RESEARCH ARTICLE

S100A14 Promotes the Growth and Metastasis of Hepatocellular Carcinoma

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Abstract

Background: S100A14 has recently been implicated in the progress of several types of cancers. This study aimed to investigate the clinical significance and possible mechanisms of action of S100A14 in the invasion and metastasis of hepatocellular carcinoma (HCC). Methods: S100A14 expression in HCC was detected at mRNA and protein levels and its prognostic significance was assessed. Functional roles of S100A14 in HCC were investigated using MTT, BrdU, wound healing, transwell invasion assay and HCC metastatic mouse model. Results: S100A14 was significantly elevated in HCC tissues, correlated with multiple tumor nodes, high Edmondson-Steiner grade and vascular invasion. Multivariate Cox analysis showed that the S100A14 expression level was a significant and independent prognostic factor for overall survival (OS) of HCC patients (hazard ratio=1.98, 95% confidence interval=1.14-3.46, *P*=0.013). S100A14 promoted cell proliferation, invasion and metastasis of HCC *in vitro* and *in vivo*. Conclusion: These results suggest S100A14 is a novel prognostic marker and therapeutic target for HCC.

Keywords: Hepatocellular carcinoma - S100A14 - metastasis - prognosis - marker

Asian Pacific J Cancer Prev, **14 (6)**, 3831-3836

Introduction

Hepatocellular carcinoma (HCC) is the one of the most common and aggressive cancers worldwide (Pu et al., 2013). The primary etiology of HCC is cirrhosis resulting from chronic infection by hepatitis B virus and hepatitis C virus as well as alcoholic or non-alcoholic liver injury (Gao et al., 2012). More than 80% of HCC cases are from the Asian and African continents, and more than 50% of cases are from mainland China with a vast majority of viral hepatitis patients (McClune et al., 2010). And, of note, its incidence in the Western world is showing an alarming rise (Nordenstedt et al., 2010). The social and economic burdens of HCC are an escalating public health problem (El-Serag et al., 2012). Although recent advances in understanding of HCC, the molecular pathogenesis of HCC remains rather elusive. Indeed, the clinical heterogeneity of HCC and lack of good treatment strategies have rendered this disease a great challenge (Yang et al., 2007).

Long-term survival after hepatic resection remains very poor for the majority of HCC patients who develop recurrence or metastasis (Zhou et al., 2006). Therefore, targeting invasion and metastasis is an attractive strategy for HCC therapy. However, recurrence and metastasis of HCC is a multistep process and the molecular mechanisms that contribute to the metastasis of HCC are poorly understood.

Accumulating evidence has shown that S100 proteins have been implicated in tumorigenesis and metastasis (Lukanidin et al., 2012). As a relatively new member of S100 proteins (Chen et al., 2012), the role of S100A14 in carcinogenesis has recently started to be understood. A recent study has indicated that ectopic over-expression of S100A14 promotes motility and invasiveness of esophageal squamous cell carcinoma cells (Chen et al., 2009). On the contrary, conflicting data also suggest that S100A14 inhibits proliferation of oral carcinoma derived cells through G1-arrest in vitro (Sapkota et al., 2012). These results are inconsistent. Therefore, we carried out the present study to investigate the clinical significance and molecular mechanism of S100A14 in the invasion and metastasis of HCC.

Materials and Methods

Study population and tissue samples

HCC tissues and their para-carcinomatous normal liver tissues (PCNLTs) were subjected to immunohistochemistry from 120 HCC patients, with median age 47 years (range 21–76 years), who underwent hepatectomy procedures from December 2003 to December 2006 at the Wuhan General Hospital and Tangdu Hospital Affiliated to the Fourth Military Medical University. Among these 120 cases, fresh specimens with vascular invasion (n=30) and without vascular invasion (n=32) were collected for real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) and Western blot analysis.

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The diagnoses of all subjects were confirmed by two pathologists in a blind manner. Samples of normal liver tissue (n=14) obtained from patients with cavernous haemangioma who underwent hepatic resection were used as control. Prior written informed consent was obtained. All study protocols were approved by the Ethics Committee of Wuhan General Hospital and Tangdu Hospital.

Immunohistochemistry

Formalin-fixed paraffin sections (4μm) were analyzed by immunohistochemical staining as previously reported (Li et al., 2008). The sections were incubated with anti-S100A14 antibody (Sigma, 1:100) at 4 °C overnight. Tissues were counterstained with haematoxylin and mounted for examination. Control slides were probed with normal goat serum under the same conditions.

