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Original Article

Prognostic value of Thy1 in non-small cell lung cancer: a RNA-Seq transcriptome analysis

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Abstract: Thy1 is a membrane glycoprotein, which can regulate cell-matrix and cell-cell interactions. Thy1 plays an important role in oncogenesis, tumor metastasis and survival. Thy1 has also been considered a major marker in many kinds of cancer stem cells. In order to investigate the expression and prognostic values of Thy1 in non small cell lung cancer (NSCLC), we cultured the stem cell-like sphere 95D cell lines in serum-free DMEM/F12. Colony formation assay, cell cycle and apoptosis were performed in vitro. Tumorigenicity was analyzed in vivo. The RNA expression status of Thy1 in sphere 95D cell lines was investigated by RNA-Seq. Then we examined Thy1 expression via immunohistochemistry (IHC) in 183 NSCLC samples and 96 normal lung samples. The stem cell-like sphere 95D cells had a higher efficiency of cloning than adherence 95D in vitro, and a better tumorigenic potential in vivo. Sphere 95D cells were blocked in G0-G1 in Sphere 95D group significantly, but have no significant effect on the apoptosis compared with adherence 95D cells. The RNA expression of Thy1 in Sphere 95D cells was significantly up-regulated compared with adherence 95D cells by RNA-seq. Of all NSCLC specimens, Thy1 was detected in the mesenchyme or nucleus by IHC, and the expression rates of Thy1 positive cells were much higher in NSCLC specimens than in normal lung samples. Mesenchymal Thy1 positive expression was an adverse predictor of prognosis in NSCLC patients, while nuclear Thy1 positive expression had no significant associations with overall survival. However, neither mesenchymal nor nuclear Thy1 positive expression was significantly related to clinicopathological characteristics in NSCLC. Thus, Thy1 might serve as a vital therapeutic target for NSCLC.

Keywords: Thy1 (CD90), cancer stem cells, lung cancer, prognosis

Introduction

Lung cancer is the leading contributor to morbidity and mortality from cancer worldwide, with its 5-year overall survival (OS) being only about 17% [1]. Non small cell lung cancer (NSCLC) is the most common form and accounts for more than 85% of all lung cancer. Although there was great progress in the therapy of NSCLC during the past years, many patients still died due to tumor metastases and resistant to conventional treatments. Recent studies reported that tumors had heterogeneity, and only about 0.1%-2% cancer cells, named cancer stem cells (CSCs), were responsible for carcinogenesis [2]. The existence of CSCs is thought to contribute to cancer metastasis, recurrence and drug resistance. Thus, identifying lung CSCs may help to develop novel, effective cancer diagnosis and treatment strategies.

Thy1 (CD90) as a membrane glycoprotein is primarily expressed in neurological and immunological systems through the glycosylphosphatidylinositol anchor. Thy1 plays multiple roles in oncogenesis, tumor metastasis and survival [3]. Saalbach et al. found interaction of Thy1 with alphavbeta3 integrin on melanoma cells could activate endothelial cells and promote melanoma cells adhesion and transmigration [4]. Study by Fiegel et al demonstrated that Thy1 (+) hepatic cells had a higher growth ability compared with Thy1 (-) hepatic cells and the Thy1 expression would decline with maturation of the liver [5]. However, contradictory data were also reported that Thy1 might inhibit tumor growth and metastasis. Abeysinghe et al.

found in human ovarian cancer cell lines, the growth rates and tumor sizes of the xenograft tumor with Thy1 transfectants were significantly reduced compared with their null counterparts [6]. So far, the function of Thy1 still remains inconsistent. Further, there are limited studies about the role of Thy1 in patients with NSCLC.

In this study, the RNA expression status of Thy1 in stem cell-like sphere 95D cell line was investigated by RNA-Seq. Then, relationships of Thy1 expression with clinicopathological features and prognostic value were analyzed in NSCLC patients.

Materials and methods

Patients and tissue specimens

One hundred and ninety-five patients of West China Hospital during 2007 were subsequently recruited. All of these NSCLC patients received complete surgical resection for primary NSCLC without previous neoadjuvant therapy. Ninety-six normal lung tissues nearby the primary tumors were considered as controls. After surgical resection, patients underwent standard therapies according to the Clinical Oncology Information Network Guidelines on the nonsurgical treatments for lung cancer [7]. Finally, One hundred and eighty-three patients with complete data were enrolled after a five-year follow-up. The stages of tumors were assessed on the basis of the American Joint Committee on Cancer (AJCC)'s tumor-node-metastasis (TNM) staging system [8], while their histological types and differentiation were based on the World Health Organization (WHO)'s classification for NSCLC [9]. This study was carried out by the permission of the Committee on Medical Ethics of West China Hospital, and all the enrolled patients signed a written informed consent.

