doi:10.4149/neo_2021_210118N81

Co-expression of CD24 and Hsp70 as a prognostic biomarker for lung cancer

Xiao-Juan QIAO[1], Yan GU[2], Hua DU[3], Zhi-Wei XING[1], Bu-Huan MA[1], Wei-Ting SUN[1], Jing-Feng YU[4], Cai-Xia LIU[1,*]

[1]Department of Medical Oncology, The Affiliated Hospital of Inner Mongolia Medical University, Inner Mongolia Medical University, Hohhot, China; [2]Department of Respiratory Medicine, The Affiliated Hospital of Inner Mongolia Medical University, Inner Mongolia Medical University, Hohhot, China; [3]Department of Pathology, The Affiliated Hospital of Inner Mongolia Medical University, Inner Mongolia Medical University, Hohhot, China; [4]College of Basic Medicine, Inner Mongolia Medical University, Hohhot, China

**Correspondence: qq13314876607@163.com*

Received January 18, 2021 / Accepted May 18, 2021

Lung cancer is one of the most common malignant neoplasms worldwide. CD24 is a marker of tumor stem cells that plays an important role in tumorigenesis. Hsp70 is an important molecular chaperone. However, the co-expression and interaction of CD24 and Hsp70, as well as the significance for the prognosis of lung cancer are still unclear. The expression levels of CD24 and Hsp70 were detected by immunohistochemistry and their correlation was analyzed. The expression levels of CD24 mRNA and protein were examined using qRT-PCR and western blotting in SPCA1, A549, H1975, and H1650 cell lines. A CD24-overexpressing cell model was established. The interaction between CD24 and Hsp70 was verified by co-immunoprecipitation and western blotting. CD24 and Hsp70 expression were significantly higher in lung cancer tissues than in adjacent tissues (CD24: $p=0.008$; Hsp70: $p<0.001$). CD24 protein expression showed a positive correlation with lymph node metastasis, TNM stage, and vascular cancer thrombus. Hsp70 protein expression showed a positive correlation with differentiation, lymph node metastasis, and TNM stage. CD24 and Hsp70 high expression were also correlated with poor survival. The positive co-expression rate of CD24 and Hsp70 in lung cancer tissues was 52.7% (49/93). CD24 and Hsp70 expression in lung cancer were positively correlated ($r=0.368$, $p<0.001$), and co-immunoprecipitation was verified that both endogenous and exogenous CD24 co-precipitated with Hsp70 directly or indirectly. When Hsp70 inhibitor VER15508 was added to A549 cells, Hsp70 and CD24 protein expression were significantly decreased. The present study demonstrated that CD24 and Hsp70 were highly expressed in lung cancer tissues, and associated with invasion, metastasis, and poor survival. Hsp70 may regulate CD24 expression. Co-expression of CD24 and Hsp70 may be a prognostic biomarker for lung cancer.

Key words: CD24, Hsp70, lung cancer, interaction

Lung cancer is one of the most common tumors, with the highest morbidity and mortality rates worldwide. The effective treatment for advanced lung cancer with distant metastasis is lacking, and the 5-year survival rate of these patients remains at 15–30% [1]. Recently, there has been increasing support for the cancer stem cell (CSC) theory, which states that tumor recurrence and metastasis stem from the activation of CSCs. CD24 (cluster of differentiation 24) is a tumor stem cell marker that is highly expressed in many tumors [2–4], and CD24 expression levels are correlated with tumor invasion, metastasis, and prognosis. CD24 is a glycosylphosphatidylinositol-anchored membrane protein that lacks intracellular structure and is unable to transmit transmembrane signals. Lipid rafts are microregions in the cell membrane and platforms for transmembrane signaling [5], which may explain the role of CD24 in transmembrane signaling pathways. Therefore, we screen for new molecules interacting with CD24 to study how it regulates tumor invasion and metastasis. It was found that high CD24 expression was significantly associated with worse survival in breast cancer patients. CD24 may play a role in tumorigenesis and cancer progression. CD24 overexpression was significantly associated with shortened overall survival and disease-free survival [6].

Constitutively expressed heat shock protein 70 (Hsp70) is a kind of highly conserved protein produced under stress. Hsp70, as a molecular chaperone, plays an important role in the process of protein folding, assembly, degradation, and controlling the activity of regulatory proteins. In some rodent models, high Hsp70 expression increases tumor growth and metastatic potential. Depletion or inhibition of Hsp70 frequently reduces the size of the tumors and even causes their complete involution [7]. It can regulate tumor cell apoptosis in different links during the programmed cell

death pathway and can affect endogenous and exogenous apoptosis [8]. Studies reported that CD24 interacts with heat shock proteins (Hsps) and may be an Hsp client [9].

This study was done to examine CD24 and Hsp70 expression in paraffin sections of clinical lung cancer tissues by immunohistochemical staining and to analyze the co-expression and interaction of CD24 and Hsp70, as well as the prognosis of lung cancer. We hope to provide new ideas and experimental basis for the treatment of lung cancer and to lay the foundation for the later research on the interaction between Hsp70 and its client protein CD24, which may activate downstream signal molecules and affect the biological characteristics of lung tumorigenesis.

Patients and methods

Patient samples. Samples were collected from 93 lung cancer patients who were treated at the Affiliated Hospital of Inner Mongolia Medical University between March 1, 2013 and June 30, 2014. The study was approved by the ethics committee of the Affiliated Hospital of Inner Mongolia Medical University (Inner Mongolia, China; approval no. YKD2019098), and informed consent was signed by all patients. All patients were diagnosed with lung cancer according to pathological characteristics. The study cohort included 73 male and 20 female patients, among whom the age range was 30–75 years, with an average age of (61.1±7.77) years and a median age of 62 years. According to the 7th edition of the AJCC cancer staging manual published in 2010, 34 cases were stage I, 29 cases were stage II, and 30 cases were stage III. The pathological types were: 30 cases of adenocarcinoma, 47 cases of squamous cell carcinoma, and 16 cases of other types (including adenoid cystic carcinoma, sarcomatous carcinoma, salivary gland carcinoma). The patients had not received radiotherapy, chemotherapy, or immunotherapy before surgery. Tumor tissues and normal tissues 5 cm away from tumor tissues were immediately obtained after surgery.

Cell lines. The human lung cancer cell lines SPCA1, A549, H1975, and H1650 were purchased from Shanghai Guandao Bioengineering Co., Ltd. (Shanghai, China).

