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Expression Characteristics of SODD and ALG-2 as Possible Biomarkers for Evaluating Lymphatic Metastasis Potential of Hepatocarcinoma in a Mouse Model

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ABSTRACT

Annexin A7 has been confirmed in our previous research to be an important factor in lymph node metastasis (LNM) of hepatocellular carcinoma (HCC). SODD and ALG-2 are the binding proteins of Annexin A7 and can work in protein complexes. The present study was carried out with the constructed cell lines in mouse model of metastasis for further elaboration of possible mechanisms and identification of associated genes in the LNM of HCC. This experiment used inbred Chinese 615 mice, as well as Hca-F and Hca-P cells. Quantification of the relative messenger RNA (mRNA) expression of SODD and ALG-2 was realized by using qRT-PCR. Quantification of the protein expressions of SODD and ALG-2 was achieved by using western blot. Experimental mice (n=160) (6-8weeks old, 18-22g, SCXK [LIAO] 2008-0002) were randomly classified into four groups equally, which were separately inoculated with Hca-F, Hca-P, FAnxa7-upregulated, and PAnxa7-upregulated cells. Serum levels of SODD and ALG-2 were measured by ELISA. Immunohistochemical analysis of SODD and ALG-2 was further conducted. Tumor LNM-related factors of SODD and ALG-2 showed the same tendency in their expression correspondingly with the up-regulated expression of Annexin A7. Our experiment further explored the roles of SODD and ALG-2 based on Annexin A7 up-regulation vectors construction and the establishment of corresponding controls in vivo. Furthermore, the mouse model of primary tumors was constructed by injecting Hca-F, FAnxa7-upregulated and Hca-P, PAnxa7-upregulated cells into the mouse footpad. Mice were sacrificed at the designated time points for detecting SODD and ALG-2 expression in tumor tissue and serum samples. Collectively, our work indicates SODD in tumors and in serum and ALG-2 in serum are valuable in evaluating LNM in mice with HCC.

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Introduction

Hepatocellular carcinoma (HCC) has been recognized to be a worldwide public health concern (1, 2). Lymph node metastasis (LNM) is a major prognostic factor for many tumor types (3-6). However, there is an absence of an accurate assay to predict the LNM potential of HCC.

Acting as two common mouse hepatocarcinoma ascites synergetic cell lines, Hca-F and Hca-P cells have been identified to be beneficial for constructing ideal models for studying LNM of HCC (7-10). According to our previous research outcome, Annexin A7 has a role in tumors related to LNM. The suppressor of death domains (SODD) is a 60kDa protein that plays important role in various activities of cells, such as apoptosis (11). Meanwhile, the apoptosis linked gene-2 (ALG-2) is a 22 kDa protein, and is the most conserved protein among the penta-EF-hand (PEF) protein family, with the detection of extensive distribution of its homologs in eukaryotes (12). Significantly, Annexin A7 can interact with both SODD and ALG-2 (13, 14). However, there is a limited understanding concerning the mechanism of how Annexin A7, SODD and ALG-2 regulate tumors. In this regard, research on binding proteins (SODD and ALG-2) of Annexin A7 may contribute to revealing the role of Annexin A7 in the mechanism of tumorigenesis and LNM, so as to exert potential therapeutic benefit in human tumors.

In order to gain an insight into the role of SODD and ALG-2 with Annexin A7 playing in Hca-F and Hca-P cells on the basis of our prior research (15), through the construction of Annexin A7cDNA vectors to upregulate ('Knock-in') the expression of Annexin A7, our experiment established two cell lines with stable 'Knock-in' of Annexin A7 in HCC cells (FAnxa7-upregulated and PAnxa7-upregulated cells) (16).

On the basis of the above, for further elaboration of possible mechanism and identification of associated genes in LNM of HCC, the present study was carried out with the constructed cell lines in mouse model of metastasis. Our

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study focused on the following key points:

·To observe the expression of SODD and ALG-2 after up-regulation of Annexin A7 in vitro;

·To inject constructed vectors into the experimental mice for the observation of subsequent effects;

·To observe the dynamic changes of SODD and ALG-2 in primary tumors in different tumor groups, and to clarify the correlation with LNM potential;

·To detect the concentration of SODD and ALG-2 in the serum in different tumor groups and to identify the correlation with LNM potential.