Cell lines and animal models

Human lung cancer cell line 95D was obtained from the Laboratory of Stem Cell Biology, West China Hospital, Sichuan University. The adherence 95D cell lines were cultured in PRMI1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Invitrogen, Carsbald, CA, USA) at 37°C in a humidified atmosphere with 5% CO_2 and 95% air. The sphere 95D cells were cultured under the stem cell conditions: serum-free DMEM/F12 containing 0.4% BSA (sigma), 20 ng/ml basic fibroblast growth factor (bFGF) (Peprotech Rocky Hill, NJ, USA), 20 ng/ml insulin growth factor (IGF) (Peprotech), 20 ng/ml epidermal growth factor (EGF) (Peprotech), and 1% N-2 Supplement (Invitrogen). And the sphere 95D cells were maintained on ultra low attachment plates (Corning, Corning, NY, USA). Four-six weeks old nude mice, male or female, were purchased from the Chengdu Experimental Animal Center of DaShuo (Chengdu, China). Each of them was injected with 1×10^4 sphere 95D cells subcutaneously in the right flank and 1×10^4 adherence 95D cells in the left flank. All the mice were housed under pathogen-free environment, and all animal studies were performed on the basis of animal protocol approved by Animal Care and Use Committee of Sichuan University.

Colony formation assay

Approximately 500 cells were inoculated into a 6-well plate growing for 14 days, then the single cell containing wells were used for cell counting by using crystal violet exclusion. Every well was identified and analyzed for the ability of the cells to generate spheres.

Cell cycle analysis

About 1×10^6 cells were gathered and washed by phosphate-buffered saline (PBS), and then fixed with 70% ethanol overnight at 4°C. Then the cells were incubated in staining buffer (Sigma, USA) with propidium iodide (PI) (1 mg/ml), R Nase A (10 mg/ml) and 0.1% (vol/vol) Triton X-100 in PBS for 30 min in the dark at room temperature. The cells' percentages in different phases of cell cycles were determined via flow cytometry equipped with CXP Modifit software (BD, USA) [10].

Apoptosis analysis

Approximately 5×10^5 cells were harvested using EDTA-free trypsin, and then incubated with 5 μl FITC and 5 μl PI (KeyGen, Nanjing, China) for 15 min at room temperature. FlowJo software and FACS were used for the FITC test. Apoptotic rates were calculated by flow cytometry. Only the cells with FITC positive and PI negative were taken as apoptosis.

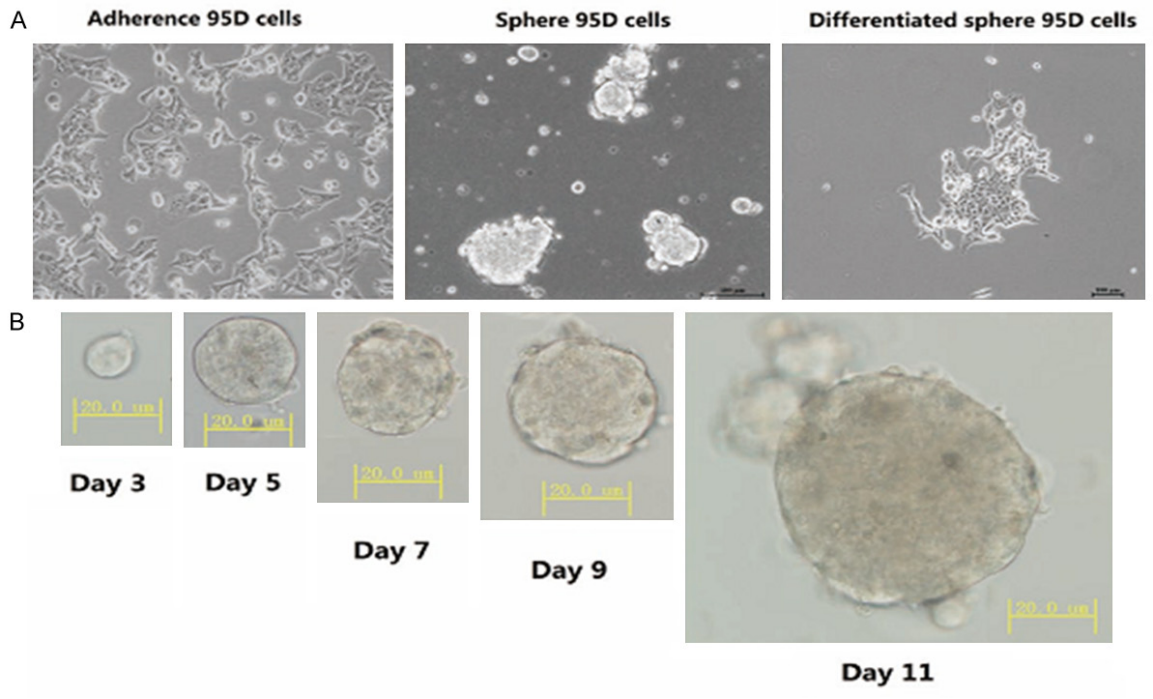

Figure 1. Phase-contrast microscopy representative images of 95D cell lines. A. Adherence 95D cells: were cultured by 10% fetal bovine serum. Sphere 95D cells: were cultured without fetal bovine serum. Differentiated sphere 95D cells: spheroids were induced by 20% fetal bovine serum. B. Images taken at different time points confirmed spheroid growth.

