Significance of stem cell marker *Nanog* gene in the diagnosis and prognosis of lung cancer

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Abstract. The aim of the present study was to analyze the stem cell marker, *Nanog* gene, for the diagnosis and prognosis of lung cancer cases, and to study its application in the diagnosis of lung cancer. In total, 100 patients diagnosed with lung cancer between April, 2013 and May, 2015 were included in the present study. The patients were randomly divided into group A (lung cancer) and group B (squamous cell lung carcinoma). RT-PCR was used to detect the cancer and adjacent tissues, and *Nanog* gene expression was detected in groups A and B in cells. The results showed that, analysis of *Nanog* gene expression in the two groups of patients varied to different degrees. There was no significant difference between the two groups with regard to age, gender, disease stage and lymph node metastasis. *Nanog* gene expression in patients with carcinoma were significantly higher than that in the adjacent tissues ($p<0.05$). By contrast, differentiated and well-differentiated carcinoma tissue showed a significantly higher *Nanog* gene expression than poorly differentiated and undifferentiated carcinoma ($p<0.05$). The expression of *Nanog* in normal cells was significantly higher than that in normal lung tissues and benign lesions in lung cancer stem cells. *Nanog* was highly expressed in $CD44^+$ cells, and *Nanog* expression in lung cancer stem cells was significantly higher ($p<0.05$). In conclusion, for groups A (lung cancer) and B (squamous cell lung carcinoma) the *Nanog* gene expression was significantly higher. The data of the present study show that the patients with stage III and IV lung cancer had a higher *Nanog* gene expression. In addition, there was a higher expression of *Nanog* in lung cancer patients. By contrast, a lower degree of cell differentiation was associated with strong *Nanog* gene expression in lung cancer.

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Introduction

Tumors possess a self-renewing ability that can generate heterogeneous cells in tumor cells. Tumors are composed of somatic mutations, each of which can be grown without restriction. However, this does not explain the phenomenon that cancer cells seem to have unlimited viability, and that not all tumor cells are capable of unlimited growth. The characteristics of tumor cell growth, metastasis and recurrence are similar to the basic characteristics of stem cells. Therefore, the theory of tumor stem cells (TSCs) has been suggested (1). This theory provides a new direction and a visual angle to us to gain a new understanding of the origin and nature of the tumor, as well as the clinical treatment of cancer. In recent years, studies conducted in China have increasingly focused on cancer stem cells. The main reason leading to tumor is abnormality of stem cells, leading to diseases, such as lung and colorectal cancer (1). Current studies have shown that malignant tumor growth leads to the expression of stem cells in molecules that play an important role in gene regulation (2).

Investigations regarding *Nanog* gene have shown that it promotes cell induction, leading to analysis of *Nanog* gene expression in tumor (3), although its expression is relatively decreased in lung cancer (4). This study primarily investigated the role of *Nanog* gene in the two groups of patients with pulmonary adenocarcinoma and squamous lung carcinoma.

Patients and methods

Patients. In total, 100 cases of tumor patients diagnosed with lung cancer between April, 2010 and May, 2012 were selected for the present study. Patient age was 22-76 years, with an average age of 58.43±10.44 years. The study included 50 men, aged 22-73 years, with an average age of 59.12±9.06 years, and 50 women, aged 23-76 years, with an average age of 58.54±9.43 years. A CT scan, MRI, chest X-ray, flexible bronchofiberscope examination and sputamentum cell examination were performed on the patients, for confirmation of lung cancer. In the 100 patients, there were 50 cases in group A (pulmonary adenocarcinoma) for whom the diagnosed age for 17 cases was <40 years, 16 cases were 40-60 years, and 17 cases were >60 years. The tested diseases of this study were divided into 9 cases in phase I, 13 cases in phase II, 11 cases

Table I. *Nanog* gene expression at different phases in pulmonary adenocarcinoma and squamous lung carcinoma patients.

