Exosomal S100A4 derived from highly metastatic hepatocellular carcinoma cells promotes metastasis by activating STAT3

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INTRODUCTION
Metastasis and recurrence, causing about 90% of deaths of cancer patients, are the most significant characteristics of malignant cancers.1,2 Tumor recurrence occurs in over half of hepatocellular carcinoma (HCC) patients at 5 years after resection and is a common cause of poor prognosis.3 Intratumor heterogeneity contributes to drug resistance and tumor relapse following therapy.4 Understanding phenotypical intratumor heterogeneity of HCC should provide critical knowledge about its diverse metastatic potential.

Intercellular cross-talk plays important roles in cancer progression and metastasis. Yet how these cancer cells interact with each other is still largely unknown. Exosomes released by tumor cells have been proved to be effective cell-to-cell signal mediators. We explored the functional roles of exosomes in metastasis and the potential prognostic values for hepatocellular carcinoma (HCC). Exosomes were extracted from HCC cells of different metastatic potentials. The metastatic effects of exosomes derived from highly metastatic HCC cells (HMH) were evaluated both in vitro and in vivo. Exosomal proteins were identified with iTRAQ mass spectrum and verified in cell lines, xenograft tumor samples, and functional analyses. Exosomes released by HMH significantly enhanced the in vitro invasion and in vivo metastasis of low metastatic HCC cells (LMH). S100 calcium-binding protein A4 (S100A4) was identified as a functional factor in exosomes derived from HMH. S100A4high exosomes significantly promoted tumor metastasis both in vitro and in vivo compared with S100A4low exosomes or controls. Moreover, exosomal S100A4 could induce expression of osteopontin (OPN), along with other tumor metastasis/stemness-related genes. Exosomal S100A4 activated OPN transcription via STAT3 phosphorylation. HCC patients with high exosomal S100A4 in plasma also had a poorer prognosis. In conclusion, exosomes from HMH could promote the metastatic potential of LMH, and exosomal S100A4 is a key enhancer for HCC metastasis, activating STAT3 phosphorylation and up-regulating OPN expression. This suggested exosomal S100A4 to be a novel prognostic marker and therapeutic target for HCC metastasis.

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Signal Transduction and Targeted Therapy
RESULTS

Isolation and identification of exosomes released and up-taken by HCC cells

Exosomes were extracted from supernatant of HCC cells by ExoQuick reagent kit. Then morphological observation was performed by transmission electron microscope and shapes of exosomes were observed (Fig. 1a). Furthermore, we utilized nanoparticle tracking analysis (NTA) to determine exosomes diameters, which were mostly ranged between 40 and 200 nm (Fig. 1a). Relatively specific exosomal markers, CD63, CD9, and Alix, were detected by Western blot in six HCC cell lines (Fig. 1b). In order to verify the reliability of extracted exosomes, we adopted methylated-β-Cyclodextrin (MβCD) treatment,

Fig. 1  Isolation and identification of exosomes released and up-taken by HCC cells. a Transmission electron microscopy of isolated exosomes from HCC cells (HCC-LM3, MHCC97-H, PLC, and HepG2. Scale bar: 200 nm). The concentration and size distribution were determined by nanoparticle tracking analysis (NTA). b Western blot of exosomal marker CD63, CD9, and Alix in exosomes isolated from HCC cell lines (HCC-LM3, MHCC97-H, MHCC-97-L, HepG2, Huh7, and PLC). c Laser scanning confocal microscope images of DIO treated exosomes (MHCC97H exo and HepG2 exo) up-taken by low metastatic HCC cells (MHCC97-L).
which can destruct exosomal lipid membrane. After M8CD treatment, exosomal markers were much less detected by Western blot, which further verified the reliability and purity of exosomes extracted by ExoQuick reagent kit (Supplementary Fig. S1c). Next, we used DIO green to separately dye exosomes released by highly metastatic HCC cells (MHCC97-H, the exosomes were marked as HMH-exosomes) and low metastatic HCC cells (HepG2, the exosomes were marked as LMH-exosomes). We also observed with laser scanning confocal microscope that both green coated HMH-exosomes and LMH-exosomes could be up taken efficiently by lowly metastatic HCC cells, MHCC97-L, and HepG2 (Fig. 1c). These indicate that we have successfully isolated and purified exosomes form HCC cells, and demonstrated that they can be up-taken by the other HCC cells.

**HMH-exosomes enhance the metastatic potential of low metastatic HCC cells**

To investigate the possible role of exosomes in cell-to-cell crosstalk of HCC metastasis, we used HMH-exosomes to pre-treat low metastatic HCC cells (MHCC97-L, Huh7, and HepG2) for 24 h (LMH-exosomes and PBS as control, respectively). After pretreatment, MHCC97-L cells underwent both migration and invasion assays, while Huh7 and HepG2 cells only invasion assays, to test their metastatic potential in vitro. It was noted that significantly more HMH-exosomes pre-treated MHCC97-L cells passed through the Transwell chamber, but not those treated with LMH-exosomes or PBS (Fig. 2a). Similar results were observed in HMH-exosomes pre-treated Huh7 and HepG2 cells, with an exception of LMH-exosomes pre-treated HepG2 cells. They also saw a slightly enhanced invasion, compared to PBS ones (Fig. 2b, Supplementary Fig. S1a, b). These suggest that HMH-exosomes, exosomes released by highly metastatic HCC cells, MHCC97-H, and HCC-LM3, could enhance the in vitro migration and invasion abilities of low metastatic HCC cells.

