Research Paper

Expression of Sox2, Oct4, and Nanog in Human Lung Cancer Non-small-cell

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ABSTRACT

Background: Cancer stem cells are a subpopulation of tumor cells with self-renewal capacity that promote tumorigenesis, resistance to chemotherapy, and metastasis. Sox2, Oct4, and Nanog are three pluripotent transcription factors expressed in embryonic stem cells and cancer stem cells.

Materials and Methods: This study aimed to evaluate the expression of Sox2, Nanog, and Oct4, and analyze their clinical significance in human non-small-cell lung cancer (NSCLC). Expression of Sox2, Nanog, and Oct4 was assayed in cancer tissues and their corresponding paracancerous tissues from 30 patients with NSCLC. RT-PCR was used to analyze the expression of these genes. The correlation between the expression of these three genes and clinical parameters including disease stage, smoking, lymph node, and cancer subgroups (adenocarcinoma and squamous) were analyzed.

Results: All three genes were expressed simultaneously in 76.6% of tumor samples. A significant correlation was observed between the expression of Sox2, Nanog, and Oct4 in the cancer tissues in comparison to the paracancerous tissues (P<0.000). Expression of Sox2 and Oct4 gene had a positive correlation with the stage of cancer (Sox2 P=0.01, Oct4 P=0.0007), while the expression profile of Nanog showed a significant positive correlation with sex (P=0.0063), smoking (P=0.0253), tumor stages (P=0.0003), and tumor type (P=0.0085).

Conclusion: Evaluating the expression of Sox2, Nanog, and Oct4 genes in NSCLC might have some implications for diagnosis and prognosis; they might be also promising treatment targets. The correlations between prognosis and pathological features and Nanog overexpression in NSCLC suggest Nanog is a potential indicator of the early stage of NSCLC.

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1. Introduction

Lung cancer is one of the leading causes of cancer-related death worldwide, accounting for nearly 1.8 million deaths in 2020 [1-4]. Non-small cell lung cancer (NSCLC) is the most common type of lung cancer (approx. 85% of cases) [5, 6]. Patients who are diagnosed and treated at an early stage have a favorable prognosis, with a 5-year survival rate of 70–90% for small localized tumors (stage I) [7]. However, most patients are diagnosed at advanced stages (stage III-IV) and have a low survival rate (In the United States, 5-year survival between 2008-2014 was 24% for patients with NSCLC, and 5.5% for those with distant metastases) [8, 9]. Clinical characteristics such as age, sex, stage of the disease, and metastatic disease burden are still the only basis to determine the prognosis of NSCLC [10, 11]. Recent efforts in studying biomarkers in tumor tissues resulted in identifying candidate prognostic and predictive molecular biomarkers associated with cancer stem cells (CSCs) [12, 13].

CSCs are a rare subpopulation of tumor cells, which can generate various differentiated cells within the tumor [14]. These cells exhibit drug resistance and have self-renewal capacity [15-17]. The sex-determining region Y (SRY), box 2 (SOX2), homeobox protein NANOG (named after the Celtic word Tír na nÓg meaning the land of the young), and octamer-binding transcription factor 4 (OCT4) are among the critical regulators of ESCs for self-renewal [18, 19]. Since CSC seems to be enriched in tumors resistance to conventional systemic therapy and radiotherapy, recent studies suggest that Sox2, Nanog, and Oct4 genes are potential diagnostic and prognostic markers in lung cancer [20]. However, so far, the predictive value for life survival for Sox2, Oct4, and Nanog gene expression changes in comparison to the classical pathological predictors has not been entirely understood [21].

This study aimed to determine the expression profile of Nanog, Sox2, and Oct4, and correlated gene expression changes in the expression profile of these genes with clinical and pathological parameters including age, sex, smoking, cancer subgroups (adenocarcinoma and squamous), lymph node involvement, and the stage of cancer in a group of lung cancer patients.

