Experimental Research

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S100A6 could not promote the differentiation of Calu-6 lung cancer cell line

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Background: Our previous study demonstrated that S100 calcium binding protein A6 (S100A6) impairs tumorigenesis by Calu-6 lung cancer cells, as well as inhibit their growth. However, the role that S100A6 plays in tumor cell differentiation has not been previously explored. This study aimed to confirm the effect of S100A6 on the direction of differentiation in the human lung cancer cell linem Calu-6m based on our previous published research.

Materials and methods: A S100A6-overexpressing lentiviral vector was successfully constructed in our previous study. Nude mouse tumorigenicity was then applied successfully, and 15 mice were divided into three groups (Calu-6, Calu-6/neo, Calu-6/S100A6). After 5 weeks, we detected lung cancer markers with immunohistochemistry in mice tumor tissues, including the adenocarcinoma markers, TTF-1 and NapsinA, the squamous cell carcinoma markers, P40, CK5/6 and P63, and the small cell lung cancer markers CD56, Syn, CgA, TTF-1, CK, and Ki-67. Differences among the three groups were statistically compared.

Results: All the above-mentioned markers were positive in the tumor tissues of all three groups, and there were no significant differences.

Conclusion: S100A6 cannot promote differentiation of the undifferentiated human lung cancer cell line, Calu-6, into adenocarcinoma, squamous, or small cell carcinoma cell lines.

Keywords: biomarker, Calu-6, differentiation, immunohistochemistry, S100A6

Introduction

Lung cancer is a leading cause of cancer-related mortality and shows a relatively high incidence in both sexes[1]. Predisposition to risk factors such as smoking, air pollution, and aging increase the occurrence of this carcinoma[2]. Adenocarcinoma and squamous cell carcinoma are two common pathological types of lung cancer[3], also known as nonsmall cell lung cancer (NSCLC). Due to a low early diagnosis rate and resistance to radiotherapy and chemotherapy, the overall prognosis of NSCLC is poor[4,5]. In recent years, the prognosis of some lung adenocarcinoma patients with positive driver genes, such as EGFR, ALK, ROS, PD-1/PD-L1 has significantly improved due to the use of targeted and immunotherapy drugs[6,7]. However, the prognosis of many patients diagnosed with NSCLC with negative driver genes has not significantly improved. Therefore, current research on lung cancer focuses significantly on driver genes and pathogenesis.

HIGHLIGHTS

- Our experiment identify S100A6 could not promote the differentiation of undifferentiated human lung cancer cell line Calu-6 into adenocarcinoma, squamous, or small cell carcinoma cells.
- This is first study to clarify S100A6's function on the differentiation of lung cancer cell line.
- In future, we look forward to the inclusion of more lung cell lines in the follow-up study to illustrate the effects of this protein in lung cancer.

The S100 protein family is the largest subfamily of calcium-binding proteins of EF-hand type. It contains a total of 25 known members, each coded by a separate gene in humans[8]. Some members in this group are found to be multifunctional proteins and have a close relationship with tumors[9]. S100A6 is a significant member of the S100 protein family but there are conflicting results about its function in lung cancer. Our previous study showed that overexpressed S100A6 inhibits tumorigenicity of Calu-6 lung cancer cells. The tumor sizes in Calu-6/S100A6 mice were smaller than that of Calu-6 mice and Calu-6/neo mice[10]. However, previous data in our study drew an opposite conclusion compared to other research using A549 cells[11]. Since A549 is a lung adenocarcinoma cell line, while Calu-6 is an undifferentiated lung cancer cell line, we suggested that S100A6 may take on various functions in different pathological subtypes of lung cancer. Additionally, we speculated that S100A6 may be involved in the differentiation of Calu-6 lung cancer cells. In this study, we used immunohistochemistry to detect classic and

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commonly used lung cancer markers, such as the lung adenocarcinoma markers TTF-1, and NapsinA, the squamous cell carcinoma markers, P40, CK5/6, and P63, and the small cell lung cancer markers CD56, Syn, CgA, TTF-1, CK, and Ki-67, in the tumor tissue of mice established from our previous study, to evaluate the relationship between S100A6 and lung cancer differentiation.