Follow-up and prognosis

Follow-up data were obtained by reviewing the hospital records and direct communication with all patients. The follow-up period was defined from the date of surgical excision of the tumor to the date of death or last follow-up. The disease-free survival was defined as the length of time after hepatectomy for HCC during which a patient survived with no sign of HCC. The median follow-up time was 986 days (range 187–2810 days). Clinical and pathological variables were also collected, including age, sex, cirrhosis, histological grading, capsular formation, tumor size, number of tumor nodes and vascular invasion.

qRT-PCR

The primers were as follows: S100A14: forward, 5'-TCACCAAAGGACCAG ACACA-3', reverse,5'-GCCCTCTCCACATCACTGAA-3';GAPDH: forward, 5'-GAC CCTTCATTGACCTC-3'; reverse, GCAATGCCAGCCCCAG. PCR amplification was performed using the SYBR Premix PCR kit (Takara, China) according to the instructions. Reactions were carried out in a 96-well plate using the ABI Prism 7300 Real-Time PCR System (Applied Biosystems, USA). The results were analyzed using the ΔΔCt method.

Western blot

Total protein was extracted from fresh tissues and determined by a bicinchoninic acid protein assay kit (Millipore). Total protein (80 μg) was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto PVDF membranes (Millipore, Bedford, MA). The blotted membranes were incubated first with the anti-S100A14 antibody, and then with the secondary antibody (all from Abcam). β-actin protein(Santa Cruz) was selected as the loading control.

Cell lines, cell culture and vector construction

HepG2, Huh7, MHCC97-L and HCCLM3 cell lines were purchased from the American Type Culture Collection (ATCC). Cells were cultured in Dulbecco's modified Eagle's medium and supplemented with 10% fetal bovine serum at 37°C with 5% CO_2.Vector construction was constructed by OriGene Technologies (Rockville, USA). The pcDNA3.1-S100A14 and short hairpin RNA (shRNA) expressing vector pGFP-V-RS-S100A14 was commercially obtained (OriGene Technologies).

Cell proliferation assay

The tetrazolium-based colorimetric assay (MTT test) was used to evaluate cell proliferation. In brief, cells were seeded in 96-well plates at a density of 5×10^3 cells/well. One plate was taken out at the same time every day after the cells adhered. 20 μl of MTT (5 mg/mL) was added to each well, and the cells were incubated for another 4h. The medium was removed, and the formazan precipitate was solubilized in 150mL dimethylsulfoxide (DMSO). The absorbance at 490 nm was measured using a microplate reader. A BrdU assay was conducted as per the manufacturer's instructions (Cell Signaling Technology). Cells were seeded at 5×10^3 cells/well in a 96-well plate and incubated overnight. The cells were then incubated for 24h. Finally, BrdU (10 μM) was added to the plate, and the cells were incubated for 4h. The absorbance at 450 nm was measured using a microplate reader. All experiments were performed in triplicate.

Transwell assay

Transwell chamber consisting of 8μm membrane filter inserts (Corning) was used. 1×10^5 cells in serum-free medium containing 0.1% bovine serum albumin were placed into the upper chamber with Matrigel (BD Biosciences). After 48 h of incubation at 37°C, the cells in the upper chamber or on the upper membrane were removed with a cotton swab. After staining with a solution containing 0.1% crystal violet and 20% methanol, the number of cells adhering to the lower membrane of the inserts was counted. The invasion assay was conducted in triplicate, and three randomly selected fields in each replicate were chosen for the cell number quantification.

Wound healing assay

Cells were seeded into 35 mm dishes (Corning, NY), and wound healing assays were performed with a sterile pipette tip to make a scratch through the confluent monolayer. The cells were then washed with fresh medium and cultured for another 48 h. The percent wound closure was calculated for five randomly chosen fields.

HCC metastatic model

Male CB17-severe combined immunodeficiency (SCID) mice (Charles River) were maintained under pathogen-free conditions in accordance with guidelines by Guangzhou Medical College Animal Care and Use Committee. Four-week-old mice were subcutaneously injected with 5×10^5 HCCLM3 or HCCLM3S100A14-cells in the back (n=8 mice per group). One month later, obvious tumors were present in the hypodermal tissue and the mice were sacrificed. The subcutaneous tumors were obtained, tumor size was and tumor volume was measured. The lungs of the mice were dissected, fixed with phosphate-buffered neutral formalin, sectioned serially and stained for standard histological examination.