RNA-Seq library preparation and sequencing

Briefly, total RNA samples were extracted from the sphere 95D cells and adherence 95D cells using TRIzol Reagent (Invitrogen, USA). Then the mRNA was enriched and purified from total RNA by the oligo (dT) magnetic beads. Cleaved mRNA fragments (about 200 bp) were synthesized the first strand of cDNA via using random hexamer-primer. Magnetic beads were then used to purify the double strand cDNA. Next, end reparation, 3'-end single nucleotide, adaptor ligation, cDNA templates purification and enrichment were performed. Constructed libraries were sequenced by Illumina HiSeqTM 2000 according to the manufacturer's instructions. TopHat version 1.2.0 was used to aligne RNA-seq reads to genome reference (hg19) and identified splice variants of each sample. The genes were considered to be differentially expressed if their false discovery rates (FDRs) < 0.001 [11]. The gene expression levels were normalized to reads per kilowatt of exon model (RPKM) per million mapped reads in order to help for the comparison of transcripts between groups. Furthermore, genes were defined as upregulated if their mean log2 fold change ratio > 1.

Immunohistochemistry

The immunohistochemical staining of Thy1 were conducted with 4-μm-thick paraffin-embedded samples. Primary antibody was rabbit monoclonal antibody Thy1 (Abcan, USA) diluted 1:200. Secondary antibody was goat anti-rabbit IgG (Dako, Shanghai, China). All samples were incubated at 4°C with the primary antibody overnight. Immunohistochemistry was fulfilled according to the standard ChemMate™ Peroxidase/DAB Detection Kit techniques following the manufacturer's recommendations as reported before [12, 13]. We replaced the primary antibody by PBS as negative controls. The positive expression of Thy1 mesenchymal expression was visually evaluated and scored using a dual rate semi-quantitative method [14]. As for the nuclear Thy1 expression, positive staining cells > 10% were considered as positive expression.

Statistical analysis

All data was analyzed by SPSS 19.0 Software (IBM SPSS Statistics, New York, NY, USA). Chi-squared test and variance analysis were used to determine the significant statistical differ-