Reagents and instruments. Mouse anti-human CD24 monoclonal antibody was purchased from Abcam (Cambridge, UK). Rabbit anti-human Hsp70 monoclonal antibody (11660-H07B) was purchased from Sino Biological Co., Ltd. (Beijing, China). MaxVision™ kit and DAB developer were purchased from Fuzhou Maixin Biotechnology Co., Ltd. (Fuzhou, China). The CD24 overexpressing plasmid pLenti-CMV-CD24-Flag-GFP-Puro was purchased from PPL Therapeutics Inc. (Blacksburg, VA, USA). Protein A/G immunoprecipitation magnetic beads were purchased from Bimake (Houston, TX, USA). Puromycin was from Sigma-Aldrich (St. Louis, MO, USA) and the Hsp70 inhibitor VER155008 was purchased from Dalian Meilun Biotechnology Co., Ltd. (Dalian, China). The BCA protein quantitation kit was purchased from Nanjing Biyuntian Biotechnology Co., Ltd. (Nanjing, China), and rat anti-human GAPDH monoclonal antibody was purchased from Cell Signaling Technology (Danvers, MA, USA). TRIzol reagent was purchased from Invitrogen (Carlsbad, CA, USA), and the cDNA synthesis kit was the product of Tiangen Biotech (Shanghai, China).

The Smartscape 2002 biological micrograph analysis system was purchased from Shanghai Furi Technology Co., Ltd. (Shanghai, China), and an Olympus CX41 optical microscope was used for imaging (Tokyo, Japan). The CO_2 thermoelectric incubator was from Thermo Fisher Scientific (Waltham, MA, USA). The stable and steady current electrophoresis apparatus was purchased from Bio-Rad (Hercules, CA, USA). The thermal cycler Veriti96 was used for PCR and was the product of Applied Biosystems (Thermo Fisher Scientific). The automatic gel imaging analyzer (BG-GDSAUTO) was the product of Beijing Biotechnology Co., Ltd. (Beijing, China).

Immunohistochemical staining and analysis. Immunohistochemistry was performed strictly in accordance with the kit's instructions. Briefly, paraffin sections were dewaxed with xylene, rehydrated in a gradient alcohol series, washed with PBS three times for 3 min, incubated with 3% H_2O_2, incubated at room temperature for 10 min, and washed with PBS again three times for 3 min. Antigens were restored with citrate buffer, and then the sections were washed with PBS three times for 3 min, and then incubated with mouse anti-human CD24 monoclonal antibody (1:100) (LS-C35485, LSBio, Inc, USA) and rabbit anti-human Hsp70 monoclonal antibody (1:700) (ab181606, Abcam, USA) overnight at 4 °C. The following day, the sections were washed with PBS three times for 3 min, and then incubated with the secondary antibody at room temperature for 15 min. After washing with PBS three times for 3 min, the sections were incubated with DAB for color rendering for 3 min, washed with flowing water, re-stained with hematoxylin, dehydrated, cleared, and sealed with neutral gum. Pathological diagnoses were made independently by two pathologists. A known positive tissue was used as a positive control, and PBS was used as a negative control for antibody staining. Positive CD24 expression was noted as yellow or brownish-yellow granules, found along the cell membrane or in the cytoplasm. Positive Hsp70 protein expression was noted when the staining was observed in the cytoplasm, a small amount in the nucleus or membrane. Sections were scored according to the proportion of stained cells and the intensity of staining. Under a 400× light microscope, 5–10 fields were randomly selected, in which 100–200 cancer cells were counted. Those with yellow cytoplasmic or nuclear staining were positive, and the percentage of positive cells was calculated. The sections were scored between 1–5 according to the percentage of positive cells: 0 (<5%), 1 (5–25%), 2 (26–50%), 3 (51–75%), and 4 (>75%). The sections were also scored based on staining intensity as: 1 (light yellow), 2 (yellow or dark yellow), and 3 (brown). CD24 protein expression was positive when the

product of the scores was more than 1. Scores between 1–3 were weak positive (+), between 4–6 were positive (++), and >6 were strong positive (+++). For the scores, (−) and (+) were negative, while (++) and (+++) were positive.

***In vitro* transfection.** Cells in the logarithmic growth phase and in good condition were digested with trypsin, collected, and centrifuged at 400×g for 5 min. Then, the cells were resuspended in 1 ml of complete medium to prepare a single-cell suspension. The cell density was 2×10^6/ml. Lentivirus was placed on ice and dissolved for use. According to the instruction provided by PPL Therapeutics Inc., shRNA-Hsp70, pLenti-CMV-CD24-Flag-GFP-Puro virus, or sham vehicle were transfected into the cells. Positive cells were selected by puromycin resistance, and stable cells lines with CD24 overexpression and shRNA-Hsp70 were established.

Quantitative real-time polymerase chain reaction (qRT-PCR). Total RNA was extracted using TRIzol reagent, then cDNA was synthesized by reverse transcription. RNA was reverse-transcribed into cDNA using the PrimeScript II 1st Strand cDNA Synthesis Kit (Takara, GlenBurnie, MD, USA) according to the manufacturer's protocol. The cDNA was amplified with GAPDH as a control. The primer sequences were as follows: CD24 forward, 5'-TTCTC-CAAGCACCCAGCA-3'; and reverse, 5'-TGGAATAAATCT-GCGTGGGTA-3'; Hsp70 forward, 5'-GGGCCTTTC-CAAGATTGCTGT3', and reverse, 5'-ATCTCTGCATG-TAGAAACCGGAAA3'. GAPDH target sequence was as follows: forward, 5'-GGAGCGAGATCCCTCCAAAAT-3'; and reverse, 5'-GGCTGTTGTCATACTTCTCATGG-3'. The PCR reaction conditions were as follows: 94 °C for 5 min, 94 °C for 30 s, 54 °C for 45 s, and 72 °C for 50 s, for a total of 30 cycles, followed by 72 °C for 10 min. PCR products were detected by 2% agarose gel electrophoresis and gel imaging. Relative expression levels were calculated as ratios normalized to GAPDH. The Cq-value for each sample was examined using the ΔΔCq method. The results were expressed as $2^{-\Delta\Delta Cq}$ [10]. The relative expression of CD24 mRNA and Hsp70 mRNA were calculated performed in triplicate. Primers were synthesized by Shanghai Bioengineering Co., Ltd. (Shanghai, China).

Western blotting. Total protein was extracted from cells by a lysis buffer. The BCA assay was used to determine the concentration of protein samples. After SDS-PAGE was performed, protein samples were transferred to PVDF membranes. Membranes were then incubated with hybridization buffer in sealed hybridization bags according to the filter area (0.1 ml/cm^2) at room temperature for 1–2 h. Next, the buffer was discarded and the membranes were incubated overnight at 4 °C with primary antibody (CD24: 1:500, Hsp70: 1:1000 dilution prepared at 0.1 ml/cm^2) (CD24: ab151707, Abcam, USA; Hsp70: ab181606, Abcam, USA). Membranes were then washed with PBS 3 times for 10 min and then incubated with secondary antibody (1:2500) (ab96899, Abcam, USA) at room temperature for 3 h. The membranes were washed with PBS 3 times for 10 min and the signal was detected using ECL chemiluminescence. Each experiment was repeated three times.