Materials and methods

The lung cancer cell line, Calu-6, was purchased from the Chinese Academy of Sciences (https://cellbank.org.cn/, serial number: TCHu144, identifier: CSTR:19375.09.3101HUMTCHu144). The cells were preserved at 37°C and 5% CO2, maintained and expanded in growth medium containing high glucose Dulbecco's modified Eagle medium (DMEM;Gibco) combined with 15% heated-inactivated fetal bovine serum (FBS, Zhengbo) and 100 U/ml streptomycin/penicillin (Gibco) for one to two weeks. Other steps, including construction, production and concentration of lentivirus vector, pLVX-AcGFP1-N1-S100A6, cell infection, RNA extraction and qPCR, Western Blot, and animal experiments, have been described in our previously published paper[11]. At the last step, all 15 mice were divided into three groups (Calu-6, Calu-6/neo, Calu-6/S100A6) and sacrificed, followed by tumor tissue collection.

Immunohistochemistry

We used immunohistochemical staining with hematoxylin/eosin to detect the expression of selected biomarkers, including TTF-1, NapsinA, P40, CK5/6, P63, CD56, Syn, CgA, CK, Ki-67. Specific information of the primary antibodies were as follows: TTF-1 rabbit monoclonal antibody (Abcam, ab76013), NapsinA rabbit monoclonal antibody (Abcam, ab248340), P40 Rabbit monoclonal antibody (Abcam, ab76158), CK5/6 mouse monoclonal antibody (Abcam, ab17133), P63 rabbit monoclonal antibody (Abcam, ab124762), CD56 rabbit monoclonal antibody (Abcam, ab220360), Syn Rabbit poly-clonal antibody (Abcam, ab214316), CgA Rabbit monoclonal antibody (Abcam, ab92738), CK Mouse monoclonal antibody (Abcam, ab215838), Ki-67 Rabbit polyclonal antibody (Abcam, ab15580), the diluted concentration was 1:250. The antigen was retrieved by hydration and deparaffinization. To quench endogenous peroxidase activity, the sections were blocked with 3% hydrogen peroxide at room temperature for 15 min. Subsequently, they were incubated with primary antibodies at 4°C. Secondary antibodies were labeled by horseradish peroxidase were then added to all sections at room temperature for 30 min. At the last step, the tissue sections were counterstained with hematoxylin after the peroxidase reaction.

We used a microscope and analyzed 10 random fields to determine the number of positive cells. The biomarker expression was graded according to the standard as follows: <25% positive cells, 1; 26–50% positive cells, 2; 51–75% positive cells, 3; > 76% positive cells, 4. The

