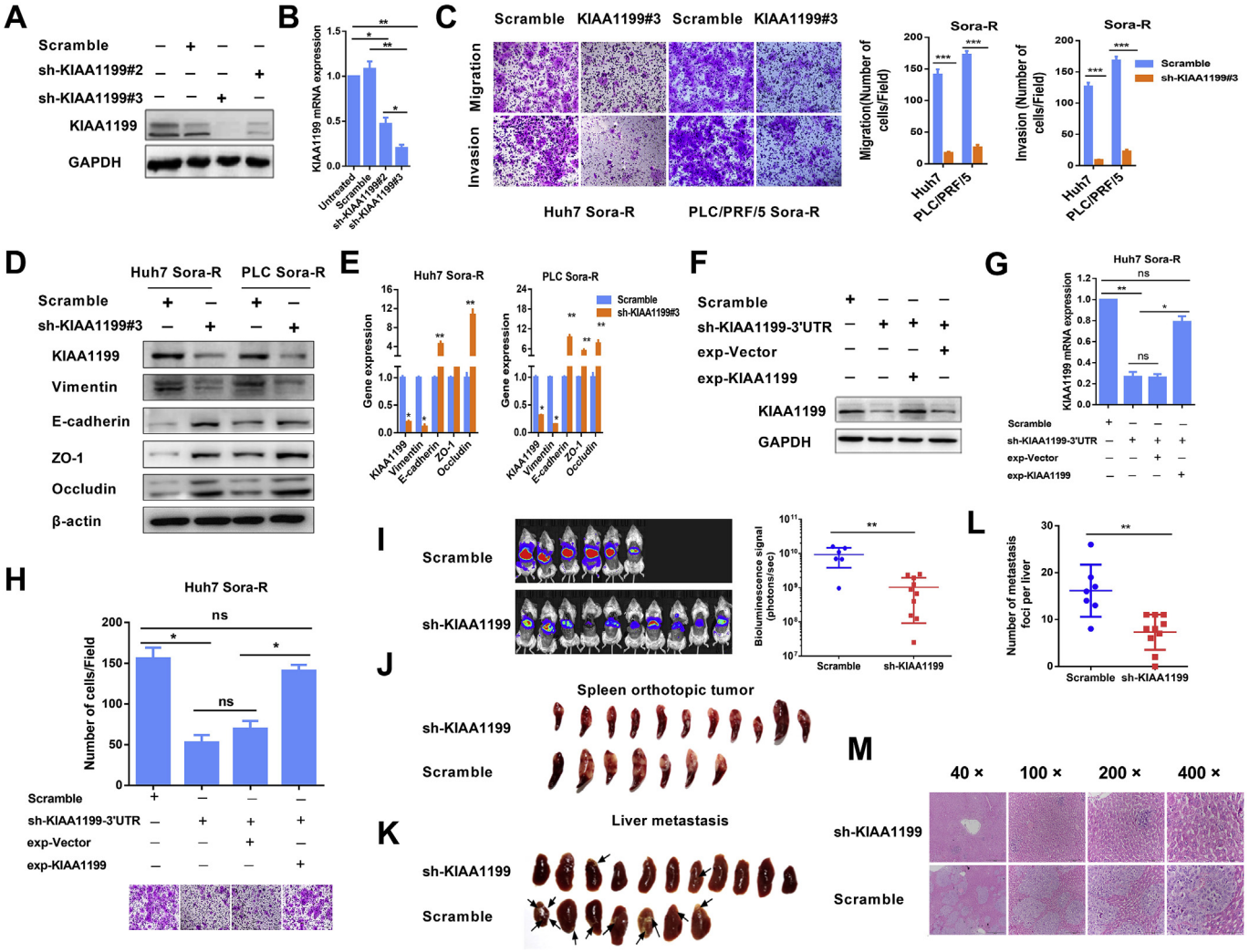

Fig. 5. Depletion of KIAA1199 expression attenuates sorafenib-tolerant HCC cell invasion and metastasis. (A, B). KIAA1199 expression in sorafenib-resistant HCC cells after stable KIAA1199 knockdown was assessed by western blotting (A) and RT-PCR (B). (C). Transwell assays show the migration and invasion abilities of KIAA1199-depleted sorafenib-resistant HCC cells. Scale bar = 100 μm (***, $P < 0.001$). (D, E). The expression of EMT markers in KIAA1199-depleted sorafenib-resistant PLC/PRF/5 and Huh7 cells was detected by western blotting (D) and RT-PCR (E) (*, $P < 0.05$; **, $P < 0.01$). (F–H). KIAA1199 expression was first silenced by the shKIAA1199-3′ UTR-expressing lentivirus in sorafenib-resistant HCC cells, and these cells were then infected with the KIAA1199 overexpression lentivirus or the corresponding control vector. KIAA1199 expression and migration abilities were evaluated. Scale bar = 100 μm (*, $P < 0.05$; **, $P < 0.01$). (I). A total of 1×10^6 KIAA1199-depleted sorafenib-resistant HCC cells (sh-KIAA1199) and the corresponding counterparts (Scramble) were implanted into the spleens of NOD/SCID mice. Metastatic foci were examined by an *in vivo* imaging system, and signal intensity was quantified (**, $P < 0.01$). (J, K). Gross morphology of the spleen (J) and liver (K) implanted with sh-KIAA1199 or Scramble cells is shown, and the arrow indicates the metastatic foci of the livers. (L). Statistical analysis of the number of metastatic foci in mice from the Scramble and sh-KIAA1199 groups (**, $P < 0.01$). (M). Liver metastatic foci were detected by HE staining, and images were taken at the indicated magnifications and times.

eleven mice bearing KIAA1199-depleted cells (18.2 %) (Fig. 5K). Moreover, we further determined that the numbers of liver metastasis foci were much higher in the Scramble group than in mice bearing KIAA1199-depleted cells (Fig. 5L and M). Taken together, our data establish a link between KIAA1199 expression, sorafenib tolerance and human HCC metastasis.

3.6. KIAA1199 promotes HCC cell resistance to sorafenib by activating the EGF/EGFR-dependent EMT program

Increasing knowledge has linked cell motile potential with the EMT process [12]. Therefore, we further investigated whether the involvement of KIAA1199 in multiple microenvironment factors induced the