Co-immunoprecipitation (Co-IP). Total cellular protein was extracted and a small amount of lysate was used as a control. The remaining lysate was incubated with 1 µg of the corresponding antibody overnight with slow shaking at 4 °C. Then, 10 µl protein A/G was washed with the appropriate buffer three times with centrifugation at 1,000×g for 3 min each time and then incubated with the cell lysate under slow shaking at 4 °C for 2–4 h. The protein A/G beads were collected in the magnetic frame and washed with lysate 3–4 times. After washing, 15 µl SDS loading buffer (2×) was added, and the mixture was boiled for 5 min. Finally, SDS-PAGE was performed and western blotting was used to observe the results.

Statistical analysis. All data were analyzed with SPSS 19.0 (IBM Corp., Armonk, NY, USA), and relationships between variables were analyzed with χ^2 tests. The data were expressed as $\bar{x}\pm s$, and the t-test was used for comparisons between two groups, while ANOVA was used for comparisons between more than two groups. Kaplan-Meier curves were used for overall survival (OS) analysis, and the log-rank test was used to examine differences. In this study, $p<0.05$ (two-tailed test) was statistically significant.

Results

CD24 and Hsp70 expression in lung cancer. Positive CD24 expression was found in 62.4% (58/93) of lung cancer tissues and in 43.0% (40/93) of para-cancerous tissues. Positive Hsp70 expression was found in 76.3% (71/93) of lung cancer tissues and in 21.5% (20/93) of para-cancerous tissues. CD24 and Hsp70 expression were significantly higher in lung cancer tissues than in para-cancerous tissues (CD24: $p=0.008$; Hsp70: $p<0.001$) (Figure 1).

Relationship between CD24 and Hsp70 expression and clinicopathological features in lung cancer. CD24 expression was correlated with lymph node metastasis, TNM stage, and the existence of vascular cancer thrombus ($p<0.05$), but not with age, gender, smoking status, histological type, differentiation degree, or T stage ($p>0.05$) (Table 1). CD24 expression was significantly higher in patients with lymph node metastasis than in patients without lymph node metastasis ($p<0.001$). CD24 expression was also significantly higher in stage III patients than in stage II and stage I patients (I vs. II: $p=0.018$; II vs. III: $p=0.001$; I vs. III: $p<0.001$). Finally, CD24 expression was significantly higher in the vascular tumor thrombus than in those without tumor thrombus ($p=0.007$).

Hsp70 protein levels were correlated with cancer differentiation, lymph node metastasis, and TNM stage ($p<0.05$), but not with age, gender, smoking status, histological type, T stage, or the existence of vascular cancer thrombus ($p>0.05$) (Table 1). Hsp70 expression levels in patients with moderately and poorly differentiated cancer were significantly higher than in patients with highly differentiated cancer ($p=0.036$).

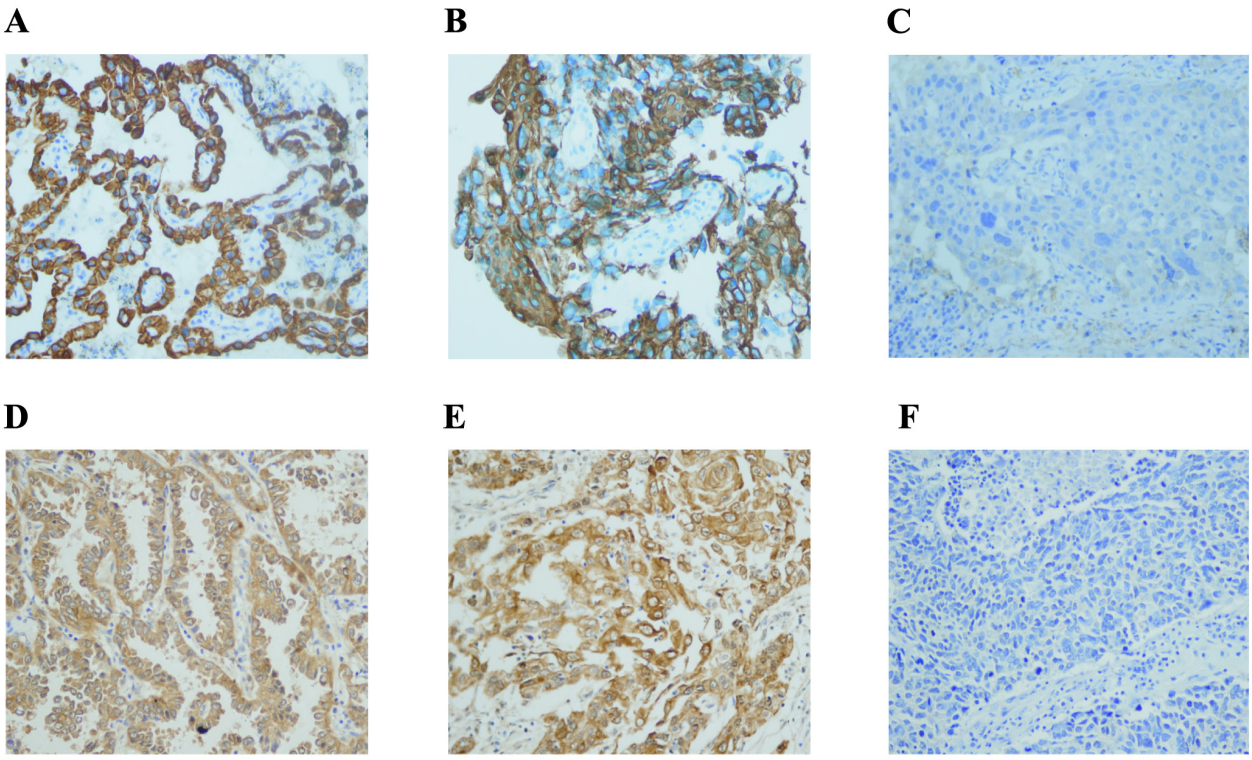

Figure 1. CD24 and Hsp70 expression in lung cancer. A) CD24 in an adenocarcinoma. B) CD24 in a squamous cell carcinoma. C) CD24 in para-cancerous tissues. D) Hsp70 in an adenocarcinoma. E) Hsp70 in a squamous cell carcinoma. F) Hsp70 in para-cancerous tissues (magnification: 400×).