2. Materials and Methods

Subjects

Eligible cases were identified in the Masih Daneshvari Hospital, Tehran, Iran. Tumor samples and paired adjacent paracanserous tissues were collected from 30 NSCLC patients, before any treatments or interventions by surgical resection. Histopathological confirmation was performed for all patients enrolled in the present study, and the pathological diagnosis and TNM staging were determined according to the WHO classification of lung cancer [22]. Informed consent was obtained from all patients, and the study was approved by the Ethics Committees of Masih Hospital.

Sample collection and tumor RNA extraction

Samples were collected from patients by an experienced cardiothoracic surgeon. The tumor and adjacent tissue samples were rapidly frozen in liquid nitrogen and then stored at -80°C for further processing. For control, blood samples (5 cc) were taken from 30 normal individuals.

Blood samples were diluted in PBS and then separated into blood components using Ficoll solution at 1:1 dilution. The samples were centrifuged at 2,000 rpm for 10 min, followed by multiple PBS washes. The layer containing nucleated cells (PBMC) was collected, and the total RNA was extracted. total RNA was extracted from the tumor and the adjacent tissues according to the manufacturer's guidelines using TriPure isolation reagent (Roche, Germany). For RNA extraction, the tissues were placed in TriPure isolation reagent and homogenized with a scalpel. Centrifugation at 11300 rpm for 15 min at 4°C was done, and the top layer clear aqueous phase including RNA was separated. The quality of the extracted RNA was measured via NanoDrop spectrophotometer at 260 nm (A260) to reach high-quality RNA for cDNA synthesis. Similarly, for the JEG-3 cell line, 1 mL Tripure isolation reagent was added to 2×10^6 cells to extract RNA.

RT-PCR and agarose gel clectrophoresis

The cDNA synthesis was done according to the kit protocol (Fermentase, cat#K1622). RT-PCR runs were conducted on ABI thermal cycler using a total volume of 25 µl containing the following reagents: 0.5 IU Taq DNA polymerase (5 units/µl), 1 µl dNTPs (10 mM), 1 µl forward primer (10 pM), 1 µl reverse primer (10 pM), 1.5 µl $MgCl_2$ (50 mM), 2.5 µl PCR buffer (10x), 5 µl cDNA (5 ng), and 13.25 µl ddH_2O.

Table 1. RT-PCR primers for Sox2, Oct4 ,NANOG and GAPDH

Gene		Primer Sequence	Temperature (°C)	Product Length (bp)	Accession Number
SOX2	Forward	CAACATGATGGAGACGGAGC	59	247	NM_003106
	Reverse	CTCCGACAAAAGTTTCCACTC			
OCT-4	Forward	TCTTCAGGAGATATGCAAAGC	58	357	NM_002701.5
	Reverse	GAGTACAGTGCAGTGAAGTG			
NANOG	Forward	CACCCAGCTGTGTGTACTC	59	193	NM_024865.3
	Reverse	CTTCTGCGTCACACCATT			
GAPDH	Forward	GCCACATCGCTCAGACAC	58	426	NM_002046.5
	Reverse	TTCACACCCATGACGAACAT			

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The reactions were conducted under the following conditions: 94°C for 1.5 min, followed by 94°C for 40 s, 56°C-62°C for 45 s, and 72°C for 1.5 min repeated for 35 cycles. Each sample was analyzed in duplicate, and the mean value was used for quantification. The sequence of primers for the amplification of target genes in multiplex PCR is summarized in Table 1; and, the binding site between primers and studied genes are detailed in Figure 1. The GAPDH gene was used as the reference gene in RT-PCR. The PCR products were subsequently run on a 2% agarose gel (Merck, Germany), and then stained with ethidium bromide and visualized under UV light.

Statistical analysis

A criterion α level of 0.05 was adopted in all experiments. SPSS software, version 26, was employed to analyze data obtained in this study. McNemar's test was used for paired nominal non-parametric data, between the two groups of tumor and adjacent tissue. Other statistical analyses were performed using GraphPad Prism software version 8.4.

3. Results

Clinical characteristics

The relevant clinical characteristics of lung cancer patients including, age, sex, smoking status, TNM stage, lymph node involvement, tumor stage, and tumor pathology are summarized in Table 2.