Then, we established in vivo orthotopic implantation xenograft model with MHCC97-L cells. First, we implanted small tumor tissues into the left liver of nude mice. After one week, PBS, HepG2-exosomes, HCC-LM3-exosomes, and MHCC97-H exosomes were respectively injected into tail veins of these nude mice twice a week for 6 weeks. Then they were euthanized for liver tumor and lung metastasis evaluation. We found that liver tumors of HMH-exosomes groups (HCC-LM3 exosomes group and MHCC97-H exosomes group) were larger than the other two groups (Fig. 2c). Similarly, number of lung metastasis in the HMH-exosomes groups were also significantly larger than the other two groups (Fig. 2d). These demonstrate that HMH-exosomes could significantly enhance in vivo growth and lung metastasis of low metastatic HCC cells.

**Exosomal S100A4 is a key enhancer of metastatic potential in HCC cells**

In order to identify the element via which HMH-exosomes work to enhance the metastatic potential of low metastatic HCC cells, we adopted iTRAQ mass spectrum screening for HMH-exosomes and LMH-exosomes. Results showed that 116 proteins were significantly up-regulated in HMH-exosomes group while 43 down-regulated (Fig. 3a, b). Proteins were clustered at the 2-fold changes with a p value less than 0.05. The volcano plots revealed that 4 protein families, Apo, PSM, EEF/EIF, and S100 calcium binding protein family, were clustered. After reviewing literatures, we focused on four members of S100 calcium binding protein family with the most differentiated expression, S100A4 (means ratio: 9.642), followed sequentially by S100A11 (8.393), S100A10 (6.132), and S100A6 (5.101). Furthermore, we conducted comparison of the expression abundance of S100A4, S100A6, S100A10, and S100A11 in 5 HCC cells lines, as well as the abundance of S100A4 in exosomes derived from these cell lines. Results were consistent with the iTRAQ data (Fig. 3c). Immunohistochemistry (IHC) staining of S100A4 in nude mice tumor models also confirmed that S100A4 was significantly more highly expressed in MHCC97-H derived tumors (Supplementary Fig. S1d). Therefore, we hypothesized that S100A4 may be one of the most potentially functional factors in HMH-exosomes and we selected it for further analyses.

We knocked down S100A4 in HCC-LM3 and MHCC97-H cells, and overexpressed S100A4 in PLC, Huh7, and HepG2 cells (Supplementary Fig. S2a). We then repeated in vitro migration and invasion assays. As a result, after S100A4 was down-regulated, the abilities of migration and invasion of HCC-LM3 and MHCC97-H cells were both inhibited. On the other hand, S100A4 overexpression greatly enhanced Huh7 and HepG2 migration and invasion (Supplementary Fig. S2b). These support that S100A4 is important for in vitro migration and invasion of HCC cells.

Since S100A4 protein could maintain cell stemness, which is important in promoting cancer metastasis, therefore, we performed in vitro sphere formation and in vivo tumor initiation assays. After S100A4 was knocked down, HCC-LM3 and MHCC97-H cells formed much fewer and smaller spheres than the controls (Supplementary Fig. S2c). As expected, when S100A4 was over-expressed in Huh7 and HepG2 cells, they formed significantly more and larger spheres (Supplementary Fig. S2c). In vivo models showed that the tumor initiation time of S100A4 down-regulated HCC-LM3 and MHCC97-H cells was sharply delayed for 1 to 2 weeks depending on the number of tumor cells implanted, and tumor sizes were also smaller as expected (Supplementary Fig. S3). These provide further support that S100A4 could enhance the stemness of HCC cells.

We then further investigated the function of exosomal S100A4 protein on metastatic potential and stemness enhancement. Firstly, we extracted exosomes from supernatants of S100A4 knock-down HCC-LM3 and MHCC97-H cells, and verified that S100A4 expression in those exosomes was significantly lower than the controls (Supplementary Fig. S4). We defined them as S100A4low and S100A4rich exosomes. S100A4rich exosomes were used to pre-treat low metastatic HCC cells, MHCC97-L and HepG2, for 24 h, controlled by S100A4low exosomes and PBS. After pre-treatment, MHCC97-L and HepG2 cells were harvested for migration and invasion assays, to test their metastatic potential in vitro. We observed significantly more S100A4rich exosomes pre-treated MHCC97-L and HepG2 cells passed through the Transwell chamber than controls (Fig. 3e). Then, we conducted in vivo experiment with tail vein injection lung-metastasis model of MHCC97-L cells. First, we pre-treated MHCC97-L cells by PBS, S100A4rich exosomes or S100A4low exosomes for 24 h. Then 50,000 of these pre-treated cells, with their corresponding exosomes or PBS, were injected into tail veins of nude mice. After that, exosomes or PBS was injected into tail vein twice a week. Four weeks later, mice were euthanized and their lungs harvested for paraffin fixation. Results showed that lung metastasis was significantly higher in S100A4rich exosomes treated group than the other two groups (Fig. 3f). In addition to metastatic promotion, in vitro sphere formation rates of MHCC97-L and HepG2 cells were also elevated by S100A4rich exosomes treatment (Supplementary Fig. S5a), so was the in vivo tumor initiation ability of MHCC97-L (Supplementary Fig. S5b). Taken together, these findings further demonstrate the functional roles of exosomal S100A4 in enhancement of the metastatic potential and stemness of HCC cells.