Hsp70 expression was significantly higher in patients with lymph node metastasis than in those without lymph node metastasis (p=0.001). Hsp70 expression was significantly higher in patients with stage III cancer than in patients with stage I cancer (p<0.001), but there was no significant difference compared with patients with stage II cancer (p=0.051). Hsp70 expression levels were higher in patients with stage II cancer than in patients with stage I cancer, while the difference was not statistically significant (p=0.153).

Relationship between CD24 and Hsp70 expression and the prognosis of lung cancer. The median OS of the 93 patients was 55 months, and 41 patients survived to the last follow-up. The median OS of the CD24 high expression group was 35 months (95% confidence interval [CI]: 8.9–61.1 months), and the median OS of the CD24 low expression group was 65 months (95% CI: 64.1–65.9 months); the differences were statistically significant (p=0.043) (Figure 2A). The median OS of the Hsp70 high expression group was 36 months (95% CI: 5.0–67.0 months), and the median OS of the Hsp70 low expression group was 65 months (95% CI: 64.0–66.1 months); the differences were significant (p=0.033) (Figure 2B). These results suggested that CD24 and Hsp70 protein expression in lung cancer were related to patient prognosis, where high expression of CD24 and Hsp70 may indicate a poor prognosis.

Co-expression and correlation analysis of CD24 and Hsp70 protein expression in lung cancer. The positive co-expression rate of CD24 and Hsp70 in lung cancer tissues was 52.7% (49/93) (Table 2). The positive rate of co-expression of CD24 and Hsp70 in different clinical features was compared. There was no statistically significant difference in the positive co-expression rate of CD24 and Hsp70 in different age, gender, smoking status, histological type, differentiation degree, and T stage (p>0.05). The positive co-expression rate of CD24 and Hsp70 was significantly higher in patients with lymph node metastasis than in patients without lymph node metastasis (p<0.001). The positive co-expression rate of CD24 and Hsp70 was also significantly higher in stage III patients than in stage II and stage I patients (p<0.001). The positive co-expression rate of CD24 and Hsp70 was significantly higher in the vascular tumor thrombus than in those without tumor thrombus (p=0.001) (Table 1). The expression of CD24 and Hsp70 were positively correlated in lung cancer (r=0.368, p<0.001) (Table 3).

CD24 and Hsp70 expression in different lung cancer cell lines. Western blotting and qRT-PCR were used to analyze CD24 and Hsp70 expression in SPCA1, A549, H1975, and H1650 cells. As shown in Figure 3A and 3B, the expression of CD24 protein and mRNA were weakly expressed in A549 cells and highly expressed in SPCA1 cells. As shown in

Table 1. CD24 and Hsp70 protein expression in lung cancer and associations with clinical characteristics.

Characteristics	n	CD24 positive (%)	p-value	Hsp70 positive (%)	p-value	co-expression of CD24, Hsp70 (%)	p-value
Age (years)			0.810		0.457		0.350
<60	57	35 (61.4)		45 (78.9)		31 (54.4)	
≥60	36	23 (63.9)		26 (72.2)		16 (44.4)	
Gender			0.784		0.891		0.576
male	73	45 (61.6)		55 (75.3)		38 (52.1)	
female	20	13 (65.0)		16 (80.0)		9 (45.0)	
Smoking status			0.349		0.685		0.911
yes	50	29 (58.0)		39 (78.0)		25 (50.0)	
no	43	29 (67.4)		32 (74.4)		22 (51.2)	
Pathological types			0.692		0.329		0.806
adenocarcinoma	30	17 (56.7)		23 (76.7)		15 (50.0)	
squamous cell carcinoma	47	30 (63.8)		38 (80.9)		25 (53.2)	
others	16	11 (68.8)		10 (62.5)		7 (43.8)	
Differentiation			0.717		0.036		0.667
well	22	13 (59.1)		10 (45.5)		12 (54.5)	
moderate or poor	71	45 (63.4)		51 (69.9)		35 (49.3)	
T stage			0.262		0.178		0.084
T1	29	13 (44.8)		20 (69.0)		10 (34.5)	
T2	49	30 (61.2)		41 (83.7)		27 (55.1)	
T3+4	15	10 (66.7)		10 (66.7)		10 (66.7)	
Lymph node metastases					0.001		<0.001
No (N0)	53	20 (37.7)	<0.001	31 (58.5)		9 (17.0)	
Yes (N1+N2)	40	38 (95.0)		36 (90.0)		36 (90.0)	
TNM stage			<0.001		0.002		<0.001
I	34	11 (32.4)		20 (58.8)		6 (17.6)	
II	29	18 (62.1)		22 (75.9)		12 (41.4)	
III	30	29 (96.7)		29 (96.7)		28 (93.3)	
Vascular cancer thrombus			0.007		0.268		0.001
no	80	45 (56.3)		59 (73.8)		35 (43.8)	
yes	13	13 (100)		12 (92.3)		12 (92.3)	

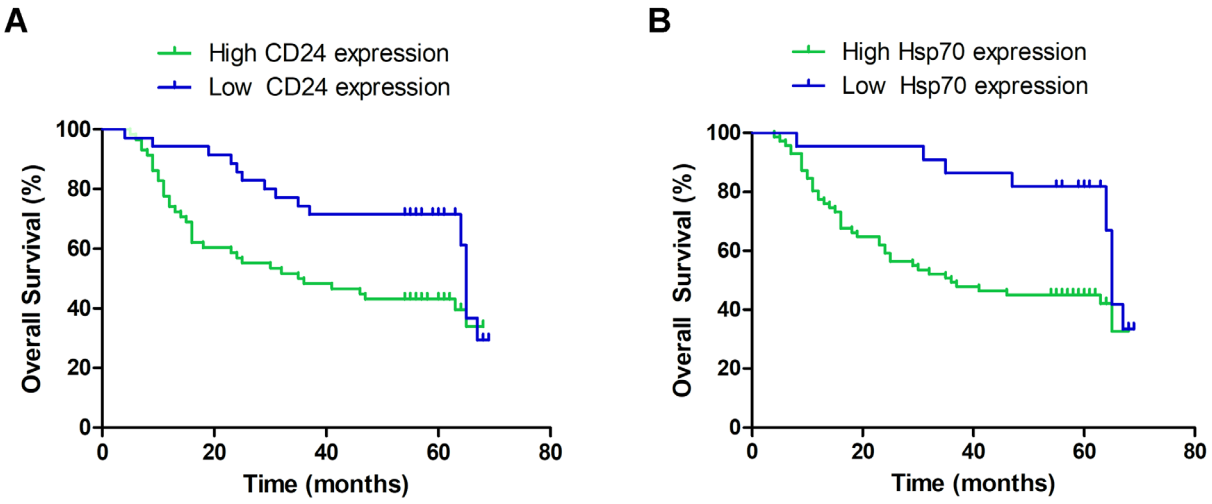

Figure 2. The relationship between CD24 and Hsp70 expression and the prognosis of lung cancer patients. A) Total survival rate of the high CD24 expression group versus the low expression group. B) Total survival rate of the high Hsp70 expression group versus the low expression group.