Confirmation of pluripotent gene expression between tumor and adjacent tissue in NSCLC patients

Using RT-PCR, the expression of Nanog, Sox2, and Oct4 genes was investigated in 30 patient-derived tumor samples and adjacent tissues. The lengths of primers for Nanog, Sox2, and Oct4 were 193, 247, and 357 bp, respectively (Figure 1A). These bands were not present in the blood samples of healthy individuals (negative control) and were present in the JEG-3 embryonic cell line (positive control). Most patients (n=23) expressed all three transcription factors in their tumor samples. Four patients expressed Oct4 and Sox2, one expressed Nanog and Sox2, and two were positive for Nanog and Oct4 (Figure 1B).

Within the cohort, Oct4 was the most prevalent gene expressed in 96.6% of samples, while Nanog was the least prevalent (86.6%). The analyzed genes were found in the adjacent tissue samples with a prevalence of 30%, 43.3%, and 40% for Nanog, Sox2, and Oct4, respectively (Figure 1C). In the present study, 76% of tumor samples and 26.6% of adjacent tissues expressed all three transcription factors. Among the adjacent normal tissue samples, only those derived from patients in advanced disease stages (II-III) expressed genes of interest, and none of the adjacent tissue samples from stage I tumors expressed these pluripotent genes.

Comparison of CSC-related genes and classical pathological predictors of survival

As reported in Table 2, samples from 23 males (76%) and 7 (23%) females were analyzed in this study. The whole samples (100%) derived from female patients were Nanog positive, while 17.3% of male samples were

Table 2. Clinical information of 30 studied patients with NSCLC

Row	Age	Sex	Smoker	Stage	Pack in Year	Lymph Node Involvement	Tumor Pathology
1	62	Male	No	3	-	Positive	Adenocarcinoma
2	59	Male	Yes	1	80	Negative	Squamous cell carcinoma
3	60	Female	No	1	-	Negative	Adenocarcinoma
4	53	Male	No	3	-	Positive	Adenocarcinoma
5	62	Male	Yes	2	30	Negative	Adenocarcinoma
6	61	Male	Yes	2	4	Positive	Squamous cell carcinoma
7	54	Female	No	2	-	Positive	Adenocarcinoma
8	59	Male	Yes	2	40	Positive	Adenocarcinoma
9	50	Male	No	3	-	Positive	Squamous cell carcinoma
10	80	Male	Yes	3	-	Negative	Adenocarcinoma
11	65	Male	Yes	1	-	Negative	Squamous cell carcinoma
12	56	Male	Yes	2	120	Negative	Squamous cell carcinoma
13	54	Female	No	3	-	Positive	Adenocarcinoma
14	62	Male	Yes	3	-	Positive	Squamous cell carcinoma
15	60	Male	Yes	3	-	Positive	Squamous cell carcinoma
16	64	Male	Yes	1	50	Negative	Squamous cell carcinoma
17	61	Male	Yes	1	80	Negative	Squamous cell carcinoma
18	55	Female	No	1	-	Negative	Adenocarcinoma
19	37	Male	Yes	2	-	Positive	Adenocarcinoma
20	60	Female	Yes	2	-	Positive	Adenocarcinoma
21	76	Male	Yes	2	15	Negative	Adenocarcinoma
22	62	Female	No	3	-	Positive	Squamous cell carcinoma
23	66	Male	Yes	3	-	Negative	Squamous cell carcinoma
24	52	Male	Yes	2	8	Negative	Adenocarcinoma
25	58	Male	Yes	2	-	Negative	Adenocarcinoma
26	53	Female	Yes	1	-	Negative	Adenocarcinoma
27	56	Male	No	2	-	Negative	Adenocarcinoma
28	58	Male	Yes	3	-	Positive	Adenocarcinoma
29	58	Male	No	1	-	Negative	Adenocarcinoma
30	69	Male	Yes	2	-	Negative	Squamous cell carcinoma