Materials and methods

Ethics statement

This experiment used inbred Chinese 615 mice provided by the SPF Experimental Animal Center of Dalian Medical University. Mice were raised and treated routinely in accordance with relevant protocols formulated to standardize the use of experimental animals in the laboratory. All procedures related to the experiment have been approved by the Experimental Animal Ethical Committee of Dalian Medical University (Permit No.: L2012012).

Cell line and cell culture

Hca-F and Hca-P cells were established and maintained in our laboratory, as described before (17, 18). The cells were cultured in 90% RPMI 1640 (Gibco, USA) supplemented with 10% fetal bovine serum (FBS; PAA, USA) and maintained in a humidified incubator under a 5% CO_2 atmosphere at 37°C. For cell populations related to the transfection of Annexin A7 gene, cells were cultured in a similar condition, except for cultivation in RPMI-1640 supplemented with 20% FBS and 400μg/ml G418 (Gibco, USA).

Quantification of the relative messenger RNA (mRNA) expression of Sodd and Alg-2

TRIzol (Invitrogen, USA) was used for the extraction of total RNA from cells, followed by reverse transcription with PrimeScript ® RT reagent kit. Gene transcripts were then quantified by qRT-PCR using SYBR® Premix Ex TaqTM II (Takara, Japan). The detected quantitative gene expressions of Sodd and Alg-2 were normalized with that of GAPDH. The primer sequences for Sodd, Alg-2 and GAPDH were shown in Table 1. Following the undermentioned conditions, PCR was performed: (1) predenaturation (30s, 95°C); (2) denaturation, (5s, 95°C), and annealing (30s, 60°C), in a total of 40 cycles. By using software MXP for analysis, the differences in mRNA expressions of the detected genes were calculated following the ΔΔCt method (19) and data were displayed as $2^{-\Delta\Delta Ct}$.

Table1. The primer sequences for *Sodd*, *Alg-2* and GAPDH

Genes	Primer sequences
Sodd	Forward: 5′-GGTAACAGCCCAACTCCAATGTC -3′
	Reverse: 5′-AGGCATCGTCTTGTGGTCGTC -3′
Alg-2	Forward: 5′-ATGAGCTTCAGCAAGCATTATCCA-3′
	Reverse: 5′-TCCCAGAGTTGTCCCTGTCGTAG-3′
GAPDH	Forward: 5′-AAGGGTTTGGGACAGACGA-3′
	Reverse: 5′-CATGAACAGCGCAAGGATTA-3′

Quantification of the protein expressions of SODD and ALG-2

After the extraction of total protein from cells and primary tumor tissues, protein concentration determination was realized by BCA protein measuring kit (Thermo, USA). Samples of protein prepared in an equal amount/volume were loaded onto a PAGE gel, which were then trans-blotted a PVDF membrane (Millipore, USA) after the above reaction. The next step was labeling with primary antibodies including SODD (Abcam, England; 1:1000), ALG-2 (1:1500; Abcam, England) and GAPDH (1:1000; ZSGB-Bio, China; internal reference) overnight at 4°C, respectively. Odyssey infrared imaging system (LI-COR, USA) was the tool to visualize the results.