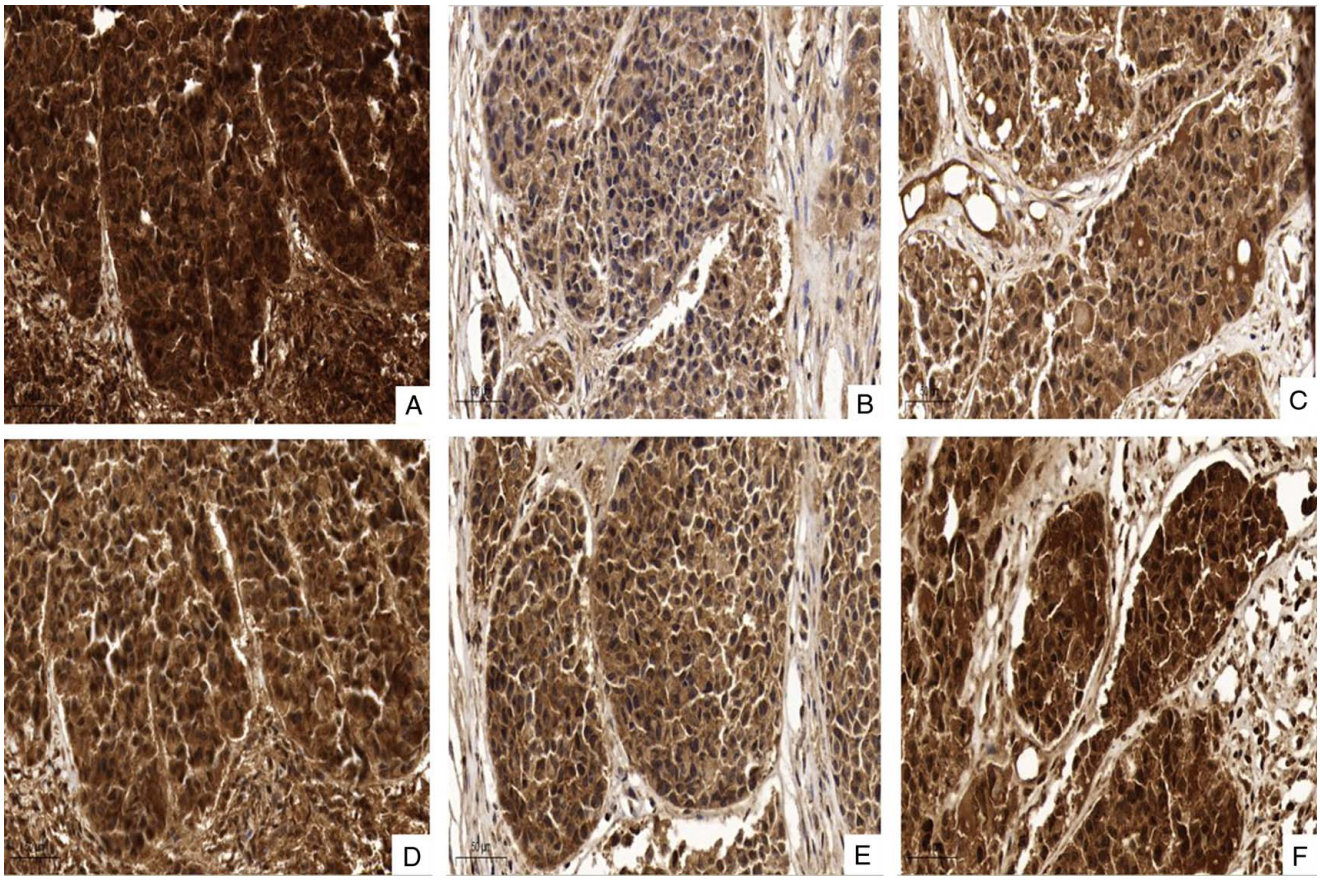

Figure 1. Immunohistochemical expression of TTF-1 (A) Calu-6, (B) Calu-6/neo, (C) Calu-6/S100A6), NapsinA (D) Calu-6, (E) Calu-6/neo, (F) Calu-6/S100A6.

Table 1

The selected markers' immunohistochemical expression score of each mouse in three groups (Calu-6, Calu-6/neo, Calu-6/S100A6)

	Calu-6				Calu-6/neo				Calu-6/S100A6						
Group marker (score)	**No.1**	**No.2**	**No.3**	**No.4**	**No.1**	**No.2**	**No.3**	**No.4**	**No.1**	**No.2**	**No.3**	**No.4**	**No.5**	**No.6**	***P***
TTF-1	6	7	7	6	7	6	7	7	7	7	5	7	7	7	>0.05
NapsinA	7	7	7	6	7	5	7	7	6	7	7	7	6	7	>0.05
P40	6	7	6	5	6	6	7	7	7	7	6	7	7	7	>0.05
CK5/6	7	7	6	7	5	7	7	7	7	7	6	7	6	7	>0.05
P63	7	7	7	7	7	6	7	7	7	6	6	7	7	7	>0.05
CD56	7	7	7	5	7	7	7	5	7	7	7	6	7	7	>0.05
Syn	7	7	6	7	7	6	7	6	5	5	6	6	7	7	>0.05
CgA	7	7	7	7	7	6	7	7	7	7	7	6	7	7	>0.05
TTF-1	6	7	7	7	7	7	7	7	7	5	6	7	5	7	>0.05
CK	7	7	7	7	7	7	7	6	6	7	7	7	7	6	>0.05
Ki-67	7	7	7	7	7	7	7	7	7	7	6	7	7	7	>0.05