Table 2. The positive co-expression rate of CD24 and Hsp70 in lung cancer tissues.

CD24 expression	Hsp70 expression		p-value
	Positive	Negative	
Positive	49 (84.48%)	9 (15.52%)	0.017*
Negative	22 (62.86%)	13 (37.14%)	

Note: *p<0.05

Table 3. Relationship between CD24 and Hsp70 expression in lung cancer.

CD24	Hsp70				r	p-value
	–	+	++	+++		
–	4	7	18	0	0.368	<0.001
+	0	0	6	0		
++	1	9	33	7		
+++	0	1	4	3		

Figure 3C and 3D, Hsp70 protein and mRNA were expressed low in A549 cells and highly in SPCA1 cells.

Construction of CD24-overexpression cell lines. To further examine the biological function of CD24, A549 cells that stably overexpressed CD24 were constructed, as A549 cells originally had relatively low CD24 expression. Western blotting and qRT-PCR were used to identify the CD24 protein and mRNA levels, respectively. As shown in Figure 3E, CD24 protein levels were significantly increased in the CD24-overexpression stable cell lines. As shown in Figure 3F, cell lines, CD24 mRNA levels were also significantly increased in CD24-overexpression stable cell lines compared with A549 and A549 vector stable.

CD24 combines with Hsp70. To study the interaction between endogenous and exogenous CD24 and Hsp70, a constructed CD24-overexpression stable cell line was used as the target cell. As shown in Figure 4A and 4B, Hsp70 protein was immunoprecipitated with CD24 protein, showing that endogenous CD24 could combine with Hsp70. The interaction between exogenous CD24 protein and Hsp70 protein was verified by using CD24 protein to precipitate Hsp70 protein. Both endogenous and exogenous CD24 co-precipitated with Hsp70, suggesting a direct or indirect interaction between the two molecules.

Interaction between CD24 and Hsp70. Western blotting was used to verify the expression of Hsp70 in CD24-OE-A549, A549, and VE-A549 cells. The results showed that there were no significant changes in the expression of Hsp70 in any group, indicating that CD24 had no regulatory effect on Hsp70; and it may be a protein that was regulated downstream of Hsp70 (Figure 4C). After adding the Hsp70 inhibitor VER155008 to A549 cells, we found that both Hsp70 and CD24 protein were significantly decreased when adding shRNA-Hsp70 to A549 cells, CD24 protein expression and mRNA levels were significantly decreased, indicating that Hsp70 could regulate CD24 in some ways (Figure 4D–4F).

Discussion

CSCs are a small group of tumor cells with self-renewal ability, multi-directional differentiation potential, and high tumorigenicity. CSCs can differentiate into tumor cells and maintain tumor cell characteristics [11]. The theory of CSCs was first confirmed in hematological malignancies [12], then, researchers isolated and identified CSCs in breast cancer, colorectal cancer, lung cancer, and other solid tumors, also proving that CSCs play important roles in tumor invasion, metastasis, recurrence, and treatment resistance [13–15]. CSC markers are a promise of stem cell research. Currently, the identified molecular markers for CSCs include CD24, CD44, CD133, and CD34 [16].

CD24 is a homologous molecule of heat-stable antigen (HSA), which is located on chromosome 6q21. In 1978, Springer et al. [17] found a heat stable, organic solvent-soluble glycoprotein with a lipid-like structure on the surface of mouse leukocytes and named it HSA. In 1991, Kay et al. [18] confirmed that CD24 was the homologous molecule of HSA in humans. CD24 has a low molecular weight and is a highly glycosylated adhesion molecule composed of 27 amino acids. It is anchored on the cell membrane by glycosyl phosphatidylinositol (GPI) and is further located on lipid rafts within the cell membrane [19]. Recent studies confirmed that CD24 was highly expressed in esophageal cancer [2], thyroid papillary cancer [3], hepatic carcinomas [4], and so on, playing a key role in tumor occurrence and development related to tumor invasion and metastasis. In a study of non-small cell lung cancer, CD24 was used as an independent prognostic factor [20]. Thus, CD24 may be a potential marker of invasion and prognosis in lung cancer.

How CD24 regulates tumor invasion and metastasis remains unclear. CD24 is a GPI-anchored membrane protein that lacks intracellular structure and is unable to transmit transmembrane signals. Lipid rafts are microregions of the cell membrane and platform transmembrane signal transmission. Therefore, new molecules that interact with CD24 have been screened. It was reported that CD24 may interact with Hsp and that CD24 may be a client protein of Hsp.

Hsps are a large super-gene family of glycoproteins with many members. Hsps primarily help fold proteins into their three-dimensional structure; thus, they also participate in the activation and maturation of client proteins as well as their degradation in the proteasome [21]. Constitutively expressed Hsp70, belonging to the Hsp family, is an important molecular chaperone. As a molecular chaperone, it regulates the stability of functional proteins [22], regulating tumorigenesis and other biological characteristics by activating downstream signaling molecules [23–24]. CD24 may be regulated by Hsp70 such as a "client protein". Currently, targeting Hsp70 is one of the current strategies for the biological treatment of lung cancer, so we chose Hsp70 as a research target.