Animal studies

Experimental mice (n=160) (6-8 week old, 18-22g, SCXK [LIAO] 2008-0002) were randomly classified into four groups equally, which were separately inoculated with Hca-F, Hca-P, FAnxa7-upregulated, and PAnxa7-upregulated cells for subsequent experiments, with the specific procedures presented in our previous study (20). In addition, 10 mice were used as the control. The experimental model was constructed by subcutaneous injection the prepared cell suspension (2×106 cells/mouse in 0.05 ml PBS) with the cell viability maintained at over 95 % into the left footpad of mice. Mice in the control group received an injection of 0.05 ml PBS in the same way. After modeling, blood samples were collected per week regularly from mice in each group via retro-orbital puncture. After incubation of the obtained samples at 37°C for 1 h, serum was collected upon centrifugation in two times (10 min per time) at 1000 g at 4 °C. After that, the prepared samples were separated into 100μL aliquots and frozen at -80°C immediately or placed in liquid nitrogen. Mice in the experimental groups were killed at the designated time points (2, 3, 4, 5, 6, 7, and 8 weeks) for sampling. At least three mice were included in each group at different time points. At the same time, in the process of sampling, the primary tumor, popliteal, inguinal and iliac lymph node were removed from the sacrificed mice, which were then subjected to H&E staining and immunohistochemical staining or frozen quickly in liquid nitrogen for Western blot after weighing and fixation in 10 % buffered formalin.

The potency of LNM was assessed by prior research steps (16), with the corresponding *in vivo* experiment repeated three times.

ELISA

Before the experiment, serum samples were taken out and defrosted completely at 37°C for about 1 h. SODD (sensitivity: 5.8pg·ml−1) and ALG-2 (sensitivity: 3.9 pg·ml−1) concentrations were determined in duplicate using mouse SODD and mouse ALG-2 ELISA kits (CUSABIO Co., Ltd., China) with the instruction of the manufacturer followed, respectively. The standard curve range was 23.5-1500 pg·ml−1 and 15.6-1000 pg·ml−1 for SODD and ALG-2, respectively. Data were read with Multiskan Go spectrophotometer (Thermo, USA) at the wavelength of 450 nm. The concentrations of SODD and ALG-2 were determined based on the plotted absorbance value of the standards and the test samples in the standard curve.

Immunohistochemical analysis

Immunohistochemistry was performed by using the primary antibodies of SODD (1:100) and ALG-2 (1:50). This experiment was conducted with the use of appropriate second-step antibodies. Quantification of the expressions of SODD and ALG-2 was realized based on the intensity of staining and the uniformity of staining in the nucleus and cytoplasm (15).

Statistical analysis

Each assay was performed three times. Data were expressed as the mean ± standard deviation (SD) and were analyzed by SPSS 17 software. T-test after one-way analysis of variance (ANOVA) was used to determine statistical differences between groups. Statistical significance was set at $P < 0.05$.

Results and discussion

Quantification of Sodd and Alg-2 at mRNA and protein levels in Hca-F/Hca-P cells

Fig.1A shows the expressions of Sodd and Alg-2 at mRNA levels (Fig.1A). With Hca-P set as the calibrator in software MxPro, the relative quantity of each gene in Hca-P was regarded as 1 in each experiment. As a result, the mRNA expressions of Sodd and Alg-2 were higher in Hca-F cells than those in Hca-P cells. Meanwhile, the protein expressions of Sodd and Alg-2 expressions were measured by Western blot (Fig.1B).

In Hca-P/Hca-F cells, Sodd/GAPDH was 0.84±0.18 versus 1.75±0.13, and Alg-2/GAPDH was 0.98±0.14 versus 1.82±0.30, respectively. Both proteins were found to be lowly expressed significantly in Hca-P cells than those in Hca-F cells. It may support our hypothesis that Sodd and Alg-2 may have a relationship with tumor LNM.

Effect of Sodd and Alg-2 after up-regulation of Annexin A7 in vitro

The mRNA expressions of Sodd and Alg-2 increased greatly in FAnxa7-upregulated and PAnxa7-upregulated cells when compared to those in Hca-F and Hca-P cells, with similar change trends observed at the protein levels (all $P < 0.05$). However, there was no evident difference of Sodd and Alg-2 expressions when compared between FAnxa7-control and Hca-F cells, or between PAnxa7-control and Hca-P cells (all $P > 0.05$) (Fig. 1C and D). In this regard, the up-regulation of Annexin A7 may increase the expression levels of Sodd and Alg-2.