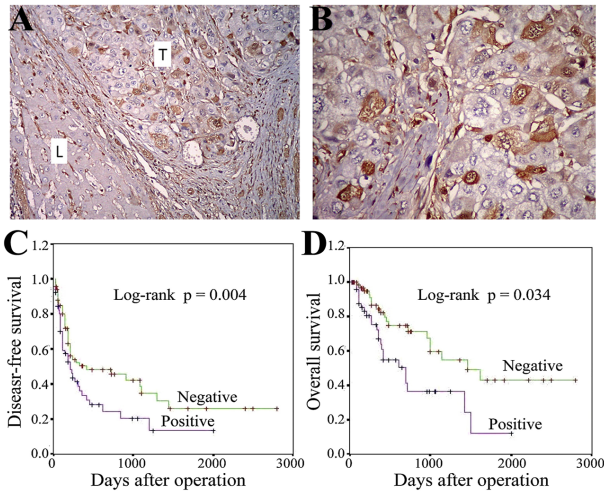

Figure 1. Expression of S100A14 by Immunohistochemical Staining in Hepatocellular Carcinoma (HCC) Tissues (A, B) and survival analysis (C, D). S100A14-positive cells are dark brown in the nuclei and/or cytoplasms. (A) S100A14 expression was present in HCC (T), but absent in the para-carcinomatous normal liver tissues (PCNLTs) (L). Original magnification ×200. (B) Another grade III tumor staining positive for S100A14. Original magnification ×400. HCC was classified into S100A14-negative group (n=68) and S100A14-positive group (n=52). Survival curves of disease-free survival (C) and overall survival (D) were analyzed using Kaplan–Meier method and Log-rank test

Statistical analysis

The Spearman rank-correlation analysis was used to analyze the correlation between S100A14 expression levels and clinicopathologic characteristics in HCC patients. The independent sample t-test was used to compare the S100A14 mRNA and protein expression levels. Survival was plotted using the Kaplan–Meier method and analyzed by the Log-rank test. Univariate and multivariate analyses were based on the Cox proportional hazards regression model. P <0.05 indicated a significant difference. All tests were processed by SPSS16.0.

Results

The expression of S100A14 in HCC tissues

The S100A14 protein expression was detected in 120 paired HCC tissues and PCNLTs by immunohistochemical staining (Figure 1A). Another grade III tumor stained positive for S100A14 (Figure 1B). The results revealed the nuclear and cytoplasmic distribution of S100A14. S100A14 protein was detected in 52 of 120 HCC tissues and in 11 of 120 PCNLTs (43.3% vs. 9.1%; P <0.001). The positive expression rate was significantly higher in HCC tissues. All the fourteen specimens of normal liver tissue showed negative staining.

Correlations of S100A14 expression with clinicopathologic characteristics and prognosis in HCC patients

Immunohistochemical staining demonstrated that the up-regulated S100A14 levels correlated with multiple tumor nodes, high Edmondson–Steiner grade and vascular invasion (Table 1). According to the immunohistochemical data, 120 patients with HCC were divided into the S100A14-positive group (n=52) and S100A14-negative group (n=68). Results indicated that patients with positive S100A14 expression had a shorter disease-free survival (DES, median disease-free survival, 208 days vs. 422 days; P =0.004; Figure 1C) and overall survival (OS, median overall survival, 730 days vs. 1520 days; P=0.034; Figure 1D) compared with patients who with negative S100A14 expression. Univariate and multivariate analysis showed that S100A14 was a significant and independent prognostic factor for the overall survival (OS) of HCC patients (Table 2, hazard ratio=1.98, 95% confidence interval=1.14-3.46, P=0.013). These findings further prove that S100A14 is an important prognostic factor for HCC patients.