**Exosomal S100A4 activates OPN transcription via STAT3 phosphorylation**

Next, we attempted to elucidate the mechanism of exosomal S100A4 on metastatic potential and stemness. First, we selected 21 cancer stemness-related genes, OPN, OCT4, NANOX, SOX2, HIF1a, BMI1, ABCG2, CK19, NOTCH1, KLF4, CD44, CD90, CD133, CD117, CD24, EPICAM, TCF3, TCL, CTNNB1, HEY1, and C-MYC. Then we downloaded a GEO datasheet of HCC samples (GSE39791) for correlation analyzes. Among those 21 stemness-related genes, S100A4 was positively correlated with OPN, HIF1a, BMI1, CK19, NOTCH1, KLF4, CD44, CD90, TCL, HEY1, and C-MYC (Fig. 4a). We
further determined the expression levels of these 11 genes (primers are shown in Supplementary Table S1) in Huh7, PLC, and HepG2 cells with S100A4 overexpression, or PBS (as negative control) for 24 h. The migration ability of MHCC97-L and invasion ability of all the three cell lines were significantly enhanced by MHCC97-H exosomes compared to the control groups. c Orthotopic implantation liver tumors from nude mice. MHCC97-L cells treated with HMH exosomes (HCC-LM3 exosomes or MHCC97-H exosomes) formed larger liver tumors. d Lung metastasis of mice injected with MHCC97-L. MHCC97-L cells treated with HMH exosomes formed significantly more lung metastatic lesions. All the in vitro assays were conducted three times with three repetitions. Error bars represent the mean ± SD, and the dots represent the value of repetitions in one experiment; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns: no significance. An unpaired t test was employed in (a) and (b), one-way ANOVA followed by Bonferroni’s post hoc test was employed in (c) and (d).

Interestingly, it was reported that S100A4 could enhance STAT3 phosphorylation,18,19 and STAT3 phosphorylation (p-stat3) was correlated with OPN expression.20 In addition, STAT3 was predicted to be a potential transcription factor of OPN.21 Therefore, we further evaluated OPN, STAT3, and STAT3 phosphorylation levels in S100A4 knockdown HCC-LM3 and MHCC97-H cells and in S100A4 overexpressed Huh7 and PLC cells, and found that OPN and STAT3 phosphorylation levels significantly changed in accordance with S100A4 levels (Fig. 5a). In addition, we knocked
down STAT3 in HCC-LM3 and MHCC97-H cells, and observed that STAT3 phosphorylation and OPN expression were both inhibited (Fig. 5b). In Huh7 and PLC cells, when S100A4 was overexpressed, STAT3 phosphorylation and OPN expression were both enhanced, which could be inhibited by stat3 knockdown or stat3 inhibitor, S3I-201 (Fig. 5c). These indicate S100A4 could activate STAT3 phosphorylation and increase OPN expression.

To further determine the influence of S100A4rich exosomes on STAT3 phosphorylation and OPN expression, we pre-treated MHCC97-L and Huh7 cells with S100A4rich exosomes before cell lysis and found that S100A4rich exosomes significantly promoted STAT3 phosphorylation and OPN expression, as well as S100A4 expression (Fig. 5d). In addition, when OPN was knocked down or the cells were treated by the STAT3 inhibitor S3I-201, the abilities of invasion and migration of HepG2 and PLC cells were significantly inhibited, even after S100A4rich exosome treatment (Supplementary Fig. S6a–d). Similarly, the sphere formation of HepG2 (Supplementary Fig. S6e) and PLC (Supplementary Fig. S6f) decreased when OPN was knocked down or p-stat3 inhibitor was added, even after with S100A4rich exosomes treatment. Moreover, in orthotopic implantation xenograft tumor of MHCC97-L, HMM-exosomes (HCC-LM3 exosomes and MHCC97-H exosomes) significantly enhanced nuclear STAT3 phosphorylation and cytoplasmic OPN expression while LMH exosomes (HepG2 exosomes) or PBS could not (Fig. 5e). Similar results were also observed with Western blot (Fig. 5f). These results support that S100A4rich exosomes from HMH promote the metastatic potential of LMH, and exosomal S100A4 is a key enhancer for HCC metastasis, by activating STAT3 phosphorylation and up-regulating OPN expression (Fig. 6a).