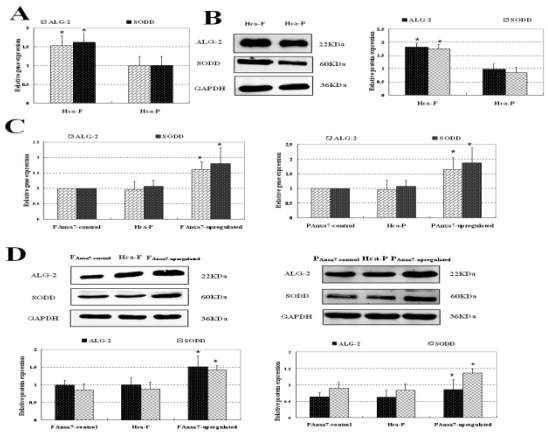

Figure 1. The mRNA and protein expressions of SODD and ALG-2; (A) Relative mRNA expression of *Sodd* and *Alg-2* in Hca-F and Hca-P cells; Data are presented as $2^{(-\Delta\Delta Ct)}$ ±SD and normalized to P cells (**P*<0.05). (B) SODD and ALG-2 protein expressions in Hca-F and Hca-P. Quantitative mRNA expressions of *Sodd* and *Alg-2* were normalized with that of GAPDH. (C) Relative mRNA expression of *Sodd* and *Alg-2* in Hca-F and $F_{Anxa7\text{-upregulated}}$ as well as Hca-P and $P_{Anxa7\text{-upregulated}}$ cells. Data are presented as $2^{(-\Delta\Delta Ct)}$ ±SD normalized to $P_{Anxa7\text{-control}}$. (**P*<0.05); (D) SODD and ALG-2 protein expressions in Hca-F and $F_{Anxa7\text{-upregulated}}$ as well as Hca-P and $P_{Anxa7\text{-upregulated}}$ cells; Quantitative protein expressions of SODD and ALG-2 were normalized with that of GAPDH. Western blot images were representative results based on three replicates of experiments separately. (**P*<0.05)

Effect of the up-regulation of Annexin A7 on LNM in HCC mouse model

For in vivo evaluation of the impact of Annexin A7 up-regulation, the aforementioned four cell lines were examined in the constructed mouse model. All mice were confirmed to have primary tumors after modeling. LNM was observed in the modeled mice at the designated time points (2, 3, 4, 5, 6, 7 and 8 weeks) (Fig. 2A). LNM potency was evaluated at 4 weeks according to our previous work [20]. Consequently, LNM rate of Hca-F group and FAnxa7-upregulated group was 80% and 40%, respectively (Fig. 2A); while that was 0% and 20% in the PAnxa7-

upregulated group and Hca-P group, respectively (Fig. 2A). It suggested that Annexin A7 protein expression could reduce LNM upon its up-regulation in Hca-F and Hca-P cells. However, there was a gradual increase in the potential of LNM along with the progression of tumors. In order to better detect the dynamic protein expression changes of SODD and ALG-2 as well as LNM potential, LNM status was detected continuously until 8 weeks (Fig. 2A).

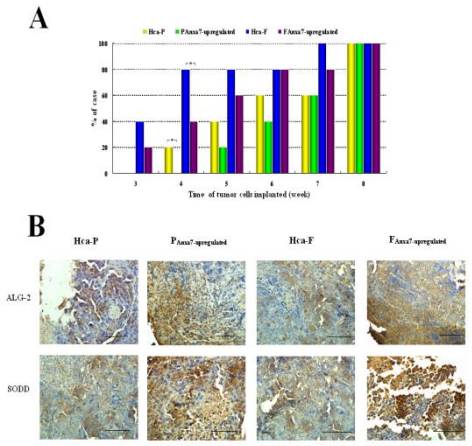

Figure 2. Status of lymph node metastasis and immunohistochemistry staining for SODD and ALG-2. (A) Status of lymph node metastasis during 8 weeks after tumor injection. (**P*<0.05). (B) Immunohistochemistry staining for SODD and ALG-2 in each tumor group. Positive expression of SODD and ALG-2 were defined as the presence of brown color in the cytoplasm, (magnification, ×200; n=20; Bar = 200 μm).