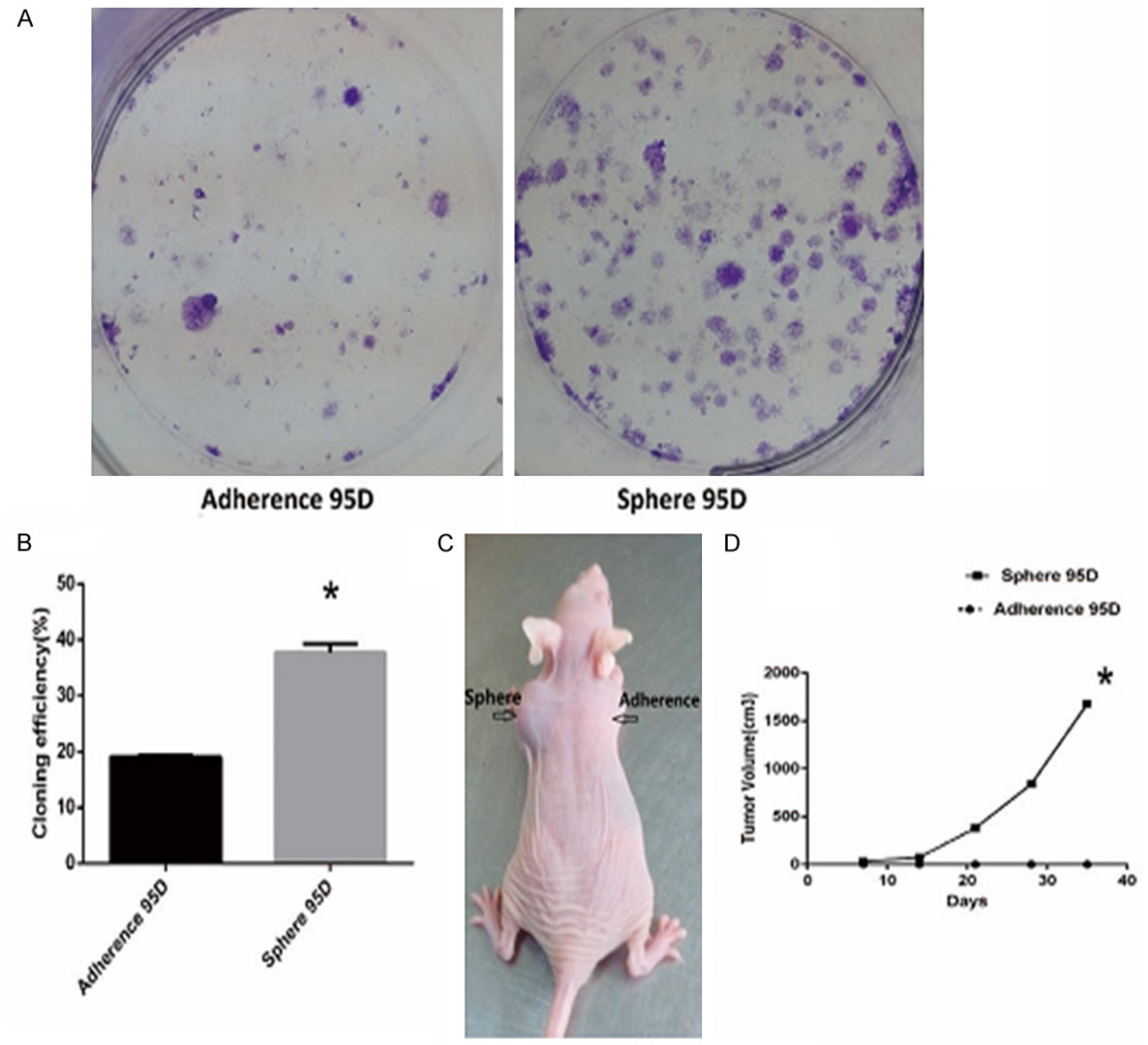

Figure 2. The cloning efficiency and tumorigenic potential of sphere 95D cells and adherence 95D cells. A, B. Colony formation assay: Approximately 500 cells were inoculated into a 6-well plate growing for 14 days. C, D. Tumorigenic potential of tumor cells; Size of tumors generated from 1×10^4 sphere 95D cells and adherence 95D cell. $^*P < 0.05$.

ences among the groups. Kaplan-Meier was performed to survival evaluate curves, while log-rank tests were conducted to estimate their significance. Figures were completed by GraphPad Prism 6.0 (GraphPad Software, Inc., San Diego, USA). All values were expressed as mean ± standard error of the mean (SEM), and levels of statistical significance of differences were set at *P* value < 0.05 (two-tailed).

Results

Stem cell-like spheres formation of 95D cells

When cultured in 10% fetal bovine serum, the 95D cells were adherent growth. Then, we cultured adherence 95D cells in a serum-free medium (SFM) supplemented with growth factors. After about 10-15 days, a small part of 95D cells formed spheres. Then, we incubated the spheres in 20% FBS containing medium for the differentiation, and the spheres regained to adherent growth (**Figure 1A**, **1B**).

Identification of the function of stem cell-like sphere 95D cells

To further identify whether the stem cell-like sphere 95D cells owned the features of tumor initiating cells, we performed some experiments in both vitro and vivo. Approximately 500 cells were inoculated into a 6-well plate growing for 14 days. The cloning efficiency of sphere 95D cells was 37.67±1.609%, while the adher-