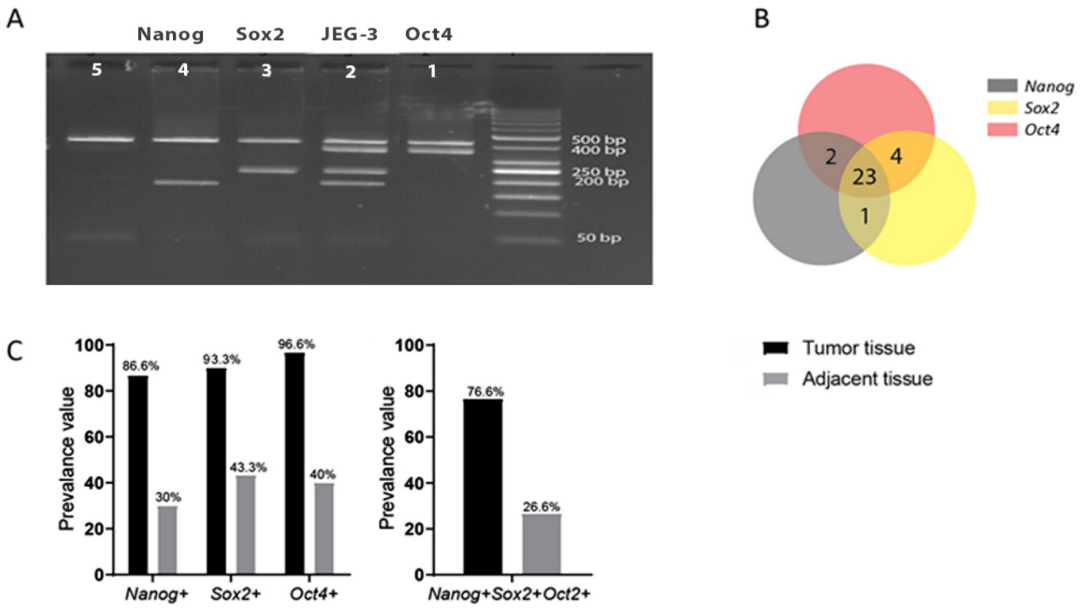

Figure 1. Gene expression profile in tumor and adjacent normal tissue samples in NSCLC patients IMMUNOREGULATION

The RT-PCR products for Nanog (193 bp), Sox2 (247 bp), Oct4 (357 bp), and GAPDH (426 bp), well number 5 as the negative control (normal blood sample) and well number 2 (JEG-3 cell line) as the positive control on the agarose gel. B, Van diagram shows the number of patients expressing different pluripotent genes. C, bar graphs show the prevalence of pluripotent gene expressions in the tumor and adjacent tissues.

Nanog negative (sex: P=0.0063). Among the Nanog negative samples, 50% were stage I squamous cell carcinoma (stage: P=0.0003, tumor pathology: P=0.0085). Notably, all Nanog negative samples were from patients with a history of smoking (P=0.0253) (Table 3). The relationships between Nanog gene expression, sex, smoking, stage, and cancer type showed significant correlations (Figure 2). However, we found no relationship between Nanog expression, lymph node involvement, and age. Our results suggested Nanog as a potential indicator of the primary stage of the disease in males with adenocarcinoma.