Dynamic protein expression changes of SODD and ALG-2 in modeled mouse tissues and correlation with LNM potential

For a clear presentation of the protein expression trends of SODD and ALG-2 during tumor progression and metastasis, the tumors of modeled mice were excised at the designated time points after the implantation of different cell lines in mice. The expression and subcellular localization of SODD and ALG-2 were analyzed firstly in modeled mouse tissues. The cytosol was observed to be the primary subcellular localization of SODD protein, with partial localization in the cell membrane as well; while ALG-2 protein was located mainly in the cytosol (Fig. 2B). In view of the protein expression levels of SODD and ALG-2 detected by Western blot, the expression of SODD appeared in Hca-P and Hca-F tumor tissues since the fourth week (Fig. 3C) and the third week (Fig. 3A), respectively; which, however, appeared since the second week in PAnxa7-upregulated and FAnxa7-upregulated tumor tissues (Fig.3B and 3D). A fluctuation was observed in the expression of SODD, which was gradual increased until 8 week (Fig.3). Similarly, the expression of ALG-2 was also fluctuant, showing a gradual increase from 2 to 8 weeks, which was in parallel with LNM potential (Fig3).

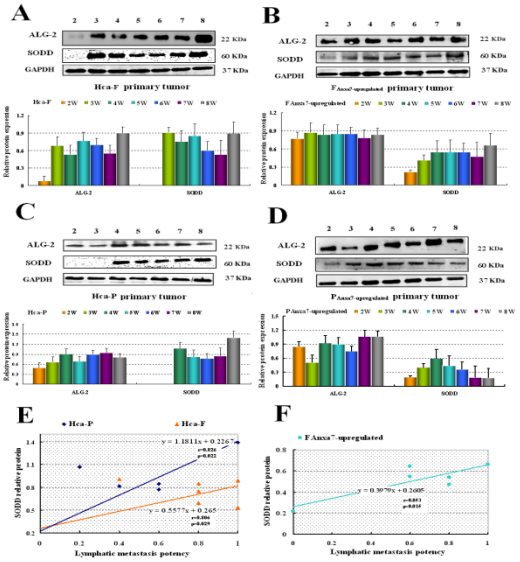

Figure 3. Expression of SODD and ALG-2 protein in primary tumors of modeled mice by western blot; (A-D) Protein expressions of SODD and ALG-2 in Hca-F (A) and $F_{Anxa7\text{-upregulated}}$ (B) as well as Hca-P (C) and $P_{Anxa7\text{-upregulated}}$ (D) groups, respectively. Quantitative protein expressions of SODD and ALG-2 were normalized with that of GAPDH, which were then analyzed by Gel-Pro Analyzer 4.0 software. Data are presented as mean ± SD. (E-F) Correlation of SODD protein expression in primary tumor tissue with lymph node metastasis potential, with the observation of statistically significant linear regression line.

Furthermore, a positive correlation was confirmed between SODD expression and LNM potential in different tumor groups except PAnxa7-upregulated group (Fig. 3E and 3F). Nevertheless, there was no correlation between ALG-2 expression and LNM potential in all different tumor groups.

Dynamic expression of SODD and ALG-2 in modeled mouse serum and correlation with LNM potential

In the control group, there was no detection of the serum concentration of SODD and ALG-2. The serum concentration of SODD appeared in Hca-P group from the fourth week (Fig. 4A) and from the third week in Hca-F group (Fig. 4A); and it was observed since the second week in both PAnxa7-upregulated and FAnxa7-upregulated groups (Fig. 4A). There was an obvious increase of serum SODD level, which was associated with the increased LNM potential in all different tumor groups (Fig. 4A). Simultaneously, in all the designated time points and in all different tumor groups, a gradual increase of serum ALG-2 level was also observed with the elevation in LNM potential from the second week (Fig. 4B).