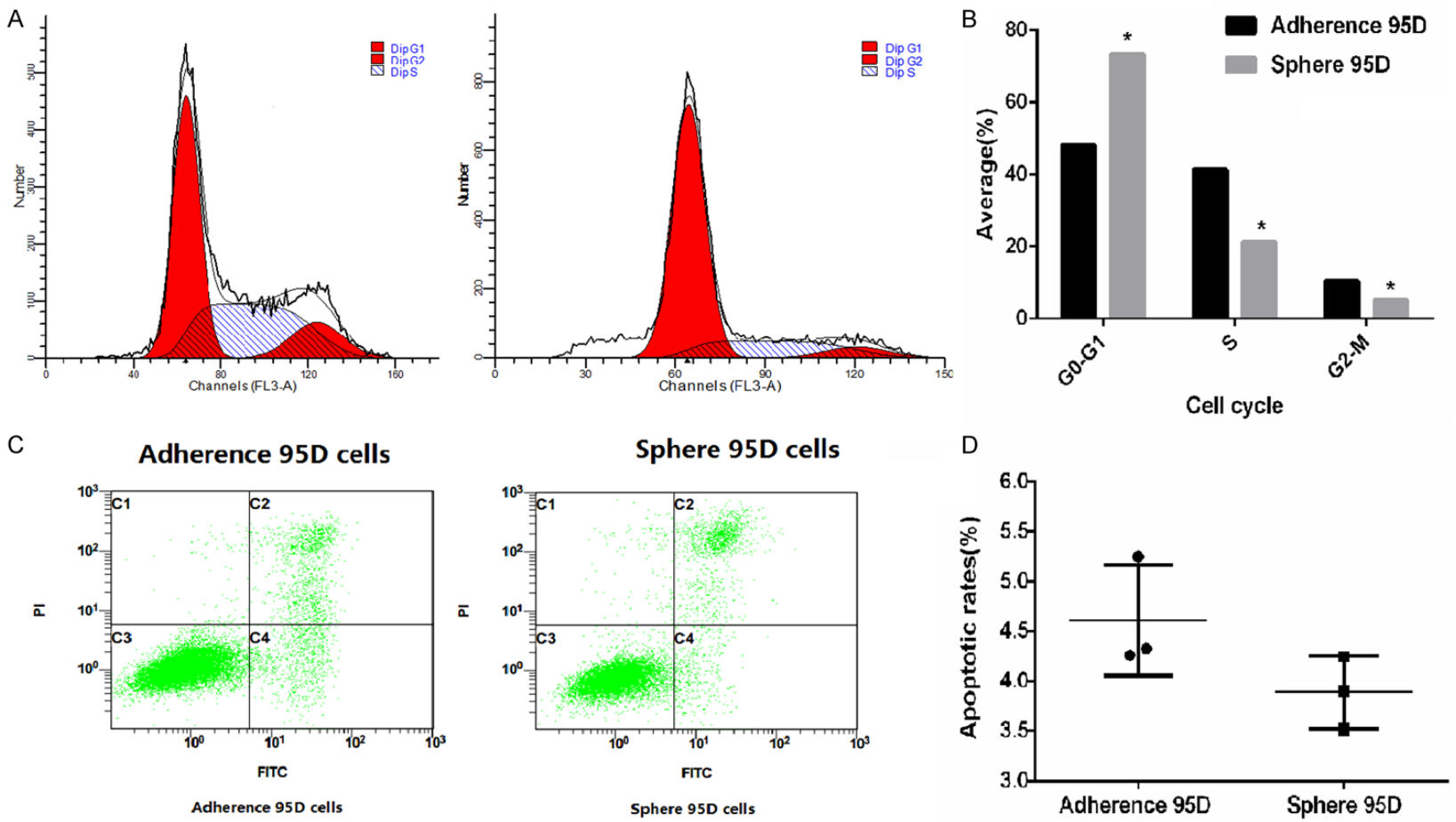

Figure 3. Cells cycle and apoptosis detection sell cycle detection. A, B. Approximately 1 × 10^6 cells were collected and stained by PI. DNA content was detected using flow cycometry. Cluster of cells were gated out, and the profiles of cell cycles were investigated to quantitate cell cycle distribution. $^*P < 0.05$. C, D. Cells were harvested and stained with FITC/PI. Apoptotic rates were calculated by flow cycometry. The FITC-positive and PI-negative cells (C4) were regarded as apoptotic.

Table 1. List of some up-regulated genes in Sphere 95D cells as compared with adherence 95D cells by RNA sequencing

GeneId	GeneSymbol	Gene Name	Mean expression (RPKM)		log2 ratio	*P* value	Function
			Adherence	Sphere			
NM_006288.3	THY1/CD90	Thy-1 cell surface antigen	6.359	21.162	1.73	< 0.0000	Cell differentiation, motility, migration
NM_000041.2	APOE	Apolipoprotein E	3.798	13.449	1.82	< 0.0000	Cell differentiation, proliferation, migration, regulation of opotosis
NM_005985.3	SNAI1	Nail family zinc finger 1	1.962	6.731	1.79	< 0.0000	Cell differentiation, motility, migration
NM_001982.3	ERBB3	V-erb-b2 avian erythroblastic leukemia viral oncogene homolog 3	0.952	3.287	1.79	< 0.0000	Cell differentiation, migration, regulation of opotosis
NM_001163213.1	FGFR3	Fibroblast growth factor receptor 3	0.914	3.016	1.72	< 0.0000	Cell differentiation, proliferation,
NM_000435.2	NOTCH3	Notch 3	4.803	18.125	1.92	< 0.0000	Cell differentiation, migration, stem cell devolopment
NM_003106.3	SOX2	SRY (sex determining region Y)-box 2	2.234	15.912	2.83	< 0.0000	Cell differentiation, migration, regulation of opotosis, stem cell devolopment
NM_001769.3	CD9	CD9 molecule	30.156	122.131	2.018	< 0.0000	Cell proliferation, differntiantion
NM_000599.3	IGFBP5	Insulin-like growth factor binding protein 5	0.467	1.336	1.52	< 0.0000	Cell growth, motility, migration