The total score was based on the intensity plus percentage of positive cells. The expression of the biomarkers was graded according to the standard as follows: <25% positive cells, 1; 26–50% positive cells, 2; 51–75% positive cells, 3; >76% positive cells, 4. The intensity was scored as follows: no signal, 0; weak, 1; moderate, 2; strong staining, 3.

intensity was scored as follows: no signal, 0; weak, 1; moderate, 2; strong staining, 3. The total score, which was divided into two groups, <4 as negative, and ≥ 4 as positive, was based on the intensity plus the percentage of positive cells. Two experienced pathologists participated in the scoring process and reported separately.

Statistical analysis

SPSS26.0 was used to collect and analyze data. We applied χ^2 test to compare qualitative variables, if a cell in the table had few expected cases (i.e. <1), Fisher's Exact test was used. A two-tailed $P < 0.05$ was considered statistically significant.

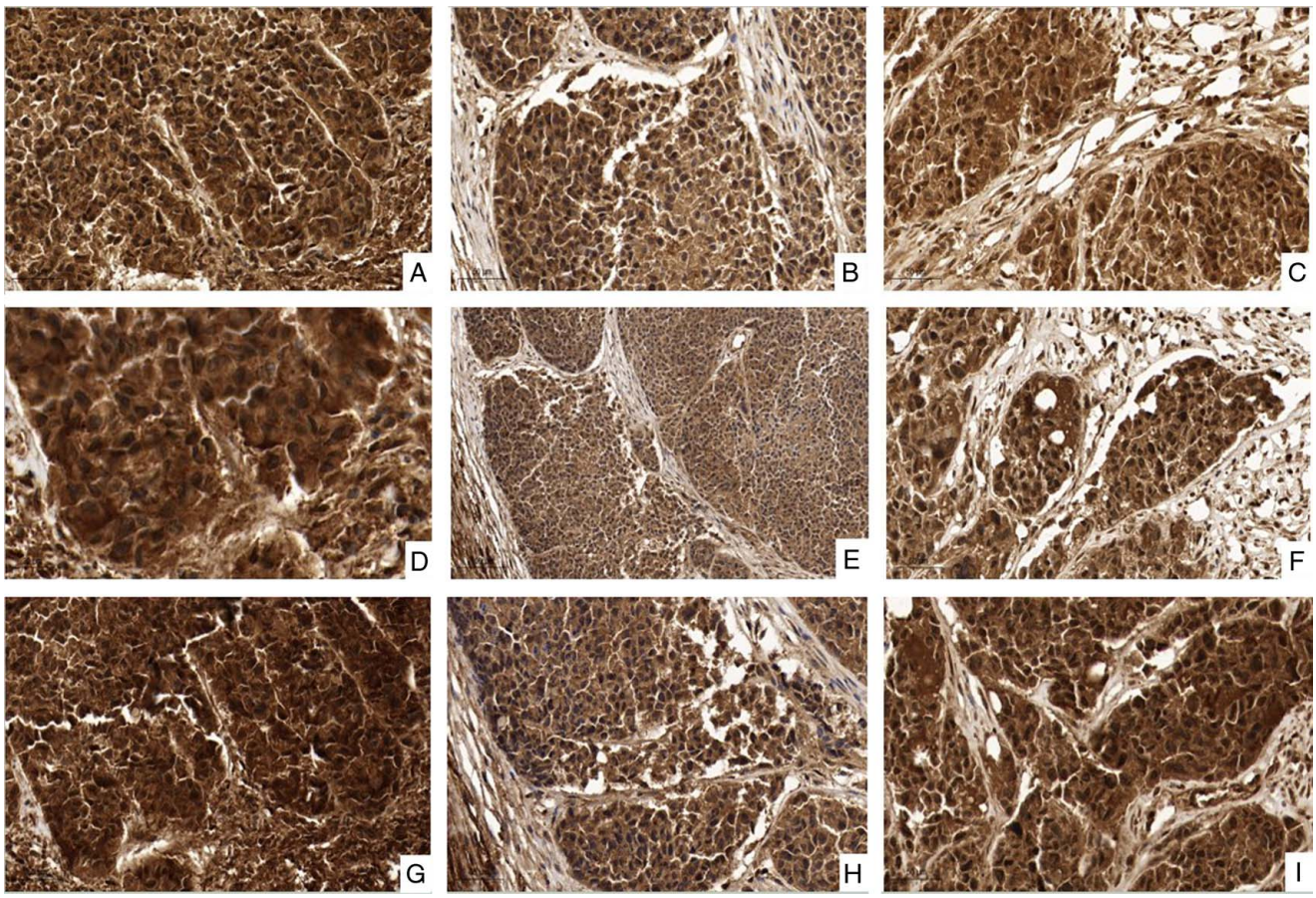

Figure 2. Immunohistochemical expression of P40 (A) Calu-6, (B) Calu-6/neo, (C) Calu-6/S100A6), CK5/6 (D) Calu-6, (E) Calu-6/neo, (F) Calu-6/S100A6 and P63 (G) Calu-6, (H) Calu-6/neo, (I) Calu-6/S100A6.