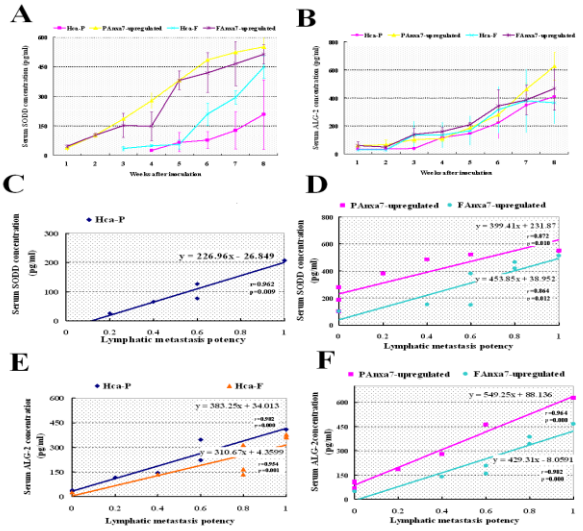

Figure 4. Expression levels of SODD and ALG-2 in serum of modeled mice using ELISA in each group at various time points. (A) SODD and (B) ALG-2 concentration in serum; Data are presented as mean ± SD. (C-D) Correlation of serum SODD and (E-F) that of serum ALG-2 concentrations with LNM potential. Statistically significant linear correlation line is shown.

In addition, there was a positive correlation between serum SODD concentration and LNM potential in different tumor groups except Hca-F group (Fig. 4C and 4D). Besides, a direct correlation was found between serum ALG-2 concentration and LNM potential in all different tumor groups (Fig. 4E and 4F). Collectively, serum SODD/ALG-2 concentration had the same tendency with the elevation of LNM potential.

HCC has caused a serious impact on human health and life, which endangers people's safety greatly since it ranks the fifth of tumor incidence globally and in the third position of cancer-related mortality worldwide (21, 22). Metastasis is still the major concern that causes death in patients suffering from HCC. Failure or low therapeutic efficacy has been recognized to be attributed to metastasis of HCC significantly. Multiple studies in the past concerning the metastasis of HCC have report that there do exist various molecular factors related to the metastasis of HCC (13, 14, 23, 24). Indeed, it is quite important to explore the internal molecular mechanism of HCC metastasis, which, however, is still unclear and there is an absence of effectual biomarkers to reveal this issue.

Considering the crucial role of LNM in HCC metastasis, our study continued to emphasize on exploring its involvement in HCC through in vivo and in vitro experiments. As it has been demonstrated in our previous research, there are higher mRNA and protein expressions of ANXA7 gene in Hca-F than in those in Hca-P cells (9, 17, 18, 25). In fact, both Hca-F and Hca-P cells are common modeling targets for hepatocarcinoma, both of which are from the same parent cell line, yet with different LNM potential. The LNM ability of Hca-F and Hca-P is about 75% and 25%, respectively, which are the ideal models for research on LNM. In this study, quantification of the mRNA and protein levels based on qRT-PCR and western blot were performed firstly, with the identification of the higher expression levels of Sodd and Alg-2 in Hca-F cells than those in Hca-P cells. Furthermore, as our previous research outcome supported, there was an involvement of Annexin A7 in LNM in vitro. Similarly, in our current work, Sodd and Alg-2, which are related to LNM, showed the same expression trends, which were in parallel with Annexin A7 expression following the alteration of Annexin A7 expression in vitro. These results may indicate both Sodd and Alg-2 may be involved in tumor LNM of HCC.

Furthermore, our study investigated the effects of Annexin A7 on HCC LNM in mouse model with the treatment of the constructed cDNA vectors to upregulate ('Knock-in') Annexin A7 expression, with Hca-F and Hca-P as the control, respectively. Consequently, after the upregulation of Annexin A7 expression, LNM rate was decreased from 20% to 0% in Hca-P cells, while from 80% to 40% in Hca-F cells, respectively. These in vivo data may provide evidence to support the critical role of Annexin A7 in HCC LNM. Besides, data provided above implied that Annexin A7 may inhibit the progression of tumors, acting as a tumor suppressor. For instance, Leighton *et al*. also reported Annexin A7 as a tumor suppressor in human prostate cancer and in Anxa7 haploinsufficient mice (26). Importantly, in terms of the association of Annexin A7 with Sodd and Alg-2, Annexin A7 have pro-beta helices repeats rich in glycine, typosice and proline within the N-terminal region, which may provide possible binding sites for both Sodd and Alg-2 (27, 28). In our study, Sodd and Alg-2 were both verified to be highly expressed in FAnxa7-upregulated and PAnxa7-upregulated cells, which may exert a synthetic, affect together Annexin A7 in LNM of HCC. Despite these findings in our present research, there is still a need to elaborate the specific mechanisms of the interaction among Anneixin A7, Sodd and Alg-2.