Table 1. Table 1. Correlations of S100A14 Expression with Clinicopathologic Features of Hepatocellular Carcinoma

Clinicopathologic variables	Number of cases	S100A14 expression		P-Value
		Negative	Positive	
Gender				
Male	104	57	47	
Female	16	11	5	0.25
Age (years)				
≤60	101	58	43	
>60	19	10	9	0.475
Liver cirrhosis				
Presence	86	47	39	
Absence	34	21	13	0.238
Capsular formation				
Presence	61	37	24	
Absence	59	31	28	0.49
Tumour size				
≤5 cm	43	22	21	
>5 cm	77	46	31	0.44
Tumour nodule number				
Multiple (≥2)	38	17	21	
Solitary	82	52	30	0.009
Edmondson–Steiner grade				
Stage I–II	66	36	30	
Stage III–IV	54	25	29	0.012
Vascular invasion				
Presence	75	35	40	
Absence	45	27	18	<0.001

Clinicopathologic variables were compared between group with negative; S100A14 expression (n=68) and positive S100A14 expression (n=52)

Table 2. Univariate and Multivariate Analysis Showing Overall Survival for Hepatocellular Carcinoma Patients

Variables	Univariate analysis			Multivariate analysis		
	HR	95% CI	P	HR	95% CI	P
S100A14	2.019	1.16-3.50	0.001	1.98	1.14-3.46	0.013
Gender	0.658	0.28-1.52	0.33			
Age	0.817	0.48-1.37	0.44			
Tumor size	1.662	1.13-2.43	0.009	1.49	0.98-2.27	0.062
Histologic grade	1.304	0.86-1.96	0.2			
Cirrhosis	0.683	0.41-1.14	0.14			
HBsAg status	1.244	0.56-2.73	0.58			
Serum AFP	1.592	0.92-2.74	0.09			
Metastasis	1.407	0.73-2.70	0.3			
Recurrence	1.349	0.80-2.26	0.25			

HR, Hazard ratio; CI, Confidence interval; AFP, α-fetoprotein

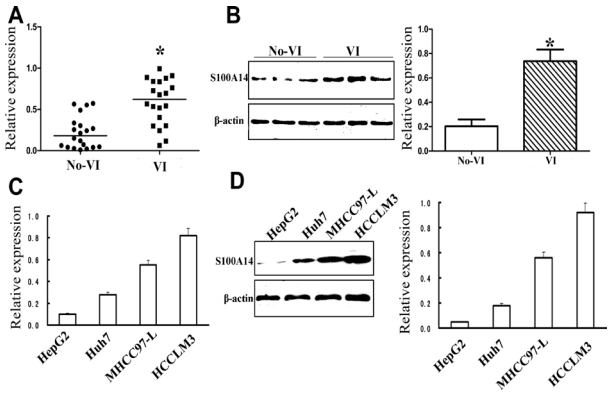

Figure 2. S100A14 mRNA and Protein Expression Level in Hepatocellular Carcinoma (HCC) Tissues and Hepatocellular Carcinoma Cell Lines. (A) Real-time quantitative reverse transcription polymerase chain reaction results of S100A14 mRNA expression in hepatocellular carcinoma tissues with vascular invasion and without vascular invasion. (B) S100A14 protein expression level was detected in HCC-No-VI and HCC-VI. β-Actin was used as loading control. The mRNA (C) and protein (D) expression of S100A14 in four different hepatocellular carcinoma cell lines (HepG2, Huh7, MHCC97-L, HCCLM3). No-VI, hepatocellular carcinoma without vascular invasion; VI, hepatocellular carcinoma tissues with vascular invasion.*p<0.01

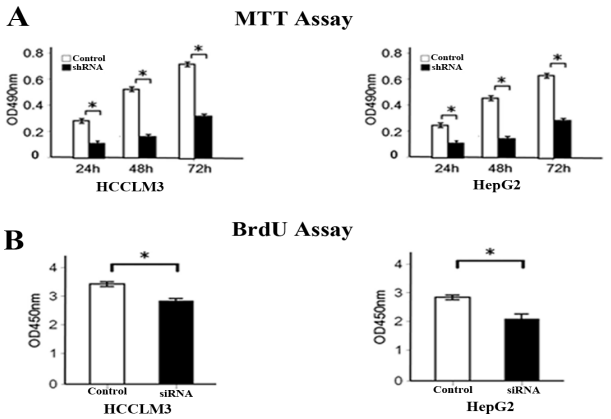

Figure 3. S100A14 Knockdown Suppressed Hepatocellular Carcinoma (HCC) Cell Proliferation. (A) Cell proliferation of HCCLM3-shS100A14, HepG2-shS100A14 cells and control cells by MTT assay analysis (*P<0.01). (B) Cell proliferation of HCCLM3-shS100A14, HepG2-shS100A14 cells and control cells after 24 h incubation by BrdU assay analysis (*P<0.01)