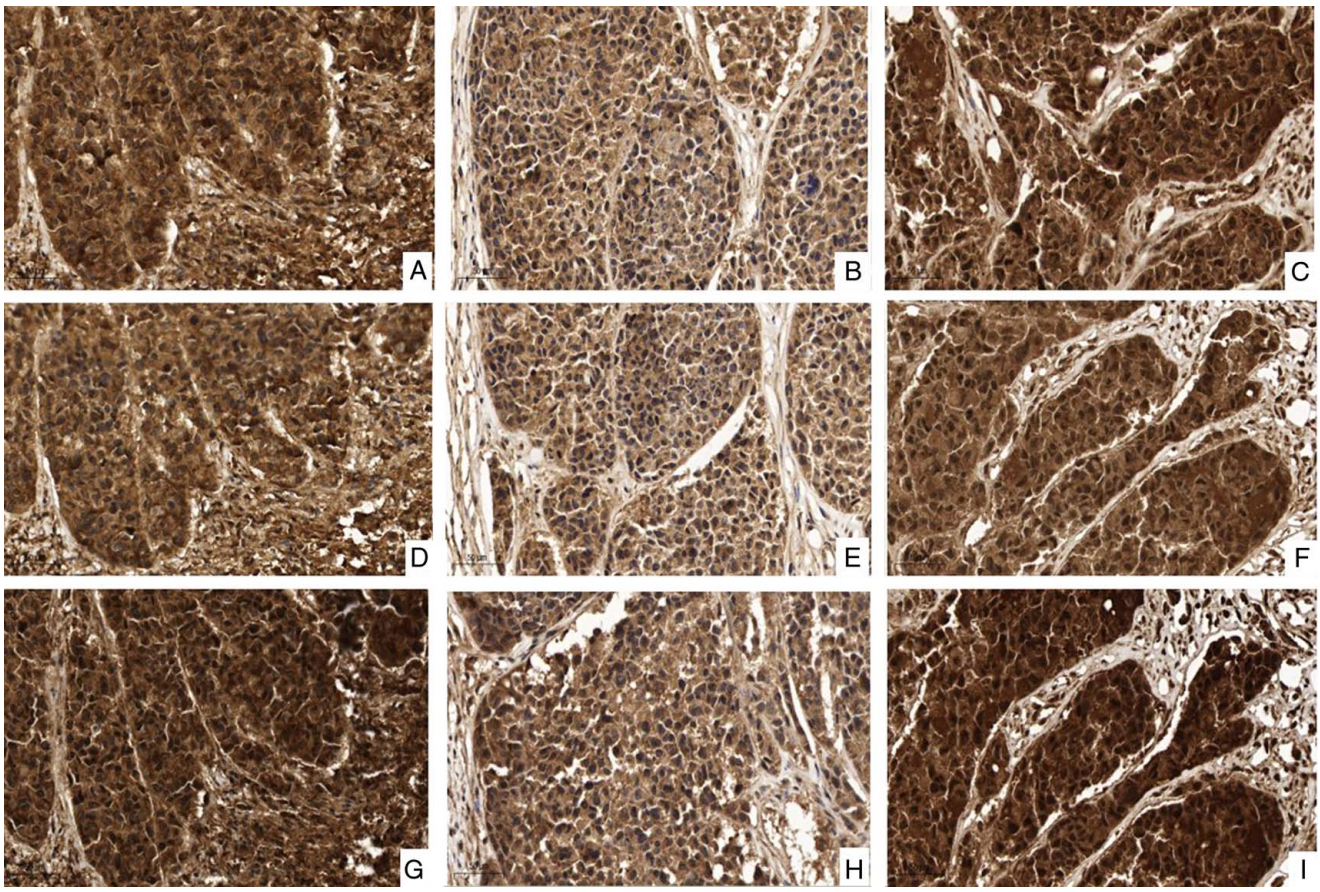

Figure 3. Immunohistochemical expression of CD56 (A) Calu-6, (B) Calu-6/neo, (C) Calu-6/S100A6), Syn (D) Calu-6, (E) Calu-6/neo, (F) Calu-6/S100A6) and CgA (G) Calu-6, (H) Calu-6/neo, (I) Calu-6/S100A6).

Results

Immunohistochemical expression of TTF-1, NapsinA

Tumor tissues from 15 mice in three groups (Calu-6, Calu-6/neo, Calu-6/S100A6) were examined. As shown in Figure 1 and Table 1, there was no statistical significance among the three groups. Both TTF-1 and NapsinA were expressed and the total score in all 15 samples were more than 4 ($P > 0.05$).

Immunohistochemical expression of P40, CK5/6, and P63

Figure 2 displays information about squamous cell carcinoma marker expression, such as P40, CK5/6 and P63, in S100A6 overexpressed tumor tissues (Calu-6/S100A6 group) and the controls (Calu-6, Calu-6/neo). Positive expressions of these three markers were detected in all samples (Table 1), and there was no statistical significance ($P > 0.05$).

Immunohistochemical expression of CD56, Syn, CgA, TTF-1, CK, and Ki-67

All markers commonly used in SCLC, including CD56, Syn, CgA, TTF-1, CK, and Ki-67, expressed positively in all 15 samples (Table 1), and there was no statistical significance among the three groups, as shown in Figures 3 and 4.

Discussion

In our preliminary experiment, we injected Calu-6 lung cancer cells, that were overexpressing S100A6, subcutaneously into mice to construct our animal model, while mice injected with Calu-6 and Calu-6/neo cells acted as control models. In this study, we detected the expression of some molecular markers in these mouse tumor tissues. Those markers included the lung adenocarcinoma markers, TTF-1, and NapsinA, the squamous cell carcinoma markers, P40, CK5/6, and P63, and the small cell lung cancer markers, CD56, Syn, CgA, TTF-1, CK, and Ki-67. These markers are commonly used in the pathological diagnosis of lung cancer[12]. By comparing the difference of these markers among the three study groups, we planned to explore the effects of S100A6 on the differentiation of Calu-6 cells. The results indicated that since these molecular markers were positively expressed in all three groups without significant differences, then S100A6 was not able to promote differentiation in Calu-6 lung cancer cells.

The S100 protein family, which is the largest subfamily of calcium-binding proteins, was firstly discovered by Moore *et al*.[13] in 1965. A majority of S100 genes, located on the human chromosome, 1q21, which is prone to rearrangement, have a close relationship with the initiation and development of many tumor types[14]. S100 proteins play critical functions in the