	Pulmonary adenocarcinoma (n=50)		Squamous lung carcinoma (n=50)	
Phase	Cancer tissue	Adjacent normal tissue	Cancer tissue	Adjacent normal tissue
I phase	1.30±0.29	0.34±0.13[c,1]	1.33±0.46	0.36±0.14[c,5]
II phase	1.38±0.32	0.36±0.14[c,2]	1.58±0.40	0.38±0.10[c,6]
III phase	2.28±0.52[a,b,9]	0.44±0.11[c,3]	2.33±0.58[a,b,11]	0.42±0.15[c,7]
IV phase	2.47±0.63[a,b,10]	0.43±0.15[c,4]	2.56±0.60[a,b,12]	0.47±0.13[c,8]

Comparison of the data in phase I, t_9=3.269, t_{10}=5.212, t_{11}=2.963, t_{12}=3.214, [a]P<0.05 to phase II, t_9=3.265, t_{10}=5.231, t_{11}=5.123, t_{12}=2.152, [b]P<0.05. Comparison to cancer tissue, t_1=2.369, t_2=2.963, t_3=3.158, t_4=5.156, t_5=2.961, t_6=2.485, t_7=2.741, t_8=2.852, [c]P<0.05.

in phase III and 10 cases in phase IV. There were 21 patients with lymph node metastasis, as indicated by test. In addition, 50 cases were included in group B (squamous cell lung carcinoma). No significant difference with regard to age, gender and diseases were observed, compared with cases in group A.

Test methods of Nanog gene expression in tumor stem cells. RT-PCR was used to quantify *Nanog* gene in real-time. The mRNA agarose gel electrophoresis was used to test 100 cases, followed by 1 μg of RNA for reverse transcription. PrimeScript RT was added to the 20 μl system and reverse transcription was initiated initially at 42˚C for 45 min, followed by incubation at 70˚C for 10 min, and cooling on ice to inactivate reverse transcriptase. Subsequently, cDNA was synthesized. Primers were designed from GeneBank data as follows: *Nanog* forward, (5'-ATGCCTGCATTTTTCATCC-3') and reverse, (5'-GAGGCAGGTCTTCAGAGGAA-3'), with a product length of 189 bp. β-actin was used as the internal control and its primers were: Forward, (5'-CAGAGCAAGAGAGGC ATCC-3') and reverse, (5'-CTGGGGTGTTGAAGGTCTC-3'), with a product length of 217 bp. PCR reaction was prepared by using 2X SYBR Premix Ex Taq 10 μl, cDNA template 2 μl, forward and reverse primers of 0.4 μl, and the total volume was brought to 20 μl with autoclaved water. Clinical SYBR-Green I fuel method was used for RT-PCR to amplify the genes. Fully automatic fluorescent quantified PCR apparatus AB17500, and Real-Time PCR system, were used and the temperature was set at 58˚C for 39 cycles. Following observation the results were recorded. The relative CT value for β-actin was calculated in detail as indicated in a previous study (5-8).

Observation index. The real-time quantified method was used to examine age, gender, and any lymph node metastasis in the two groups of patients. RT-PCR of tumor and adjacent normal tissue was used to test the gene expression level of stem cells of the two groups of patients. After obtaining the data, the gene expression amount of groups A and B was analyzed to varying extent (7). PCR gel electrophoresis was used to test Nanog expression in $CD44^+$ cells, and the living curve was used for statistics and to compare the survival rate of the patients in five years. In addition, immunostaining was performed to stain lung cancer cells in patients.

Statistical analysis. The data were presented as mean ± standard deviation. Quantified data were expressed by cases (n) and percentage. Data analysis was conducted using SPSS 15.0 software (Chicago, IL, USA). A Student's t-test and χ^2 test were used to compare data. The ranked data were compared by a non-parametric test. P<0.05 was considered to indicate a statistically significant difference.

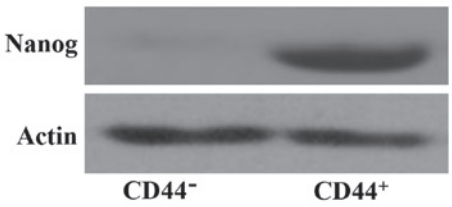

Figure 1. Nanog expression in lung cancer stem cell $CD44^+$ cells.

Results

Nanog expression in $CD44^+$ cells in lung cancer stem cell. As shown in Fig. 1, the lung cancer stem cells $CD44^+$ cells showed a higher expression of *Nanog*, suggesting that it plays an important role in lung cancer stem cells.