ence 95D was 19.00±0.3819%. The results showed sphere 95D cells had a high efficiency of cloning than adherence 95D (P=0.001) (**Figure 2A**, **2B**). To better understand the tumorigenic potential of tumor cells In vivo, xenograft models were established. After about 30 days, as few as 1×10^4 sphere 95D cells could generate xenografts and adherence 95D cells failed to do so (**Figure 2C**, **2D**). In cell cycle analysis, approximately 1×10^6 cells were collected and stained by PI. DNA content was detected using flow cytometry. Cluster of cells were gated out, and the profiles of cell cycles were investigated to quantitate cell cycle distribution. Our results demonstrated that cells were blocked in G0-G1 in Sphere 95D group significantly (all P < 0.05) (**Figure 3A**, **3B**). However, we did not observe the differences on the apoptosis between sphere 95D cells and adherence 95D cells (P=0.1315) (**Figure 3C**, **3D**).

Aberrant mRNA expression in sphere 95D cells

Total numbers of RNA-seq reads obtained from the sphere 95D cells and adherence 95D cells were 11, 863, 292 (581, 301, 308 bp) and 11, 863, 292 (565, 244, 351 bp). Our results showed 637 genes were up-regulated. Through Gene ontology analysis, we found overrepresented GO terms in the stem cell like spheres gene sets, were closely collated to cell proliferation, motility and cell differentiation (All P < 0.05) (Table S1). Furthermore, we found some genes in sphere 95D cells were significantly up-regulated compared with adherence 95D cells (**Table 1**).

Thy1 expression and association with clinical characteristics

To further verified the results of RNA-seq, we immunohistochemically analyzed the expression of Thy1 in 183 NSCLC samples (Table S2) and 96 normal lung specimens nearby the tumors. Our results demonstrated that Thy1 was detected in the mesenchyme or nuclei (**Figure 4**). As showed in **Table 2**, the expression rates of Thy1 positive cells were much higher in NSCLC specimens than in normal lung samples (P < 0.001). Of all the NACLC specimens, there were 112 (61.2%) specimens with Thy1 nucleus positive expression, and 148 (80.87%) specimens with Thy1 mesenchymal positive expression. However, neither mesenchymal nor nuclear Thy1 positive expression was significant statistically associated with clinicopathological characteristics (All P > 0.5). Then, the Kaplan-Meier survival curves were used to access the correlations between the Thy1 expression levels in mesenchymal or nuclear and patients' 5-year overall survivals (**Figure 4I**, **4J**). The results suggested mesenchymal Thy1 positive