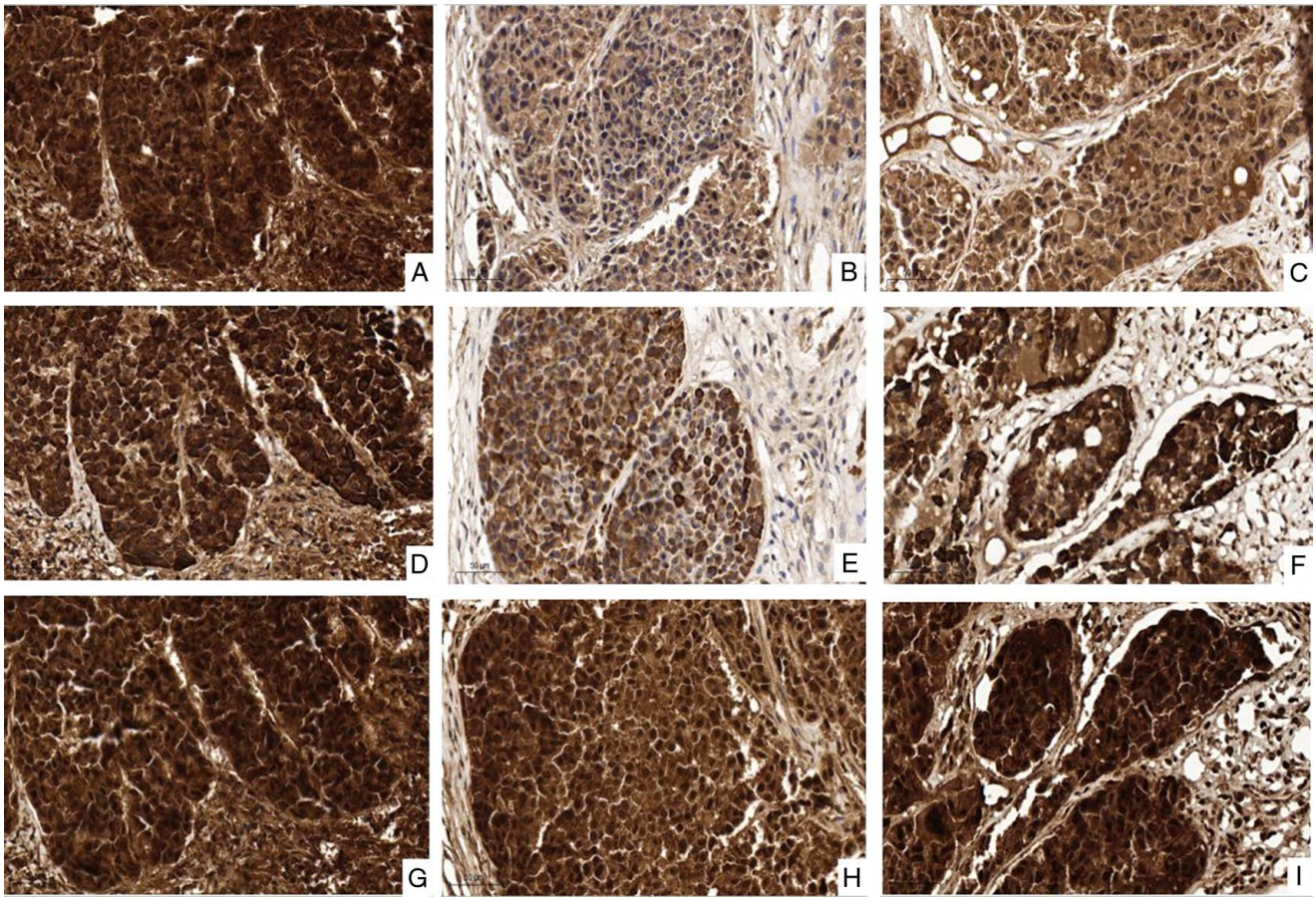

Figure 4. Immunohistochemical expression of TTF-1 (A) Calu-6, (B) Calu-6/neo, (C) Calu-6/S100A6), CK (D) Calu-6, (E) Calu-6/neo, (F) Calu-6/S100A6) and Ki-67 (G) Calu-6, (H) Calu-6/neo, (I) Calu-6/S100A6).

regulation of cell proliferation, migration, differentiation, and apoptosis via interacting with various signaling proteins[15,16].