Plasma exosomal S100A4 and OPN levels jointly serve as a powerful postoperative prognostic factor for HCC patients. The clinical significance of plasma exosomal S100A4 and OPN levels was further investigated in 168 patients who received curative liver resection for HCC. We first detected the plasma...
levels of exosomal S100A4, CD63 and CD9 for feasibility verification by Western blot in 4 HCC patients (Supplementary Fig. S7a). We also tested plasma exosomal S100A4 in 10 HCC patients and 10 healthy donors. The exosomal S100A4 was significantly higher in HCC patients (Supplementary Fig. S7b). Exosomal S100A4 was significantly higher in metastatic HCC patients than non-metastatic ones (Supplementary Fig. S7c). Then we determined the plasma exosomal S100A4 and OPN levels by ELISA assay, and found a significantly positive correlation between the exosomal S100A4 and OPN levels (Fig. 6b). Based on the median value of exosomal S100A4 (98.54 pg/ml) and OPN level (103.78 ng/ml), these patients were
divided into high - \( n = 84 \) and low-exosomal S100A4 group \( n = 84 \); as well as high - \( n = 84 \) and low-OPN group \( n = 84 \). Crosstab analyses showed that exosomal S100A4 level was significantly correlated with serum alpha-fetoprotein (AFP) level, tumor size, vascular invasion, and TNM stage, but not with BCLC stage (Table 1). Plasma OPN level was significantly correlated with vascular invasion, tumor differentiation, and TNM stage, but not with tumor size,
S100A4 and low OPN level had the best prognosis (the longest OS and TTR), and the worst survival was found in those with high S100A4 and high OPN levels ($p < 0.001$) (Fig. 6e, f).

Univariate analysis showed that exosomal S100A4 level, OPN level, tumor size, tumor capsule, and vascular invasion were significantly associated with OS and TTR of HCC patients (Table 2). Multivariate analysis showed that all of them were independent prognostic indicators for OS, and exosomal S100A4 level, OPN level, tumor size, tumor capsule, and tumor thrombus were independent predictors for TTR (Table 2). The combination of exosomal S100A4 and OPN levels had a better prognostic performance than exosomal S100A4 or OPN alone (Table 2).

**DISCUSSION**

Metastasis, causing about 90% deaths of cancer patients, is well known as the most significant characteristic of malignant cancer. Intratumor heterogeneity, which fosters tumor
Table 1. Relationship between plasma exosomal S100A4 level and clinicopathologic features

| Variable                  | Plasma exosomal S100A4 level (pg/ml) |
|---------------------------|--------------------------------------|
|                           | High (n = 84) | Low (n = 84) | P  |
|                           | No. of patients | % | No. of patients | % |
| Gender                    |               |   |               |   |
| Female                    | 14            | 16.7 | 9.5           |   |
| Male                      | 70            | 83.3 | 90.5          |   |
| Age (years)≤50            | 35            | 41.7 | 27.2          | 0.201 |
| Age (years)>50           | 49            | 58.3 | 72.8          |   |
| HBsAg                     |               |   |               |   |
| Negative                  | 6             | 7.1   | 5             | 0.755 |
| Positive                  | 78            | 92.9  | 94.0          |   |
| Cirrhosis                 |               |   |               |   |
| No                        | 13            | 15.5 | 6             | 0.088 |
| Yes                       | 71            | 84.5  | 92.9          |   |
| ALT (U/L)≤75              | 72            | 85.7  | 71            | 0.828 |
| ALT (U/L)>75             | 12            | 14.3  | 29            |   |
| AFP (ng/ml)≤20            | 15            | 17.9  | 36            | <0.001 |
| AFP (ng/ml)>20           | 69            | 82.1  | 64            |   |
| Tumor size (cm)≤5        | 30            | 35.7  | 48            | 0.005 |
| Tumor size (cm)>5        | 54            | 64.3  | 52            |   |
| Tumor number Single      | 68            | 81.0  | 73            | 0.294 |
| Tumor number Multiple    | 16            | 9.0   | 11            |   |
| Tumor capsule None       | 50            | 59.5  | 45            | 0.436 |
| Tumor capsule Complete   | 34            | 40.5  | 55            |   |
| Tumor thrombus No        | 41            | 48.8  | 55            | 0.029 |
| Tumor thrombus Yes       | 43            | 51.2  | 45            |   |
| Tumor differentiation I+II| 56           | 66.7  | 61            | 0.401 |
| Tumor differentiation III+IV| 28         | 33.3  | 23            |   |
| TNM stage                 |               |   |               | 0.045 |
| I                        | 34            | 40.5  | 47            |   |
| II+III                   | 50            | 59.5  | 37            |   |
| BCLC stage                |               |   |               | 0.087 |
| 0+I-A                    | 62            | 73.8  | 71            |   |
| B+C                      | 22            | 26.2  | 29            |   |

HBsAg hepatitis B surface antigen, HBcAb hepatitis B core antibody, AFP alpha-fetoprotein, ALT alanine aminotransferase, TNM tumor-node-metastasis, BCLC Barcelona Clinic Liver Cancer

Statistical analysis: Chi-Square
Bold values: A p-value of 0.05 or lower is considered significant

Evolution, is a key challenge for cancer treatment. Exosomes have been reported to mediate intercellular communication and serve as a powerful promotor to cancer invasiveness. Exosomes are derived from multivesicular bodies, which are formed by almost all types of cells. Tumor cells might release more exosomes than normal cells, the amount of which might be positively correlated with malignancy. Our study, based on intra-tumor heterogeneity theory, utilized HCC cell lines with different metastatic potentials for functional and mechanistic study of exosomes related HCC metastasis.