Correlations of S100A14 expression in frozen HCC tissues with vascular invasion

We also investigated the relationship between S100A14 expression and vascular invasion using qRT-PCR and Western blotting analysis. The results showed that S100A14 mRNA level in HCC tissues with vascular invasion was significantly higher than that without vascular invasion [median relative expression level: 0.16 (range 0.03–0.57) vs. 0.67 (range 0.06–0.98); P<0.01, Figure 2A]. Consistent with the mRNA expression, the S100A14 protein expression level was also significantly elevated in HCC tissues with vascular invasion (0.21 ± 0.08 vs. 0.76± 0.23, P< 0.01; Figure 2B).

Furthermore, among the four HCC cell lines analyzed, HCCLM3 cells, with the highly metastatic ability (Tao et al., 2013), had the highest expression levels of S100A14 at mRNA and protein levels, followed by MHCC97-L, Huh7 and HepG2 cells (Figure 2C and D). These data suggest that there may be a positive correlation between S100A14 expression and the metastasis potential of HCC.

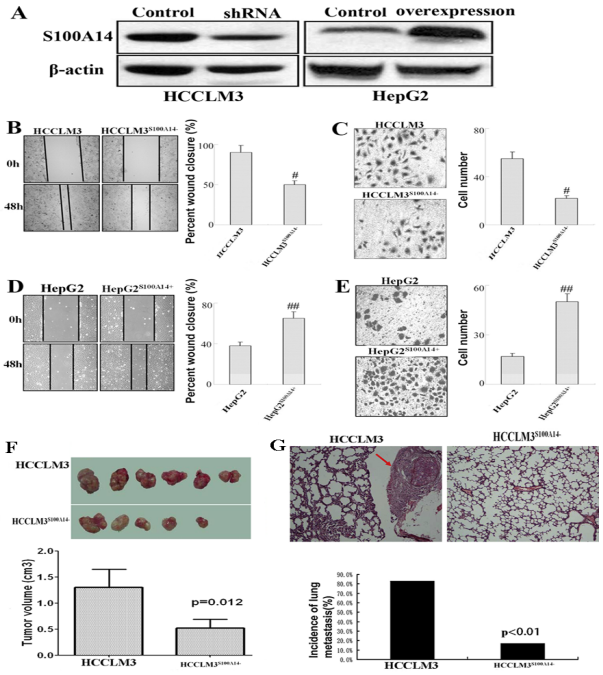

Figure 4. S100A14 Promoted the Migration and Invasion of Hepatocellular Carcinoma (HCC) Cells *in vitro* and *in vivo*. Western blot confirmed the effects of S100A14 silencing and overexpression (Figure 4A). The wound healing assay showed that the closure of HCCLM3S100A14– cells was significantly slower than that of HCCLM3 cells (92% vs. 49%, P<0.01; Figure 4B), whereas the invasion assay indicated that the number of HCCLM3S100A14– cells that passed through the Matrigel was smaller than that of HCCLM3 cells (58±11 vs. 21± 7, $^{\#}P$<0.01; Figure 4C). S100A14 overexpression promoted the migration and invasion of HepG2 cells [HepG2S100A14+ vs. HepG2: wound healing assay, 67% vs. 39%, p<0.05, Figure 3D; invasion assay, 50 ± 9 vs. 18 ± 5, $^{\#\#}P$<0.05; Figure 4E]. (F) Formation of subcutaneous tumors in the two groups of cell lines. The mean volume of subcutaneous tumor of the HCCLM3S100A14– group was 0.52±0.17cm3, while that of HCCLM3 group was 1.28±0.29 cm^3 (P=0.012). (G) The lung metastasis rate of HCCLM3 group was significantly higher than that of the HCCLM3S100A14– group (83.3% vs. 16.7%, respectively, P<0.01). The red arrow indicates the metastasis lesion in the lung of the mouse (HE staining, 100×), n=8 mice per group

Knockdown of S100A14 inhibited proliferation of HCCLM3 and HepG2 cells

We assessed the effects of S100A14 on the cell proliferation of HCCLM3 and HepG2. MTT assays showed significant reduction in cell proliferation of HCCLM3-shS100A14 and HepG2-shS100A14 cells compared with control cells at 24 h, 48h and 72h (Figure 3A). We also evaluated their proliferative capacity using a BrdU assay. The BrdU staining after incubation for 24h (Figure 3B) was in accordance with the results of MTT assay, suggesting that S100A14 silencing inhibited the proliferation of HCCLM3 and HepG2 cells compared with control cells.