S100A6 is the first member proven to play a role in tumor proliferation, progression, and invasion in this family[17]. In gastric cancer, S100A6 was demonstrated to be overexpressed in primary gastric carcinoma tissues compared with matched noncancerous mucosal tissues, and had an obvious relationship with invasive cell behaviors, such as positive lymph node involvement[18]. In cervical cancer, a study by Li *et al.*[19] indicated that S100A6 facilitates the malignant potential of cervical cancer cells, particularly EMT and metastatic ability. Lung cancer-related studies in recent years have suggested that the mechanism of S100A6 is complex, and may play different roles in different subtypes. Researchers detected S100A6 expression in archival tumor cell lysates from 39 patients with resected NSCLC, their study validated that S100A6 expression has a direct correlation with nonsquamous histology, and an inverse relationship with P53 expression. S100-positive cases show a trend towards longer survival compared with S100A6-negative cases[20]. However, in another study, 177 lung squamous cell cancer patients were divided into two groups according to S1006 level, and those with a higher S100A6 expression tended to have an unfavorable prognosis[21]. In lung adenocarcinoma, S100A6 tended to have a higher expression in a mixed subtype of bronchioloalveolar carcinoma (BAC) compared to pure BAC. Aya Ishii *et al.* used immunohistochemistry to assess S100A6 expression in tumor cell cytoplasm and nuclei of 92 lung adenocarcinomas. They proved that invasive tumors had increased cytoplasmic S100A6 expression compared to normal lung tissue and nonadvanced lesions. This trend was more significant in adenocarcinoma with a BAC component[22]. Concerning small-cell lung cancer (SCLC), S100A6 was found to be largely down-regulated in comparison with normal lung tissues[23]. At the cellular level, compared with human bronchial epithelioid cell lines (HBE) and NSCLC cell lines (H441 and H1975), A549 had the highest expression level of S100A6. Moreover, this over-expression was demonstrated to promote the proliferation, migration, invasion, and angiogenesis of A549 cells by blocking P53 acetylation[10]. In contrast, our previous study drew the conclusion that S100A6 plays an inhibitive role in the carcinogenesis and progress of Calu-6 cells by suppresing migration, invasion, proliferation, and enhancing apoptosis[11]. This may be attributed to Calu-6 being an undifferentiated lung cancer cell line, while A549 is a lung adenocarcinoma cell line. This also awakened our interest to research the effects of S100A6 on the differentiation of lung cancer cell lines. In osteosarcoma, over-expression of S100A6 has been proven to inhibit the differentiation of mesenchymal stem cells into osteoblasts, thus

promoting tumorigenesis[24]. Until now, few studies have focused on the function(s) of S100A6 on differentiation of lung cancer cells.

Conclusion

Our experiment indicates that S100A6 probably could not promote the differentiation of the undifferentiated human lung cancer cell line, Calu-6, into adenocarcinoma, squamous, or small cell carcinoma cells.

Some limitations in our study include that only one cell line was used, and the function of S100A6 in the differentiation behavior of stem cells was not explored. In the future, we plan to include more lung cell lines to illustrate the effects of this protein in the differentiation of lung cancer.

Ethical approval

Ethical approval for this study (No. 20230058) was provided by the Ethical Committee of Xi'an People's hospital (Xi'an No. 4 hospital), Xi'an, China on 23 February 2023.

Consent

Informed consents from patients were not required for this systematic review. The present study followed international, national and institutional guidelines for humane animal treatment and complied with relevant legislation. Meanwhile, the present study involved client-owned animals and demonstrated a high standard of veterinary care and involved informed client consent.

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Author contribution

All authors read and approved the manuscript. Detailed contributions are listed as follows: J.L.: performed the literature review and drafted the manuscript; T.W.: participated in the revision of manuscript, and is responsible for experiments; D.D.: interpreted the data and will pay the publication fee.

Conflicts of interest disclosures

All authors declare that they have no conflicts of interest.

Research registration unique identifying number (UIN)

Unique identifying number was not required for this systematic review.

Guarantor

All data generated or analyzed during this study are available, and could be achieved from the corresponding author Dan Dang if required.

Availability of data and materials

All data generated or analyzed during this study are available, and could be achieved from the corresponding author Dan Dang if required.

Provenance and peer review

Not applicable.

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