Previously published literature reported that exosomes could transfer mutated EGFRvIII protein to promote malignancy of EGFRvIII wildtype glioma, and HCC-derived exosomes could mobilize normal hepatocyte. We observed that HMH-exosomes could significantly enhance metastatic potential of MHC97-L and HepG2, both low metastatic HCC cell. This phenomenon was also observed by Wang et al. that exosomal circPTGR1 from LM3 cells could increase migratory and invasive ability of HepG2 cells. In order to determine the key functional factor in HMH-exosomes, we adopted iTRAQ spectrum mass for screening, and found that members of 4 protein families were significantly up-regulated in HMH-exosomes, including Apo, PSMA/B, EEF/EIF, and S100 calcium binding protein family. Recent papers have demonstrated that S100A protein family played key roles in cancer metastasis and pre-metastatic niche formation. In addition, S100A4 might be vital for cancer stemness and metastasis.

Consistently, our data showed that S100A4 expression in HCC cell lines and cell line derived exosomes were positively correlated with metastatic potential of these cell lines. S100A4 expression had a great influence on migration and invasion abilities of HCC cells, as well as sphere formation and tumor initiation assays. Furthermore, we obtained S100A4low and S100A4rich exosomes from S10A4 knockdown MHCC97-H cells and controlled counterparts, based on research reported by Peinado H et al. As expected, we observed that migration and invasion in vitro along with lung metastatic potential in vivo of MHCC97-L were significantly enhanced by S100A4rich exosomes, but not S100A4low exosomes. Similar results could be observed in sphere formation and tumor initiation assays.

We further selected 21 stemness-related genes based on 3 published studies and analyzed their relationship with S100A4 expression in HCC tissues (GSE39791). Ultimately, we focused on OPN, a typical HCC promotor, significantly upregulated by S100A4. Extracellular S100A4 was reported to upregulate OPN via NFκB pathway in osteosarcoma, and to maintain STAT3 phosphorylation level in neuron for functional protection of the brain. Coincidently, Yang XL et al. reported that S100A4 could promote STAT3 phosphorylation in HCC. Combined with another literature indicating that STAT3 phosphorylation might induce OPN expression in HCC, and with our experiment data, we proposed that S100A4rich exosomes could up-regulate OPN expression in low metastatic HCC cells via STAT3 phosphorylation. Our findings suggested that exosomal S100A4 induce the expression of OPN via Stat3 phosphorylation but not NFκB- signaling. This novel finding adds more information to how S100A4 mediates the metastatic progression. Interestingly, Jiao et al. reported that S100A4+ stromal cells could maintain HCC stemness, which suggests a crosstalk between inflammation and stemness. Our study suggested exosomal S100A4 could play a role in S100A4+ cell subpopulation in the crosstalk with cancer stem cells.

Mortality of HCC mostly results from high recurrence rate even after curative surgical resection. The predictive biomarkers for recurrence of HCC have great benefit for clinical decision making and follow-up procedure establishment. Recently studies showed great potential of exosomes as predictive biomarker. Wang et al. found that miR-21 is enriched in serum exosomes of HCC patients which may serve as a diagnostic biomarker. Decreased
expression of serum exosomal miR-718 was associated with recurrent HCC. In this study, we found that plasma exosomal S100A4 and plasma OPN levels were significantly associated with prognosis of HCC patients, and the combination of exosomal S100A4 and OPN had a better prognostic performance than each alone.

In summary, our findings demonstrated a vital role of exosomal S100A4 in regulating stemness and metastatic potential of HCC cells. Exosomal S100A4 released by highly metastatic HCC cells enhanced metastatic potential of low metastatic HCC cells via STAT3 phosphorylation and OPN up-regulation. Moreover, plasma exosomal S100A4 level combined with plasma OPN level was determined as a powerful prognostic predictor for postoperative HCC patients. Our study highlights a novel function of exosomal S100A4 in regulating HCC stemness and provides an insight into the participation of S100A4 in exosome-mediated communication in HCC.

### MATERIALS AND METHODS

**Cell lines**

HCC cell lines HCC-LM3, MHCC97-H, and MHCC97-L were established at the Liver Cancer Institute, Fudan University. They have genetically identical backgrounds and stepwise increasing metastatic potentials. The Huh7, PLC/PRF/5 (PLC), and HepG2 cell lines were purchased from the Shanghai cell bank, Chinese Academy of Sciences. All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Carlsbad, CA, USA) and maintained in a humidified incubator with 5% CO2 at 37°C.