Effects of S100A14 on the migration and invasion of HCC in vitro and in vivo

We further determined the roles of S100A14 in HCC metastasis. HepG2 (with low metastatic ability) and HCCLM3 (with high metastatic ability) cells were selected. The S100A14-stably overexpressed HepG2S100A14+ cells and silenced HCCLM3S100A14– cells were commercially constructed.

First of all, Western blot confirmed the effects of S100A14 silencing and overexpression (Figure 4A). The wound healing assay showed that the closure of HCCLM3S100A14– cells was significantly slower than that of HCCLM3 cells (92% vs. 49%, $P<0.01$; Figure 4B). The invasion assay indicated that the number of HCCLM3S100A14– cells that passed through the Matrigel was smaller than that of HCCLM3 cells (58±11 vs. 21± 7, $P<0.01$; Figure 4C). In accord with the results observed from HCCLM3 cells, S100A14 overexpression promoted the migration and invasion of HepG2 cells [HepG2S100A14+ vs. HepG2: wound healing assay, 67% vs. 39%, $p<0.05$, Figure4D; invasion assay, 50 ± 9 vs. 18 ± 5, $P<0.05$; Figure 4E].

To validate the observations obtained from in vitro studies, we examined the in vivo role of S100A14 in HCC metastasis in the HCC metastatic mouse model using HCCLM3 cells, which has the potential of lung metastasis after subcutaneous implantation into nude mice (Xue et al., 2012). The mean volume of subcutaneous tumors of the two groups differed significantly ($P<0.05$; Figure 4F). Furthermore, the lung metastasis rate of HCCLM3 was 83.3% (5/6), while that of HCCLM3S100A14– was 16.7% (1/6) ($P<0.01$; Figure 4G).

These results indicate that S100A14 can significantly promote HCC cell migration and invasion in vitro and in vivo, suggesting an important role of S100A14 in HCC metastasis.

Discussion

Increasing evidence showed the importance of S100 family in cell migration, invasion, and cancer metastasis. Among them, S100A4 has been identified as a well known metastasis marker (Boye et al., 2010; Wang et al., 2010). In addition, S100A2 has also been reported as a strong metastasis inducer in non-small cell lung cancer (Bulk et al., 2009). S100A8/S100A9 has been implicated in both myeloid cell recruitment and tumor cell invasion in lung cancer (Hiratsuka et al., 2006). Recently, S100A14 has emerged as an important molecule to promote cell proliferation and survival (Jin et al., 2011). In this study, we used an extensive collection of HCC tumors to show that S100A14 was significantly elevated in HCC tissues. The increased S100A14 expression was correlated with multiple tumor nodes, high Edmondson-Steiner grade and vascular invasion. These observations were reminiscent of previous reports in other malignancies such as esophageal squamous cell carcinoma (Chen et al., 2009) and colorectal cancer (Wang et al., 2010). Moreover, our survival analysis revealed that overexpression of S100A14 in HCC predicted shorter OS and DFS of HCC. Univariate and multivariate analysis revealed that S100A14 was an independent factor for predicting OS. These findings strongly implicate S100A14 as a marker for tumor aggressiveness and a predictor for prognosis in HCC.

Several pieces of evidence in this study showed a close association between S100A14 expression and HCC metastasis. First, the expression levels of S100A14 correlated with the metastatic potentials of the HCC cell lines.Second, functional assays showed that inhibition of S100A14 significantly inhibited the cell proliferation and invasion. Third, using a HCC metastatic mouse model, we found that knockdown of S100A14 in the implanted HCC cells markedly reduced the volume of subcutaneous tumors in vivo as well as the lung metastasis rate. These results suggest that S100A14 plays a crucial role in regulating HCC progression and metastasis.

However, the mechanism for the functional roles of S100A14 in enhancing different aspects of tumor malignancy remains elusive. Currently, there are limited reports to refer to regarding the underlying mechanisms, which needs to be explored. Taken together, this study show that overexpression of S100A14 in HCC is a strong indicator for more aggressive tumors and poorer clinical outcome. The evidence presented strongly suggests that S100A14 could be a candidate biomarker for HCC prognosis and a target for therapy.

Acknowledgements

The author(s) declare that they have no competing interests.

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