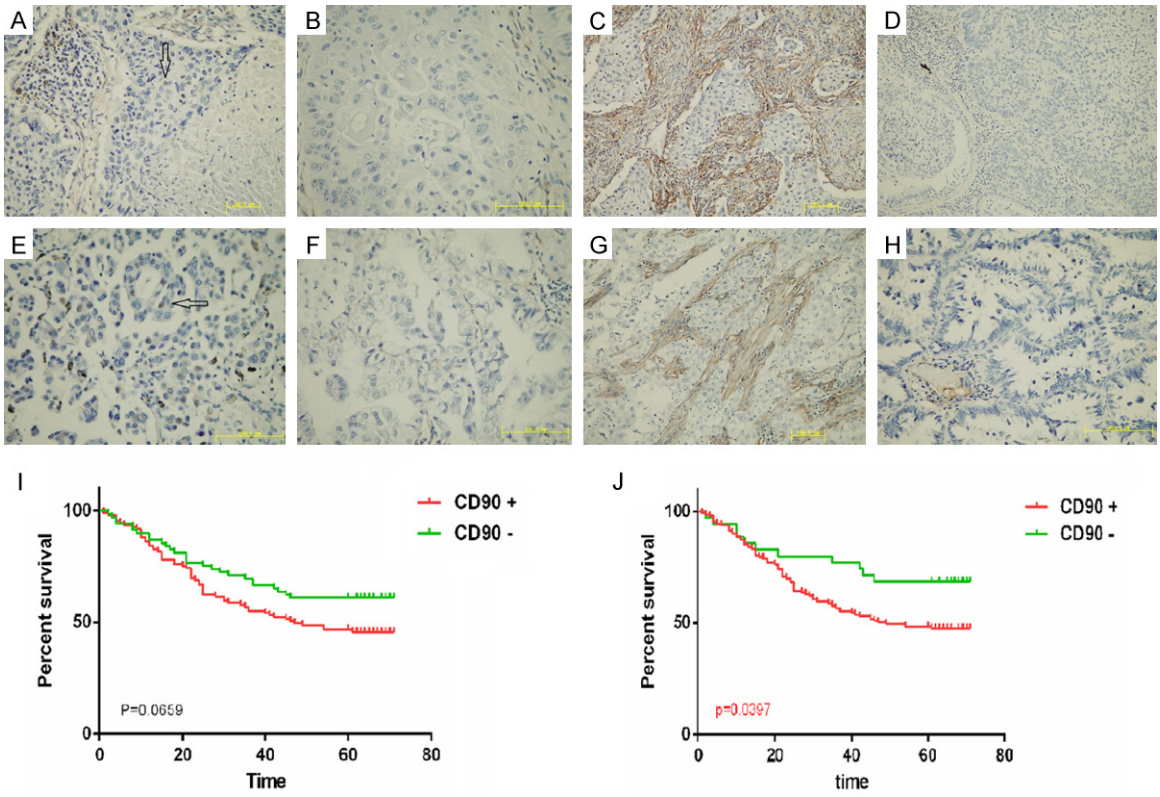

Figure 4. Expression of Thy1 staining in NSCLC by immunohistochemistry and the correlations with overall survivals. A. Positive expression of nucleus CD90 in squamous cell carcinoma (SCC). B. Negative expression of nucleus CD90 in SCC. C. Positive expression of nucleus CD90 in adenocarcinoma (ADC). D. Negative expression of nucleus CD90 in ADC. E. Positive mesenchymal expression of nucleus CD90 in SCC. F. Negative mesenchymal expression of nucleus CD90 in SCC. G. Positive mesenchymal expression of nucleus CD90 in ADC. H. Negative mesenchymal expression of nucleus CD90 in ADC. Original magnification, × 40. I. Survival of nucleus CD90 positive expression. J. Survical of mesenchymal CD90 positive expression. $^{*}P < 0.05$.

expression was an adverse predictor of prognosis in NSCLC patients (P=0.0397), while nuclear Thy1 positive expression had no significant associations with overall survival (P=0.0659).

Discussion

In the present study, we found the stem cell-like sphere 95D cells had a higher efficiency of cloning than adherence 95D in vitro and a better tumorigenic potential of tumor cells in vivo. On the other hand, sphere 95D cells were blocked in G0-G1 in Sphere 95D group significantly, but have no significant effect on the apoptosis compared with adherence 95D cells. Furthermore, we observed that Thy1 was upregulated in stem cell like 95D cell lines and was overexpression in NSCLC patients. Mesenchymal Thy1 positive expression was a poor prognostic predictor for NSCLC patients.

CSCs are a subpopulation of tumor cells that related to tumor proliferation and spreading. These cells have unlimited proliferation potential, capacity to self-renewal and ability to differentiate into a variety of specialized cells [15]. However, the methods of identifying CSCs are conflict nowadays and the amount of CSCs are too low for further analysis. Sphere formation by a conditioned serum-free culture system was reported to be an effective method for enriching stem cell-like cells in numerous studies. Sphere cells with the ability for tumorigenicity and self-renewal are thought to be CSCs [16, 17]. Previous studies also reported they may be an important factor in tumor metastasis, resistance to current radiotherapy and chemotherapy [18, 19]. In our study, we compared the capacity of tumorigenicity and self-renewal in sphere 95D cell lines with adherence 95D cell lines. The results showed the sphere 95D

Table 2. Thy1 expression and association with clinical characteristics

Characteristics	Tumor Nucleus		*P*-value	Tumor mesenchyma		*P*-value
	Thy1- (No.)	Thy1+ (No.)		Thy1- (No.)	Thy1+ (No.)	
Normal Tissue	92	4	0.000*	64	32	0.000*
Tumor Tissue	71	112		35	148	
Gender						
Female	20	29	0.735	12	37	0.265
Male	51	83		23	111	
Histology						
Adenocarcinoma	41	55	0.379	19	77	0.705
Squamous cell carcinoma	25	51		13	63	
Others	5	6		3	8	
Grade						
Median-High	48	79	0.435	25	102	0.682
Poor	22	33		9	46	
T Status						
T1	14	17	0.427	6	25	0.972
T2-4	57	95		29	123	
N Status						
N0	39	60	0.857	17	82	0.572
N1	32	52		18	66	
M Status						
M0	67	107	0.737	32	142	0.377
MX	4	5		3	6	
Stage						
I-II	54	72	0.094	25	101	0.714
III-IV	17	40		35	148	

No.: number. *P < 0.05.

cells had a higher efficiency of cloning than adherence 95D in vitro and a better tumorigenic potential of tumor cells in vivo. We also observed cell cycle was arrested in G0/G1 in Sphere 95D group markedly. This result was consistent with another study in hepatocellular carcinoma (HCC) [20]; they found human poorly differentiated HCC cell lines can form spheres, while the well differentiated ones cannot. Furthermore, they also observed that cell cycle was blocked at G0-G1 in sphere cells compared with parental cells, and P21, which plays a crucial effect on the transition of the cell cycles from the G0/G1 to the S phase [21], was upregulated in sphere cells.