### Exosomes isolation

Concerning cells were cultured in DMEM with 10% FBS until they reached 80% confluence. The cell culture medium was removed. The cells were washed once with PBS and then cultured in cell culture medium containing no serum for 48 h. Cell culture

### Table 2. Univariate and multivariate analysis of factors associated with survival and recurrence

|                                      | Overall survival | Recurrence free survival |
|--------------------------------------|------------------|--------------------------|
|                                      | HR (95%CI)       | p                        | HR (95%CI)       | p                        |
| **Univariate analysis**              |                  |                          |                  |                          |
| S100A4 (high vs. low)                | 3.056 (1.965-4.752) | <0.001               | 2.746 (1.772-4.257) | <0.001               |
| OPN (high vs. low)                   | 2.556 (1.671-3.910) | <0.001               | 1.775 (1.173-2.685) | 0.007               |
| Sex (male vs. female)                | 1.179 (0.627-2.215) | 0.609               | 0.980 (0.545-1.764) | 0.947               |
| Age (≥50 vs. <50 years)              | 0.793 (0.522-1.205) | 0.278               | 0.844 (0.554-1.287) | 0.431               |
| HBsAg (positive vs. negative)        | 0.872 (0.501-1.516) | 0.627               | 0.845 (0.486-1.470) | 0.551               |
| HBcAb (positive vs. negative)        | 1.030 (0.450-2.358) | 0.944               | 0.896 (0.391-2.055) | 0.796               |
| Cirrhosis (yes vs. no)               | 0.933 (0.496-1.754) | 0.830               | 1.172 (0.623-2.203) | 0.623               |
| ALT (>75 vs. ≤75 U/L)                | 0.798 (0.451-1.410) | 0.437               | 1.073 (0.584-1.973) | 0.819               |
| AFP (>20 vs. ≤20 ng/ml)              | 1.558 (0.965-2.519) | 0.070               | 1.504 (0.941-2.404) | 0.088               |
| Tumor size (>5 vs. ≤5 cm)            | 2.431 (1.577-3.749) | <0.001               | 1.920 (1.258-2.928) | 0.002               |
| Tumor number (multiple vs. single)    | 1.281 (0.773-2.122) | 0.337               | 0.989 (0.568-1.721) | 0.969               |
| Tumor capsule (none vs. complete)     | 1.929 (1.249-2.979) | 0.003               | 1.796 (1.168-2.762) | 0.008               |
| Tumor thrombus (yes vs. no)          | 2.511 (1.661-3.795) | <0.001               | 2.228 (1.476-3.365) | <0.001               |
| Tumor differentiationb (III-IV vs. I-II) | 1.222 (0.787-1.896) | 0.372               | 1.529 (0.988-2.367) | 0.057               |
| Combination of S100A4 and OPN         |                  |                          |                  |                          |
| Double high vs. double low           | 9.490 (4.488-20.065) | <0.001               | 5.897 (2.874-12.102) | <0.001               |
| Double high vs. (S100A4 high, OPN low) | 2.280 (1.360-3.825) | 0.002               | 1.615 (0.941-2.772) | 0.082               |
| Double high vs. (S100A4 low, OPN high) | 2.471 (1.435-4.254) | 0.001               | 1.812 (1.014-3.239) | 0.045               |
| **Multivariate analysis**             |                  |                          |                  |                          |
| S100A4 (high vs. low)                | 2.554 (1.631-3.999) | <0.001               | 2.360 (1.502-3.708) | <0.001               |
| OPN (high vs. low)                   | 2.423 (1.577-3.724) | <0.001               | 1.760 (1.153-2.685) | 0.009               |
| Tumor size (>5 vs. ≤5 cm)            | 1.730 (1.098-2.727) | 0.018               | 1.345 (0.859-2.107) | 0.195               |
| Tumor capsule (complete vs. none)     | 1.740 (1.121-2.703) | 0.014               | 1.565 (1.016-2.411) | 0.042               |
| Tumor thrombus (yes vs. no)          | 1.743 (1.132-2.685) | 0.012               | 1.766 (1.144-2.727) | 0.010               |
| **Combination of S100A4 and OPN**     |                  |                          |                  |                          |
| Double high vs. double low           | 7.697 (3.561-16.641) | <0.001               | 4.795 (2.236-10.281) | <0.001               |
| Double high vs. (S100A4 high, OPN low) | 2.265 (1.343-3.821) | 0.002               | 1.692 (0.978-2.928) | 0.060               |
| Double high vs. (S100A4 low, OPN high) | 2.150 (1.233-3.750) | 0.007               | 1.489 (0.819-2.706) | 0.192               |

**AFP** alpha-fetoprotein, **ALT** alanine aminotransferase, **HBsAg** hepatitis B surface antigen, **HBcAb** hepatitis B core antibody, **HR** hazard ratio, **CI** confidence interval

a exosomal S100A4
b Edmondson grade

1 Multivariate analysis of S100A4, OPN, Tumor size, Tumor capsule, Tumor thrombus
2 Multivariate analysis of Combination S100A4 and OPN, Tumor size, Tumor capsule, Tumor thrombus

Bold values: A p-value of 0.05 or lower is considered significant

**SPRINGER NATURE**

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medium was centrifuged at 3000 g for 15 min to eliminate cell debris. The exosomes were extracted by EXOQC50A-1 (System Biosciences, Palo Alto, CA, USA) according to manufacturer's instructions. Exosomes were isolated from plasma specimen by EXOQSTM-1 (System Biosciences), according to the manufacturer's instructions.