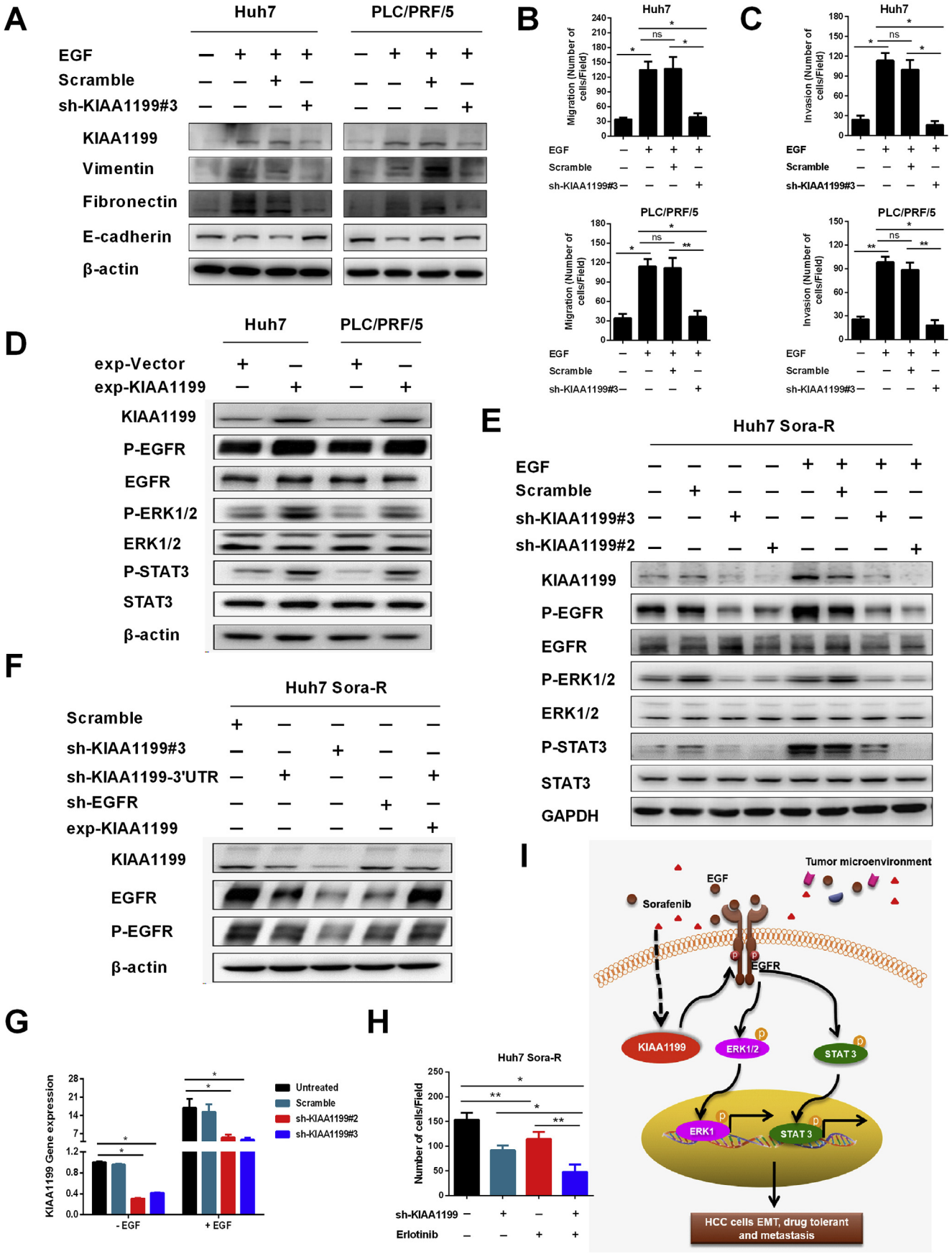

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Fig. 6. KIAA1199 promotes HCC cell resistance to sorafenib by activating the EGF/EGFR-dependent EMT program. (A). HCC cells were first serum starved for 24 h and subsequently exposed to EGF (20 ng/ml) for 24 h. Then, KIAA1199 expression was silenced for another 48 h, and the expression of KIAA1199 and EMT markers was detected by western blotting. (B, C). The migration and invasion abilities of HCC cells were determined by the Transwell assay (*, $P < 0.05$; **, $P < 0.01$). (D). Effect of KIAA1199 overexpression on EGFR activation in HCC parental cells. (E). Effect of KIAA1199 knockdown on EGF-mediated EGFR phosphorylation. KIAA1199-depleted sorafenib-resistant HCC cells were untreated or stimulated with EGF (20 ng/ml) for 24 h. The indicated cell extract was examined by western blotting using the indicated antibodies. (F). The expression of KIAA1199 and EGFR expression was first silenced by an shRNA lentivirus in Huh7 Sora-R cells, and these cells were then infected with the KIAA1199 overexpression lentivirus or the corresponding control vector. The indicated cell extract was examined using the indicated antibodies. (G). The expression of KIAA1199 was determined in both unstimulated and EGF-treated cells. (H). Huh7 Sora-R cells were treated with the EGFR inhibitor erlotinib at a dose of 1 μM for 24 h, and migration ability was examined by the Transwell assay. (I). Schematic summary of the involvement of KIAA1199 in regulation of the EMT process and drug tolerance in HCC cells.