Nanog expression in adjacent normal tissue and benign lesion cancer tissue. The results showed that *Nanog* was mainly expressed in the nucleus of lung cancer cells, and in positive control (spermatogenous cell). The expression was mainly in the nucleus, and *Nanog* expression in lung cancer cells was significantly higher than that of adjacent normal tissue and benign lesion lung tissue (Fig. 2).

Nanog gene expression. We found that the expression level of phase I patients was 1.30±0.29, phase II patients was 1.38±0.32, phase III was 2.28±0.52 and phase IV was 2.47±0.63. The patients expression levels had significant different extents of the improvement (p<0.05). The Nanog gene expression in cancer tissues significantly decreased, and the data showed that Nanog levels in pulmonary adenocarcinoma and squamous lung carcinoma patients were basically the same (Table I).

Nanog expression of cells in various differentiation condition. A comparison of the *Nanog* detection rate in differentiation cells in pulmonary adenocarcinoma and squamous lung carcinoma yielded 33.5 and 37.8%, respectively. In middle- and high-differentiation cells, the *Nanog* detection rate was

Table II. Nanog detection in adenocarcinoma and squamous lung carcinoma patients under various differentiation conditions.

Phase	Pulmonary adenocarcinoma (n=50)		Squamous lung carcinoma (n=50)	
	Detection cases/cases	Detection rate (%)	Detection cases/cases	Detection rate (%)
No differentiation	8/9	89.1	8/10	82.0
Low differentiation	7/12	70.2	9/13	69.4
Middle differentiation	4/12	33.5	4/11	36.6[a]
High differentiation	3/8	37.8	2/7	28.8[a]

Compare the two groups of differentiation, χ^2=5.31, [a]P<0.05; compared with low differentiation, χ^2=6.18, [b]P<0.05.

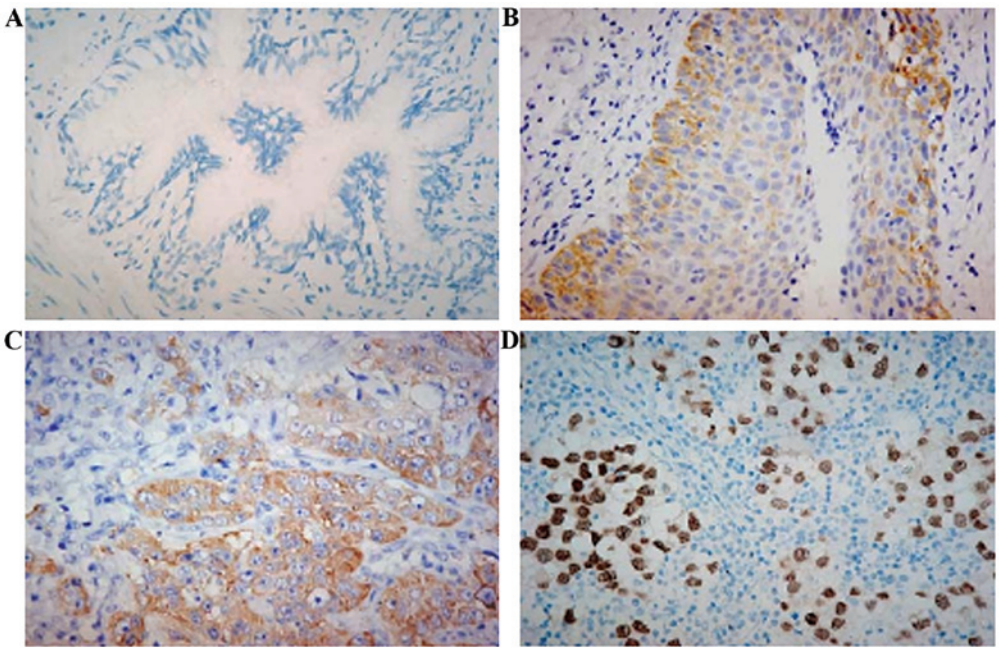

Figure 2. Nanog expression in adjacent normal tissue, benign lung cancer and lesion lung cancer tissue (immunostaining; original magnification, x400). (A) Positive staining of normal lung cells, (B) Nanog in squamous epidermis, (C) Nanog staining in squamous cell cancer, (D) Nanog staining in spermatogenous cell cancer nucleus.

relatively significantly high, while in the no and low differentiation cells, the expression was 89.1 and 70.2%, respectively (p<0.05). In the present study, we found that *Nanog* is basically the same in stem cells of pulmonary adenocarcinoma and squamous lung carcinoma patients (Table II).