Briefly, 5 mL of centrifuged supernatant was mixed with 1 mL of ExoQuick-TC solution by inverting the tube several times. The sample was incubated overnight at 4 °C then centrifuged twice at 1500 g for 30 and 5 min, respectively, in order to remove the supernatant. The pellet was re-suspended in sterilized PBS, quantified by bicinchoninic acid (BCA) assays (Thermo Fisher Scientific, Waltham, MA, USA).

In vitro sphere formation, migration, and invasion assays

Sphere formation was performed by plating 1000 cells per well into 6-well ultra-low attachment plate (Corning Incorporated Life Sciences, NY, USA) in serum-free DMEM/F12 medium (Gibco), supplemented with B27 (1:50; Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), N2 (1:100; Invitrogen), 20 ng/ml bFGF, 10 ng/ml EGF (Peprotech, Rocky Hill, NJ, USA) and 20 μg/ml exosomes or 100 μl PBS as control.

Cells were incubated in a 5% CO2 incubator at 37 °C for 1 week. Intravenously injected through the tail vein twice a week. PBS Mice were sacrificed by BCA assays.

Establishment of in vivo tumor models

For the assessment of tumor initiation abilities, 1000 cells were suspended in 100 μl of PBS (HyClone) and Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). The bottom chamber was filled with DMEM containing 10% FBS as a chemoattractant. HCC cells were co-cultured with 20 μg/ml exosomes for 24 h, PBS as control. Cells, 5 × 10^4 cells for migration and 10 × 10^4 cells for invasion, in serum-free medium were seeded into the upper chamber and maintained at 37 °C in a humidified incubator containing 5% CO2. Cells that migrated to the underside of the membrane were stained with crystal violet, imaged, and counted with light microscope (×100, Leica, Wetzlar, Germany).

All the in vitro assays were conducted three times with three repetitions.

DATA AVAILABILITY

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request. Additional methods were described in the Supplementary Information.

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AUTHOR CONTRIBUTIONS

H.S., C.W., B.H. and X.G. contributed equally to this work. L.Q., Q.D. and C.W. designed experiments; H.S., C.W., B.H., X.G., Q.L., T.Z., Y.F. and Y.G. performed experiments; K.Z., X.R., M.C., S.Y., L.Y. and Y.S. analyzed the data; Q.D. and Y.Z. supervised the research; C. W. and H.S. drafted the manuscript; L.Q. revised the manuscript.

ADDITIONAL INFORMATION

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Competing interests: The authors declare no competing interests.

Ethics approval and consent to participate: All clinical samples were collected with informed consent from patients, and the research was approved by the Ethics Committee of Huashan Hospital, Fudan University (Shanghai, China). All experimental procedures involving animals were approved by The Animal Care and Use Committee of Shanghai Medical College, Fudan University, China.

REFERENCES

1. Gupta, G. P. & Massague, J. Cancer metastasis: building a framework. Cell 127, 679–695 (2006).
2. Hanahan, D. & Weinberg, R. A. Hallmarks of cancer: the next generation. Cell 144, 646–674 (2011).
3. Villanueva, A. Hepatocellular carcinoma. N. Engl. J. Med. 360, 1450–1462 (2019).
4. Liu, J., Dang, H. & Wang, X. W. The significance of intertumor and intratumor heterogeneity in liver cancer. Exp. Mol. Med. 50, e416 (2018).
5. Becker, A. et al. Extracellular vesicles in cancer: cell-to-cell mediators of metastasis. Cancer Cell 30, 836–848 (2016).
6. Colombo, M., Raposo, G. & Thery, C. Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. Annu. Rev. Cell Dev. Biol. 30, 255–289 (2014).
7. Zhu, L. et al. Isolation and characterization of exosomes for cancer research. J. Hematol. Oncol. 13, 152 (2020).
8. Al-Nedawi, K. et al. Intercellular transfer of the oncogenic receptor EGFRVIII by microvesicles derived from tumour cells. Nat. cell Biol. 10, 619–624 (2008).
9. Peinado, H. et al. Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. Nat. Med. 18, 883–891 (2012).
10. Chang, H. J. et al. Significance of PML and p53 protein as molecular prognostic markers of gallbladder carcinomas. Pathol. Oncol. Res. 13, 326–335 (2007).
11. Cui, J. F. et al. Differential proteome analysis of human hepatocellular carcinoma cell line metastasis-associated proteins. J. Cancer Res Clin. Oncol. 130, 615–622 (2004).
12. Schmidt-Hansen, B. et al. Extracellular S100A4(mts1) stimulates invasive growth of mouse endothelial cells and modulates MMP-13 matrix metalloproteinase activity. Oncogene 23, 5487–5495 (2004).
13. Dulyaninova, N. G., Malashkevich, V. N., Almo, S. C. & Brennink, A. R. Regulation of myosin-IIA assembly and Mts1 binding by heavy chain phosphorylation. Biochemistry 44, 6867–6876 (2005).
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14. Li, Z. H. & Bresnick, A. R. The S100A4 metastasis factor regulates cellular motility via a direct interaction with myosin-IIA. *Cancer Res.* **66**, S173–S180 (2006).

15. Yan, X. L. et al. Hepatocellular carcinoma-associated mesenchymal stem cells promote hepatocarcinoma progression: role of the S100A4-miR155-5OC51-MMP9 axis. *Hepatology* **57**, 2274–2286 (2013).