EMT process. Here, we show that KIAA1199 could be stimulated by multiple EMT-induced factors in the Huh7 cell model (Supplementary Fig. S6A). To further unravel the dynamic changes of KIAA1199 in multiple microenvironment factor-induced EMT, we first selected EGF as an induced EMT model because the induction effect of EGF on KIAA1199 expression was stronger than other factors. Exogenous addition of 20 ng/ml EGF in HCC cells caused the cells to exhibit a spindle-like appearance (Supplementary Figs. S6B) and a loss of epithelial markers and a gain of mesenchymal markers (Supplementary Figs. S6C and D). We also noticed that KIAA1199 was robustly enhanced 24 h after EGF treatment; therefore, we depleted KIAA1199 and reevaluated these EMT models. Our data indicated that KIAA1199 depletion after EGF treatment could not only repress the morphological change and alleviate the loss of epithelial markers and dampen mesenchymal marker activation (Fig. 6A) but also impair migratory (Fig. 6B) and invasion (Fig. 6C) potentials. These results reveal that KIAA1199 is a required modulating factor in the EGF-induced EMT program. KIAA1199 has been characterized as an EGFR-binding protein [13]. Thus, we further evaluated the effect of KIAA1199 expression on EGFR activation. Our data showed that the overexpression of KIAA1199 in HCC parental cells significantly promoted EGFR phosphorylation, EGF-dependent STAT3 phosphorylation and ERK1/2 phosphorylation (Fig. 6D). In addition, KIAA1199 knockdown markedly inhibited the phosphorylation of EGFR, STAT3 and ERK1/2 in cells treated with or without EGF (Fig. 6E). Furthermore, we also found that the depletion of KIAA1199 expression attenuated EGFR expression; however, the inhibition of EGFR expression had no effect on KIAA1199 expression. Moreover, disrupted EGFR expression was successfully restored by rescuing KIAA1199 expression in sorafenib-resistant HCC cells, suggesting that KIAA1199 may be the upstream factor of EGFR (Fig. 6F). In addition, we found that EGF-induced KIAA1199 expression could be attenuated if KIAA1199 expression was knocked down by the KIAA1199 shRNA lentiviral construct (Fig. 6G), suggesting that KIAA1199 is required for the EGF-induced EMT program. Subsequently, we wondered whether EGFR was involved in KIAA1199-induced sorafenib-tolerant HCC cell migration and invasion, and the EGFR inhibitor erlotinib was used to confirm this. Indeed, the depletion of EGFR impaired the migration ability of sorafenib-resistant HCC cells (Fig. 6H), implying that KIAA1199-mediated EGF/EGFR cascade signaling activation regulates sorafenib-resistant HCC cell migration and invasion.

4. Discussion

Sorafenib is the first and only targeted therapy for advanced HCC [14]. However, the emergence of acquired sorafenib tolerance has prevented patients from benefiting from this drug [15,16]. At present, research on drug resistance is mainly focused on the natural resistance or induced resistance of tumor cell lines and often leads to a tumor centric drug resistance mechanism [17]. However, tumor-host interactions cannot be reflected *in vitro* using drug resistance models. Previous reports have indicated that the tumor microenvironment/tumor stroma contributes to tumor resistance [18,19]. Sorafenib-resistant HCC xenograft models contain multiple components of the tumor microenvironment, which better reveals the correlation between drug response and clinical drug efficacy [20].

Here, we demonstrated that KIAA1199 is involved in HCC cell sorafenib tolerance based on our established sorafenib-resistant HCC xenograft models. Previous studies have described that KIAA1199 is an inner ear-specific protein that is associated with auditory function [21]. Subsequent studies that addressed elevated KIAA1199 expression revealed its association with tumor progression, dissemination and poor prognosis of patients [22–25]. Moreover, KIAA1199 has also been implicated in hyaluronan depolymerization [26,27], rheumatoid arthritis [28], and metabolic reprogramming [27,29]. This evidence further verifies that KIAA1199 is a crucial gene involved in various biological processes. However, the expression pattern and function of KIAA1199 in HCC-acquired sorafenib tolerance remained unknown.