By RNA-Seq, we found the expressions of many genes were different between sphere 95D and adherence 95D cells. These genes were closely collated to cell proliferation, motility and cell differentiation through Gene Ontology analysis. Among all of these, some genes already have many studies indicating their relationships with CSCs, such as Thy1 [22], SNAI1 [23], NOTCH3 [24], SOX2, CD9 [25] and so on. However, the results were conflict sometimes. Further, most differential genes have not been studied, and their functions in cancer were still unknown. Thus, more researches are necessary.

Thy1 plays a vital role in the prognosis of many cancers, such as pancreatic adenocarcinoma [26], esophageal cancer [27], hepatocellular carcinoma [28], meningiomas [29] and so on. With respect to NSCLC, the prognostic significance of Thy1 in NSCLC was still limited. Our results demonstrated that Thy1 was detected in the mesenchyme or nucleus, and the expression rates of Thy1 positive cells were much higher in NSCLC specimens than in normal lung samples. Of the 183 specimens, mesenchymal Thy1 positive expression was an adverse predictor of prognosis in NSCLC patients, while nuclear Thy1 positive expression had no significant associations with overall survival. However, neither mesenchymal nor nuclear Thy1 positive expression was significant statistically associated with clinicopathological characteristics in NSCLC. Kawamura et al. [30] conducted immunohistochemical analysis of clinical specimens (6 mesothelioma, 28 lung adenocarcinoma, 33 lung squamous cell carcinoma), and found the positive expression of Thy1 was higher in mesothelioma than lung cancer. Study by Chen and his collaborators showed Thy1 positive expression can serve as a prognostic maker for lung cancer [31]. However, Matsuwaki et al. have opposite opinions; their study suggested that Thy1 positive expression had no significant relationships with three-year recurrence-free

survival [32]. These inconsistencies might be explained by the limited patients' number, the different cut-off scores and different primary antibody types or dilutions. Therefore, Thy1 might serve as a vital prognostic biomarker for NSCLC, and some large cohort studies with more suitable laboratory methodology are also needed to further demonstrate the relationships between Thy1 and the survival of NSCLC patients.

Thy1 operates as an important factor of regulating cell-matrix and cell-cell interactions, proliferation, adhesion, apoptosis, migration, metastasis, angiogenesis and fibrosis [33] in cancers. Wang et al. found in lung squamous cell carcinoma, CD44 (+) CD90 (+) cells have a higher ability to form spheroids, and revealed mesenchymal morphology [34]. In gastric cancer, Thy1 was overexpressed and could inhibit gastric cancer cell apoptosis through regulating the expression levels of SPARC proteins [35]. In melanoma, Thy1 could promote the adhesion and transmigration of melanoma cells to the activated endothelium via the interaction with the αvβ3-integrin [4, 36]. Furthermore, Sukowati et al. found in hepatocellular carcinoma, expression of ABCG2 was upregrulated in CD90 (+) subpopulation after exposure to DOX, indicting CD90 involved in drug resistance [37]. Thus, Thy1 plays an important role in the progress of the cancers, and might serve as a therapeutic target for NSCLC.

In summary, our results have shown that Thy1 was up-regulated in stem cell like cells, and was overexpression in NSCLC patients. Although nuclear Thy1 positive expression was not associated with overall survival, mesenchymal Thy1 positive expression was an adverse predictor of prognosis for NSCLC patients. Thus, Thy1 might serve as a vital therapeutic target for NSCLC.

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Disclosure of conflict of interest

None.

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Table S1. Enrichment of genes involved in biological process in Sphere 95D cells

Gene Ontology	Number of genes		*P* value
	Up-regulated genes	Down-regulated genes	
Cell proliferation	33	33	0.00132*
Cell motility	27	33	0.02861*
Cell migration	24	34	0.09278
Regulation of cell differentiation	26	34	0.04712*
Regulation of apoptotic process	32	29	0.38457
Regulation of cell adhesion	5	12	1
Stem cell development	5	1	1

Table S2. Patient characteristics

Characteristic	Total	%
Total	183	100
Median age (years; range)	58.5 (28-81)	
Gender		
Male	134	73.2
Female	49	26.8
Histology		
Adenocarcinoma	98	53.5
Squamous cell carcinoma	75	41.0
Other*	10	5.5
Pathological stage		
I-II	107	58.5
III-IV	76	41.5
T Status		
T1-2	117	63.9
T3-4	66	36.1
N Status		
N0	99	54.1
N1-3	84	45.9
M Status		
M0	174	95.1
Mx	9	4.9
Grade		
I	55	30.1
II	51	27.9
III	78	41.5
Not defined	1	0.5

*: Other histology included ten patients with not specified non-small-cell lung cancer.