16. Franzen, C. A. et al. Urothelial cells undergo epithelial-to-mesenchymal transition after exposure to muscle invasive bladder cancer exosomes. *Oncogenesis* **4**, e163 (2015).

17. Emmanouilidi, A., Paladin, D., Greening, D. W. & Falasca, M. Oncogenic and non-malignant pancreatic exosome cargo reveal distinct expression of oncopgenic and prognostic factors involved in tumor invasion and metastasis. *Proteomics* **19**, e1800158 (2019).

18. Dmytryieva, O. et al. The metastasis-promoting S100A4 protein confers neuro-protection in brain injury. *Nat. Commun.* **3**, 1197 (2012).

19. Grum-Schwensen, B. et al. S100A4-neutralizing antibody suppresses spontaneous tumor progression, pre-metastatic niche formation and alters T-cell polarization balance. *BMC Cancer* **15**, 44 (2015).

20. Choi, S. I. et al. Osteopontin production by TM4SF4 signaling drives a positive feedback autocrine loop with the STAT3 pathway to maintain cancer stem cell-like properties in lung cancer cells. *Oncotarget* **8**, 101286–101297 (2017).

21. Goel, S. et al. STAT3-mediated transcriptional regulation of osteopontin in STAT3 loss-of-function related hyper IgE syndrome. *Front Immunol.* **9**, 1080 (2018).

22. McGranahan, N. & Swanton, C. Clonal heterogeneity and tumor evolution: past, present, and the future. *Cell* **168**, 613–628 (2017).

23. He, M. et al. Hepatocellular carcinoma-derived exosomes promote motility of immortalized hepatocyte through transfer of oncopgenic proteins and RNAs. *Carcinogenesis* **36**, 1008–1018 (2015).

24. Wang, G. et al. Three isomers of exosomal circPTGR1 promote hepatocellular carcinoma metastasis via the miR449a-MET pathway. *EBioMedicine* **40**, 432–445 (2019).

25. Lukandin, E. & Sleeman, J. P. Building the niche: the role of the S100 proteins in metastatic growth. *Semin. cancer Biol.* **22**, 216–225 (2012).

26. Tan, J. et al. PDK1 signaling toward PLK1-MYC activation confers oncogenic transformation, tumor-initiating cell activation, and resistance to mTOR-targeted therapy. *Cancer Discov.* **3**, 1156–1171 (2013).

27. Kim, H. et al. Human hepatocellular carcinomas with “Stemness”-related marker expression: keratin 19 expression and a poor prognosis. *Hepatology* **54**, 1707–1717 (2011).

28. Fei, F. et al. S100A4 in cancer progression and metastasis: a systematic review. *Oncotarget* **8**, 73219–73239 (2017).

29. Cao, L. et al. Osteopontin promotes a cancer stem cell-like phenotype in hepatocellular carcinoma cells via an integrin-NF-kappaB-HIF-1alpha pathway. *Oncotarget* **6**, 6627–6640 (2015).

30. Wan, S. et al. Tumor-associated macrophages produce interleukin 6 and signal via STAT3 to promote expansion of human hepatocellular carcinoma stem cells. *Gastroenterology* **147**, 1393–1404 (2014).

31. Zhang, J. et al. BMP promotes venous metastases of hepatocellular carcinoma through promoting IL-6 transcription. *Oncogene* **34**, 1575–1583 (2015).

32. Wang, C. Q. et al. Interleukin-6 enhances cancer stemness and promotes metastasis of hepatocellular carcinoma via up-regulating osteopontin expression. *Am. J. Cancer Res.* **6**, 1873–1889 (2016).

33. Ye, Q. H. et al. Predicting hepatitis B virus-positive metastatic hepatocellular carcinomas using gene expression profiling and supervised machine learning. *Nat. Med.* **9**, 416–423 (2003).

34. Berge, G. et al. Osteopontin—an important downstream effector of S100A4-mediated invasion and metastasis. *Int. J. Cancer* **129**, 780–790 (2011).

35. Wang, C. J. et al. Interferon-alpha enhances antitumor activities of oncolytic adenovirus-mediated IL-24 expression in hepatocellular carcinoma. *Mol. cancer* **11**, 31 (2012).

36. Jiao, J. et al. Depletion of S100A4(+) stromal cells does not prevent HCC development but reduces the stem cell-like phenotype of the tumors. *Exp. Mol. Med.* **50**, e422 (2018).

37. Minguex, B. et al. Gene-expression signature of vascular invasion in hepatocellular carcinoma. *J. Hepatol.* **55**, 1325–1331 (2011).

38. Wang, H. et al. Expression of serum exosomal microRNA-21 in human hepatocellular carcinoma. *Biomed. Res Int.* **2014**, 864894 (2014).

39. Sugimachi, K. et al. Identification of a bona fide microRNA biomarker in serum exosomes that predicts hepatocellular carcinoma recurrence after liver transplantation. *Br. J. Cancer* **112**, 532–538 (2015).

40. Li, Y. et al. Establishment of cell clones with different metastatic potential from the metastatic hepatocellular carcinoma cell line MHCC97. *World J. Gastroenterol.* **7**, 630–636 (2001).