Investigation and observation of the patients in the present study. The patients were divided into two groups according to high or low expression of *Nanog*, and then followed-up for the survival rate of the two groups. As shown in Fig. 3, a high expression level of *Nanog* in patients had a lower meta-survival rate (44%), and a low expression level of *Nanog* patients had a higher meta-survival rate (60%), and χ^2=4.69. P<0.05 was considered to indicate a statistically significant difference.

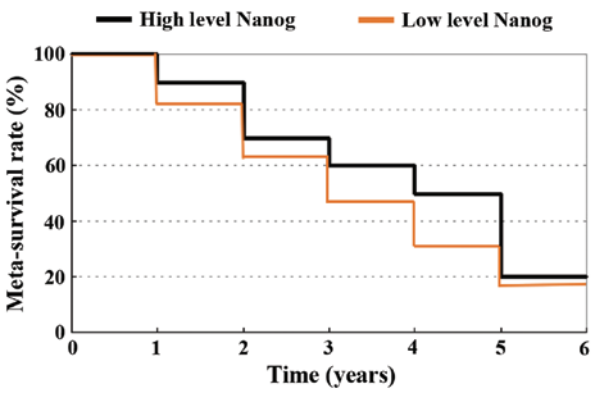

Figure 3. Observational survival rate (%) for all the patients.

Discussion

Clinical studies have found that cell heterogeneity is an important cause of tumor development (8). Certain specificity cells in the human body have a certain ability for self-renewal and differentiation (9), and these types of cells have anti-drug and drug resistance (10), showing some characteristics of stem cells (11). Tumor stem cells are tumor cells that occur clinically, and are capable of renewal and proliferation (12). They can

indirectly influence tumor growth, and therefore are of great significance in the control and prevention of tumor (13). *Nanog* expression is a transcription factor of embryonic stem cells (ESCs), and is also a type of primitive reproduction cell. It has been found that *Nanog* exists in embryonic stem cell, reproduction stem cell and other related tumor cells (14,15). Relevant studies suggested that cancer cells in human body can grows in an uncontrollable manner with low-differentiation. The results of the present study suggest that *Nanog* intervention can effectively regulate human body mechanism of tumor patients, and *Nanog* plays an important role in the treatment process. However, there is currently no evidence showing whether the diseases are associated with *Nanog* (14). In the present study, we knocked out *Nanog* gene, and found that the tumor was inhibited after the knockout, suggesting *Nanog* can directly participate in human body repair treatment (15). Previous experiments found that except for repairing human stem cells, Nanog (16) can self-renew and regulate as well as differentiate. For instance, the higher the data, the stronger the ability of low- and no-differentiation of the stem cells (17).

Besides being expressed in reproductive cells and malignant tumor, *Nanog* is also expressed in entity tumor-like breast cancer, retinoblastoma and oral squamous cell carcinoma (18). *Nanog* pseudogene expression is found in cervix cancer and breast cancer (19-21). From the data of 100 cases of lung cancer in the present study, we found that *Nanog* gene expression was significantly higher in lung cancer tissue than in adjacent normal tissue ($p<0.01$). The data showed that there may be *Nanog* gene in lung cancer stem cells (LCSCS) in tissue of lung cancer patients (22). The main factor promoting lung cancer cells in human is that it can self-renew and proliferate in its LCSCS (23). When we examined the adjacent normal tissue, we found *Nanog* is positive in 5 cases, demonstrating this part of adjacent normal tissue may contain normal lung cancer stem cells. The present study found that *Nanog* gene expression is consistence with the differentiation extent of lung cancer tissue and tumor, and a positive expression rate is evident in low- and no-differentiation, but not in high-differentiation ($p=0.0112$) (24). We found that the *Nanog* gene and differentiation condition of tumor stem cells are consistent. A high expression of Nanog can maintain the low-differentiation condition of stem cells, and maintain self-renewal and proliferation of the stem cells, which is crucial in assistance to differentiation signals.

In summary, the present study found a correlation between consistency of tumor and Nanog gene expression, showing that when human cell differentiation reaches the lowest point, *Nanog* gene was stronger. As a newly found specific marker, the *Nanog* gene contributes to potential clinical prevention of lung cancer.

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