The expression of CD24 and Hsp70 were first examined in paraffin sections of clinical lung cancer tissues by immuno-

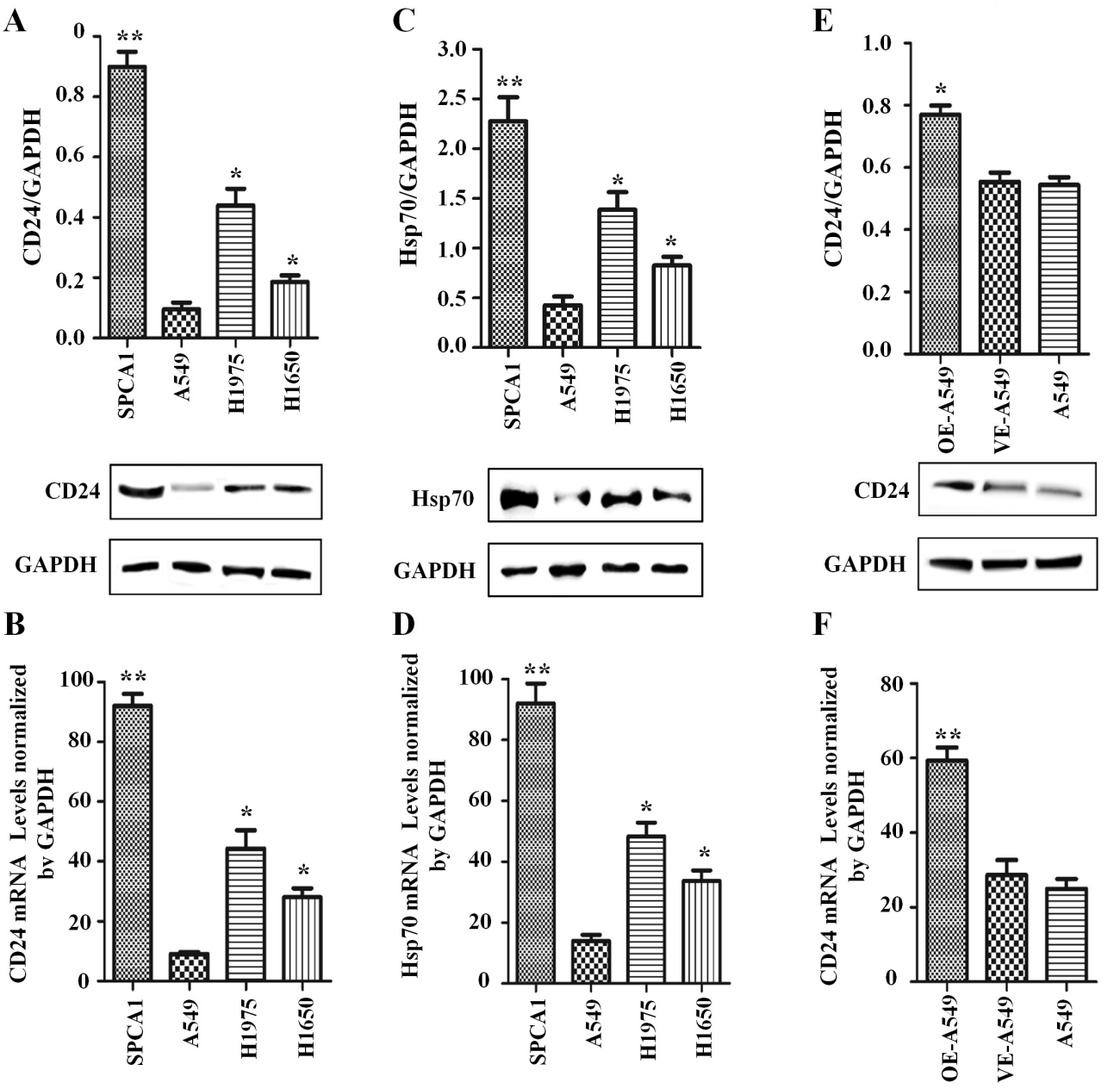

Figure 3. CD24 and Hsp70 protein and mRNA expression in lung cancer cells. A) Western blotting was used to detect CD24 protein expression in A549, SPCA1, H1975, and H1650 lung cancer cells. B) qRT-PCR was used to detect the expression of CD24 mRNA in A549, SPCA1, H1975, and H1650 lung cancer cells. C) Western blotting was used to detect Hsp70 protein expression in A549, SPCA1, H1975, and H1650 lung cancer cells. D) qRT-PCR was used to detect the expression of Hsp70 mRNA in A549, SPCA1, H1975, and H1650 lung cancer cells. E) Stable CD24-overexpressing A549 cells were constructed from parental A549 cells with a relatively low CD24 expression. Western blotting was used to detect changes in CD24 expression in OE-A549 cells, negative control, and vector control cells. F) qRT-PCR was used to detect the changes in CD24 mRNA levels in CD24-overexpressing A549 cells, negative control, and vector control cells.

histochemistry. The results showed that CD24 and Hsp70 expression were significantly higher in lung cancer tissues than in adjacent tissues, and the differences were statistically significant. CD24 and Hsp70 expression in lung cancer was related to lymph node metastasis and TNM stage. Thus, CD24 and Hsp70 may play important roles in the invasion and metastasis of lung cancer. Survival analysis showed that CD24 and Hsp70 expressions in lung cancer were related to the prognosis. The median OS of the CD24 and Hsp70 low expression group was significantly increased. The positive co-expression rate of CD24 and Hsp70 in lung cancer tissues was analyzed, which was associated with lymph node metas-