Further exploration was continued to further explore the expression characteristics of SODD and ALG-2 in tumor tissues of modeled mice. As results, SODD protein concentrated primarily in cytosol in terms of its subcellular localization and in cell membrane partially. Moreover, the expression of SODD was associated with LNM potential. In addition, the expression trends by immunohistochemistry were confirmed by western blot. Furthermore, the expression of SODD appeared from the fourth week while from the third week in tumor tissues of Hca-P group and Hca-F group, respectively, while it was observed since the second week in both PAnxa7-upregulated and FAnxa7-upregulated groups. Besides, there was a fluctuation of the

expression of SODD, showing a gradual increase until 8 weeks with the progression of LNM. A positive correlation was also confirmed between SODD and LNM potential in different tumor groups except for PAnxa7-upregulated group. Similarly, one previous experiment also stressed the important role of up-regulating SODD in distant metastasis of pancreatic cancer (29). Simultaneously, as it is known to all, SODD is a chaperone-regulating protein, also known as BAG-4, belonging to the family of BAG. Similar to the results observed in our study, one past research revealed that BAG4 (or SODD) was evidently highly expressed in HCC tissue than that in matched non-cancerous liver, and an exogenous downregulation of its expression would result in the increased apoptosis of HCC, so as to inhibit the progression of HCC (30). At the same time, ALG-2 protein was also localized mainly in the cytosol, showing a fluctuation in its expression as well, which increased gradually from 2 to 8 weeks in parallel with LNM potential, while no correlation was found between ALG-2 and LNM potential in all different tumor groups. Similarly, up-regulation of ALG-2 was also reported to be crucial in the distant metastasis of lung cancer (31).

The levels of SODD and ALG-2 in serum were also explored in our study. According to the results, serum SODD levels had a similar tendency of change with those observed in tumors. A positive correlation was confirmed between serum SODD level and LNM potential in tumor groups except for Hca-F group. Similarly, the changing trend of serum ALG-2 levels was consistent with that of the expression of ALG-2 in tumors, while a direct correlation was noticed between serum ALG-2 level and LNM potential in all different groups. Indeed, comparative proteome analyses have identified various metastasis-associated biomarkers, the majority of which, however, were conducted using HCC tissues and cell lines (32, 33). It shall be noted that biomarkers which are highly valuable for screening and monitoring tumor progression shall also be detectable in serum as well. The fact is that owing to the absence of high sensitivity and specificity, there are few of biomarkers in serum that are available for monitoring metastasis in the clinical setting (34). Significantly in our study, both SODD and ALG-2 were identified to be novel serum biomarkers for HCC metastasis using homograft mouse model.

In summary, findings in our study suggest that Annexin A7 and its protein complexes with SODD and ALG-2 may have an influence on mouse hepatocarcinoma LNM both in vitro and in vivo. SODD and ALG-2 may be new biomarkers for predicting LNM potential of HCC. Anyway, further investigation is required for in-depth and comprehensive confirmation of the dynamic alterations of SODD and ALG-2 and theirs precise role in metastasis of HCC.

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Authors' contributions

SW conceived and designed the study, and drafted the manuscript. FG, YS and XY collected, analyzed and interpreted the experimental data. QB, JT and XW revised the manuscript for important intellectual content. All authors read and approved the final manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

The study was approved by the Ethical Committee of Dalian Medical University and conducted in accordance with the ethical standards.

Informed consent

Not applicable.

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