In the current study, we first systemically characterized KIAA1199 expression in clinical HCC specimens. Our data indicated that KIAA1199 was upregulated in HCC tissues compared with adjacent noncancerous tissues. Moreover, we further revealed that elevated KIAA1199 expression positively correlated with high metastatic probability and TNM stage in HCC specimens (Table 1). In sorafenib-resistant HCC cell models, we found that KIAA1199 was involved in HCC cell resistance to sorafenib mainly through mediating cell migration and invasion. In spleen-liver metastasis models, we also elucidated that impaired KIAA1199 expression attenuated the formation of metastatic foci in sorafenib-resistant cells. These results are highly consistent with the notion that KIAA1199 is upregulated and promotes progression and metastasis in HCC patients. Interestingly, a recent published work indicated that KIAA1199 knockdown inhibited growth and metastasis by inducing HCC cell cycle arrest and cell apoptosis, further supporting a key role for KIAA1199 in HCC [30]. Our study suggests that KIAA1199 is a novel potential candidate drug-tolerant accelerant in HCC cell sorafenib resistance and tumor metastasis.

Previous studies have suggested that EMT plays an important role in numerous cellular processes [31–33]. In our study, we revealed that the increased migratory ability of sorafenib-resistant HCC cells was closely associated with EMT phenotypic changes, as indicated by decreased epithelial markers (E-cadherin, ZO-1, and Occludin) and increased mesenchymal markers (Fibronectin and Vimentin), implying that KIAA1199 is a required modulating factor for the EGF-induced EMT process. The constitutive activation of NF-kB- and EGFR-dependent signaling pathways is a typical hallmark of cancers [34]. Notably, a recent work suggested that EGFR activation may also contribute to sorafenib resistance in HCC [35]. Furthermore, we next evaluated the effect of KIAA1199 expression on EGFR activation. Our data showed that the upregulation of KIAA1199 in HCC parental cells promoted the phosphorylation of EGFR and its downstream kinases. Meanwhile, KIAA1199 knockdown markedly inhibited the phosphorylation of EGFR, STAT3 and ERK1/2 in both unstimulated and EGF-treated cells, implying that KIAA1199 is required for EGFR stability and activated the activates downstream kinases. In addition, we also found that silencing KIAA1199 attenuated EGFR expression, and disrupted EGFR expression was successfully restored by rescuing KIAA1199 expression in sorafenib-resistant HCC cells, suggesting that KIAA1199 may be the upstream factor of EGFR. Additionally, our data demonstrated that KIAA1199 may promote EGF-dependent EGFR phosphorylation, further activating the STAT3 and ERK1/2 signaling pathways and ultimately

leading to Vimentin/Fibronectin upregulation, thus mediating the metastasis of sorafenib-resistant HCC cells (Fig. 6I). Altogether, KIAA1199 is a potential critical target for modulating HCC sorafenib tolerance.

In our study, we first established links between KIAA1199 expression, sorafenib tolerance and HCC metastasis. The precise mechanism of KIAA1199-induced sorafenib tolerance and metastasis requires further elucidation. In addition, KIAA1199 consists of isoforms 1 and 2 after alternative splicing; however, the isoform involved in HCC cell resistance to sorafenib is poorly characterized. Subsequently, we will further determine which isoform of KIAA1199 could be a key druggable target in the prevention of HCC metastasis. Collectively, our results showed that the upregulation of KIAA1199 contributes to the loss of epithelial cell architecture and the acquisition of a mesenchymal cell aggressive phenotype in sorafenib-resistant HCC cells, making KIAA1199 a novel candidate for HCC sorafenib tolerance and revealing it as a metastasis-promoting gene.

Conflicts of interest

All authors declare no conflicts of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.03.049.

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