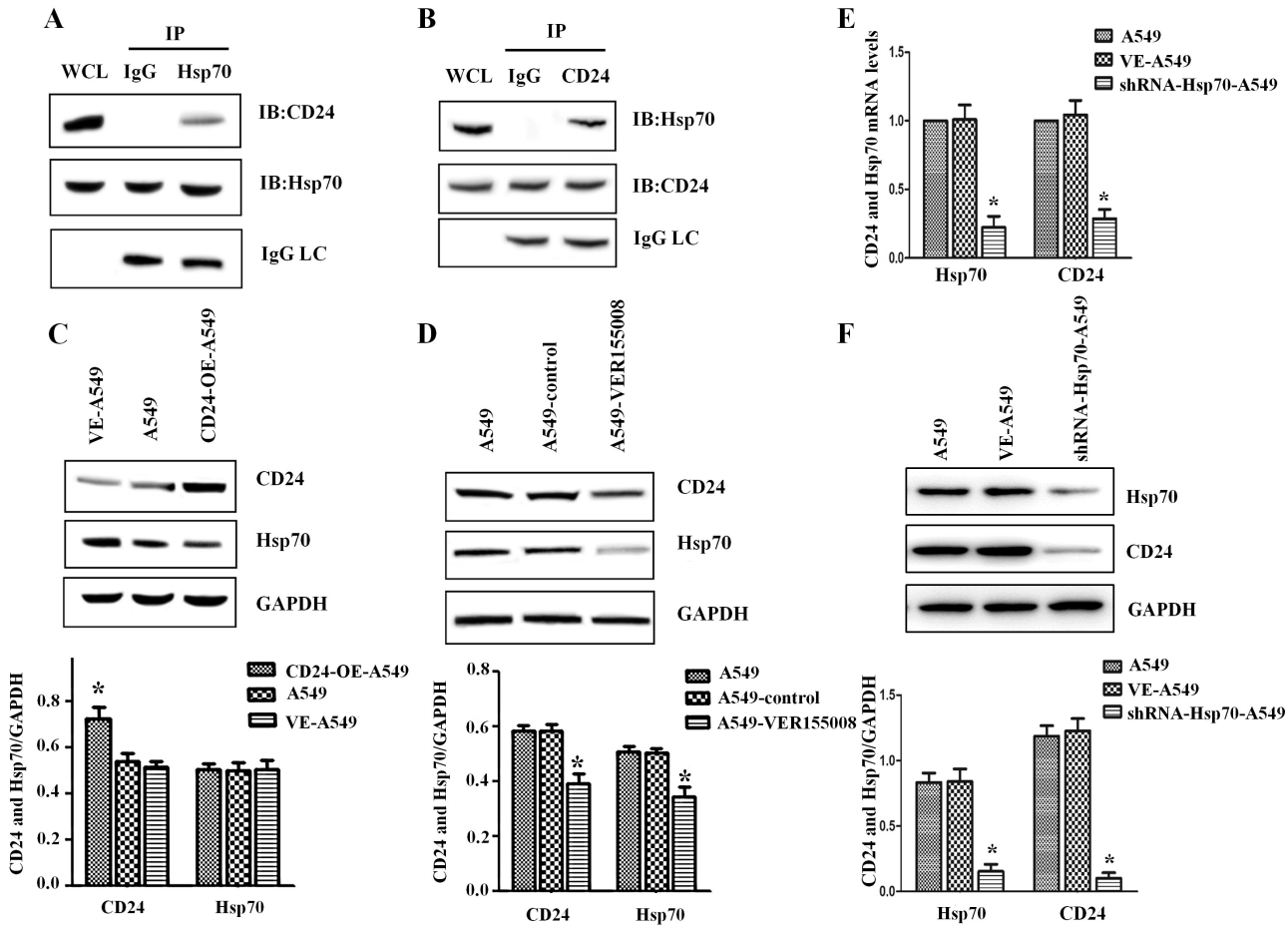

Figure 4. The interaction between Hsp70 protein and CD24 protein. A) Co-IP using Hsp70 protein to precipitate CD24 protein was used to verify the interaction between endogenous Hsp70 protein and CD24 protein. B) Co-IP using CD24 protein to precipitate Hsp70 protein was used to verify the interaction between exogenous CD24 protein and Hsp70 protein. C) After overexpressing CD24 in A549 cells, there was no significant change in Hsp70 levels. D) After adding the Hsp70 inhibitor VER155008 to A549 cells, Hsp70 and CD24 protein levels were significantly decreased compared with control cells. E, F) After adding shRNA-Hsp70 to A549 cells, the expression of CD24 protein and mRNA were decreased compared with control cells.

tasis, late stage, and vascular cancer thrombus, suggesting that co-expression of CD24 and Hsp70 may be related to invasion, metastasis, and prognosis of lung cancer The correlation analysis of CD24 and Hsp70 confirmed that CD24 and Hsp70 expression in lung cancer were positively correlated in some ways.

A CD24 overexpression cell model was constructed and used as the target cell to study the interaction between endogenous and exogenous CD24 and Hsp70 by Co-IP. The results showed that both endogenous and exogenous CD24 could precipitate with Hsp70, suggesting a direct or indirect interaction. Both endogenous and exogenous CD24 interacted with Hsp70, indicating that CD24 may be a client protein of Hsp70.

Western blotting and qRT-PCR were used to detect the expression of CD24 and Hsp70 in four lung cell lines, and the lowest expression was found in A549 cells. Therefore, this cell line was selected to construct a CD24-overexpression cell line. No significant change was found in Hsp70 expression in the CD24-overexpressing A549 cells, while when the Hsp70 inhibitor VER155008 was added to A549 cells, Hsp70 and CD24 protein expression were significantly decreased. When adding shRNA-Hsp70 to A549 cells, CD24 protein expression and mRNA levels were significantly decreased, indicating that CD24 may be a protein that was regulated downstream of Hsp70. Hsp70 had a regulatory effect on CD24. Chen et al. showed that CD24 expression is associated with high Hsp70 expression. CD24 could regulate the expression of Hsp70, and Co-IP results showed that CD24 was related to Hsp70, and CD24/HSP70 pathway regulates the immune response [8]. Keller et al. showed that exosomes from body fluids were positive for the marker proteins CD24 and Hsp70, and displayed the correct buoyant density and orientation of antigens [25]. Another study also showed that the high expressions of CD24, Hsp70, and Hsp90 were found in the progression of lymphoma for their adjuvant

activity on immune responses [26]. These data revealed a regulatory effect between Hsp70 and CD24, and we added further research to reveal the regulatory relationship between Hsp70 and CD24 in lung cancer. There were some differences in our results. We only examined Hsp70 expression in CD24-overexpressing A549 cells in the study, lacking more experimental verification. In our later study, we will construct CD24 and Hsp70 over- and low-expressing cell lines, to observe whether the expression level of CD24 protein and mRNA are consistent with the level of Hsp70 protein and mRNA, then to infer whether CD24 can regulate the expression of Hsp70 or Hsp70 can regulate the expression of CD24.

In conclusion, these results showed that CD24 and Hsp70 were highly expressed in lung cancer tissues, and associated with invasion, metastasis, and poor survival. Co-expression of CD24 and Hsp70 may be a prognostic biomarker for lung cancer. Hsp70 may be regulated by CD24 expression. How CD24 promotes the invasion and metastasis of lung cancer cells and the downstream molecular pathways are yet to be further explored.

Acknowledgments: This investigation was funded by Doctor's Compensation of the First Hospital Affiliated to Inner Mongolia Medical University (grant no. NYFY BS 2018) and was funded by General research projects of The First Hospital Affiliated to Inner Mongolia Medical University (grant no. NYFY YB 008). We thank James P. Mahaffey, PhD., from Liwen Bianji, Edanz Editing China (www.liwenbianji.cn/ac), for editing the English text of a draft of this manuscript.

References

[1] ZHOU Y, ZHENG X, CHEN LJ, XU B, JIANG JT. microRNA-181b suppresses the metastasis of lung cancer cells by targeting sex determining region Y-related high mobility group-box 6 (Sox6). Pathol Res Pract 2019; 215: 335–342. https://doi.org/10.1016/j.prp.2018.12.009

[2] TAKASHIMA K, FUJII S, KOMATSUZAK R, KOMATSU M, TAKAHASHI M et al. CD24 and CK4 Are Upregulated by SIM2, and Are Predictive Biomarkers for Chemoradiotherapy and Surgery in Esophageal Cancer. Int J Oncol 2020; 56: 835–847. https://doi.org/10.3892/ijo.2020.4963

[3] RYU YJ, CHOE JY, LEE K, AHN SH. Clinical prognostic significance of cancer stem cell markers in patients with papillary thyroid carcinoma. Oncol Lett. 2020; 19: 343–349. https://doi.org/10.3892/ol.2019.11087

[4] SANG X, WU F, WU D, LIN S, LI J et al. Human Hepatic Cancer Stem Cells (HCSCs) Markers Correlated With Immune Infiltrates Reveal Prognostic Significance of Hepatocellular Carcinoma. Front Genet 2020; 28: 112. https://doi.org/10.3389/fgene.2020.00112

[5] GILAD N, ZUKERMAN H, PICK M, GATT ME. The role of CD24 in multiple myeloma tumorigenicity and effects of the microenvironment on its expression. Oncotarget 2019; 10: 5480–5491. https://doi.org/10.18632/oncotarget.27190

[6] WANG Z, WANG Q, WANG Q, WANG Y, CHEN J. Prognostic significance of CD24 and CD44 in breast cancer: a meta-analysis. Int J Bio Markers 2017; 32: e75–e82. https://doi.org/10.5301/jbm.5000224

[7] BOUDESCO C, CAUSE S, JEGO G, GARRIDO C. Hsp70: A Cancer Target Inside and Outside the Cell. Methods Mol Biol 2018; 1709: 371–396. https://doi.org/10.1007/978-1-4939-7477-1_27

[8] ALBAKOVA Z, ARMEEV GA, KANEVSKIY LM, KOVALENKO EI, SAPOZHNIKOV AM. HSP70 Multi-Functionality in Cancer. Cells 2020; 9: E587. https://doi.org/10.3390/cells9030587

[9] CHEN GY, TANG J, ZHENG P, LIU Y. CD24 and Siglec-10 selectively repress tissue damage-induced immune responses. Science 2009; 323: 1722–1725. https://doi.org/10.1126/science.1168988

[10] LIVAK KJ, SCHMITTGEN TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001; 25: 402–408. https://doi.org/10.1006/meth.2001.1262

[11] ANDRADE NP, RODRIGUES MF, RODINI CO, NUNES FD. Cancer stem cell, cytokeratins and epithelial to mesenchymal transition markers expression in oral squamous cell carcinoma derived from ortothopic xenoimplantation of CD44(high) cells. Pathol Res Pract 2017; 213: 235–244. https://doi.org/10.1016/j.prp.2016.12.009

[12] BONNET D, DICK JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med 1997; 3: 730–737. https://doi.org/10.1038/nm0797-730

[13] GENER P, GOUVEIA LP, SABAT GR, SOUSA DF, FORT NB et al. Fluorescent CSC models evidence that targeted nanomedicines improve treatment sensitivity of breast and colon cancer stem cell. Nanomedicine 2015; 11: 1883–1892. https://doi.org/10.1016/j.nano.2015.07.009

[14] ZHAO C, SETRERRAHMANE S, XU H. Enrichment and characterization of cancer stem cells from a human non-small cell lung cancer cell line. Oncol Rep 2015; 34: 2126–2132. https://doi.org/10.3892/or.2015.4163

[15] SURESH R, ALI S, AHMAD A, PHILIP PA, SARKAR FH. The role of cancer stem cells in recurrent and drug-resistant lung cancer. Adv Exp Med Biol 2016; 890: 57–74. https://doi.org/10.1007/978-3-319-24932-2_4

[16] ALLEGRA E, TRAPASSO S, PISANI D, PUZZO L. The role of BMI1 as a biomarker of cancer stem cells in head and neck cancer: a review. Oncology 2014; 86: 199–205. https://doi.org/10.1159/000358598

[17] SPRINGER T, GALFRE G, SECHER DS, MILSTEIN C. Monoclonal xenogeneic antibodies to murine cell surface antigens: identification of novel leukocyte differentiation antigens. Eur J Immunol 1978; 8: 539–551. https://doi.org/10.1002/eji.1830080802

[18] KAY R, ROSTERN PM, HUMPHRIES RK. CD24, a signal transducer modulating B cell activation responses, is a very short peptide with a glycosyl phosphatidylinositol membrane anchor. J Immunol 1991; 147: 1412–1416.

[19] ZHANG P, ZHENG P, LIU Y. Amplification of the CD24 Gene Is an Independent Predictor for Poor Prognosis of Breast Cancer. Front Genet 2019; 10: 560. https://doi.org/10.3389/fgene.2019.00560

[20] KRISTIANSEN G, SCHIUNS K, YONGWEI Y, DENKERT C, DIETEL M et al. CD24 is an independent prognostic marker of survival in nonsmall cell lung cancer patients. Br J Cancer 2003; 88: 231–236. https://doi.org/10.1038/sj.bjc.6600702

[21] YU A, LI P, TANG T, WANG J, CHEN Y et al. Roles of Hsp70s in Stress Responses of Microorganisms, Plants, and Animals. Biomed Res Int 2015; 2015: 510319. https://doi.org/10.1155/2015/510319

[22] CHANTELOUP G, CORDONNIER M, IIAMBERT N, BERTAUT A, MARCION G et al. Membrane-bound Exosomal HSP70 as a Biomarker for Detection and Monitoring of Malignant Solid Tumours: A Pilot Study. Pilot Feasibility Stud 2020; 6: 35. https://doi.org/10.1186/s40814-020-00577-2

[23] YU A, LI P, TANG T, WANG J, CHEN Y et al. Roles of Hsp70s in Stress Responses of Microorganisms, Plants, and Animals. Biomed Res Int 2015; 2015: 510319. https://doi.org/10.1155/2015/510319

[24] ABKIN SV, OSTROUMOVA OS, KOMAROVA EY, MESHLKINA DA, SHEVTSOV MA et al. Phloretin increases the anti-tumor efficacy of intratumorally delivered heat-shock protein 70 kDa (HSP70) in a murine model of melanoma. Cancer Immunol Immunother 2016; 65: 83–92. https://doi.org/10.1007/s00262-015-1778-1

[25] KELLER S, RIDINGER J, RUPP AK, JANSSEN JW, ALTEVOGT P. Body Fluid Derived Exosomes as a Novel Template for Clinical Diagnostics. J Transl Med 2011; 9: 86. https://doi.org/10.1186/1479-5876-9-86

[26] MENAY F, HERSCHLIK L, DE TJ, COCOZA F, TSACALIAN R et al. Exosomes Isolated From Ascites of T-Cell Lymphoma-Bearing Mice Expressing Surface CD24 and HSP-90 Induce a Tumor-Specific Immune Response. Front Immunol 2017; 8: 286. https://doi.org/10.3389/fimmu.2017.00286