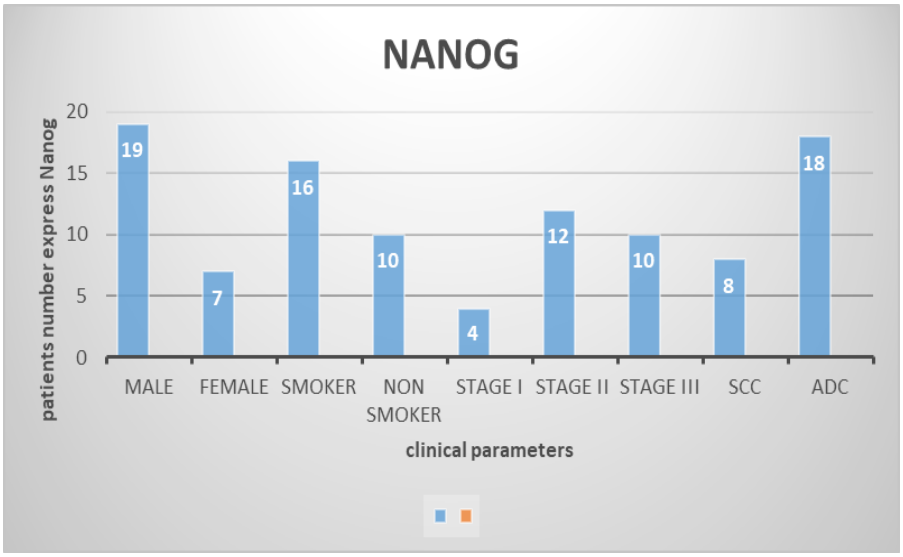

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Figure 2. Nanog expression correlated with clinical parameters in 30 patients with NSCLC, such as sex, smoking, tumor stage, and tumor type

Table 3. Correlations of tumor pluripotent genes (Nanog, Sox2, and Oct4) expression in 30 NSCLC patients with their clinical characteristics

Variables		(n=30)	P		
			Nanog	Sox2	Oct4
Sex	Females	7	0.0063	0.356	0.0614
	Males	23			
Smoking	Smokers	20	0.0253	0.1596	>0.9999
	Non-smokers	10			
Stage	I	8	0.0003	0.0102	0.0007
	II	12			
	III	10			
Lymph node involvement	Positives	12	0.0603	0.2005	0.1104
	Negatives	18			
Tumor pathology	SCC	12	0.0085	0.232	0.136
	ADC	18			

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Sox2 and Oct4 genes were expressed in 85% of stage I NSCLC patients; they were detected in 100% of the samples from more advanced stage tumor samples. They were significantly correlated with higher stages (Sox2: P=0.0102, Oct4: P=0.0007) of the disease. Furthermore, the analysis showed a significant correlation between tumor and paracancerous tissues in Nanog, Sox2, and Oct4. The expression of all three genes was significantly higher than their paracancerous tissues (Figure 3).

4. Discussion

In this report, we studied the expression profile of Sox2, Oct4, and Nanog genes in the tumor samples and adjacent normal tissues in 30 patients with NSCLC. According to the results of this investigation, the Nanog gene was expressed in 86.7% (26/30), Oct4 in 96.6% (29/30), and Sox2 in 93.3% (28/30) of tumor samples. The expression of all three genes with clinicopathological features was examined. The Oct4 gene expression showed a significant correlation to the higher stages of cancer and less differentiated tumors (P=0.01). The Oct4 gene expression in cells is reduced during differentiation, and it significantly correlates with less differentiation in tumor cells during re-expression [23, 24]. The higher expression of this gene in a tumor cell may directly relate to less differentiation and higher stages of cancer [21, 25]; also, our results revealed a significant correlation between Nanog gene expression in tumor samples and higher-stage cancer (Nanog: P=0.002). This result could be due to the nuclear translation factor activity of Nanog protein and its essential role in maintaining pluripotency and self-renewal characteristics of embryonic cells [26-29].

It is worth noting that the expression of Sox2, Oct4, and Nanog genes was also observed in adjacent normal tissues (according to our criteria as mentioned above, they were considered normal tissue in this study). To ensure that the genes were not expressed in normal somatic cells, normal peripheral blood leukocytes were investigated for the expression of Sox2, Oct4, and Nanog genes. The expression of all three Sox2, Oct4, and Nanog genes was examined in the nucleated blood cells of healthy subjects by RT-PCR; none of the genes was expressed in all 30 normal blood samples. Earlier studies have also confirmed the lack of expression of Sox2, Oct4, and Nanog genes in the nucleated blood cells [30-33]. The present investigation results were in line with the study mentioned earlier. The promoter of these genes likely continues to be hypermethylated in normal somatic cells.

An expression was observed in cancer cells, which may be caused by epigenetic changes such as methylation. Promoters of these multi-potent genes are hypomethylated during the embryonic period; however, they become hypermethylated after birth. During carcinogenesis, these genes become unmethylated again [34]. The expression of these genes in adjacent normal tissues

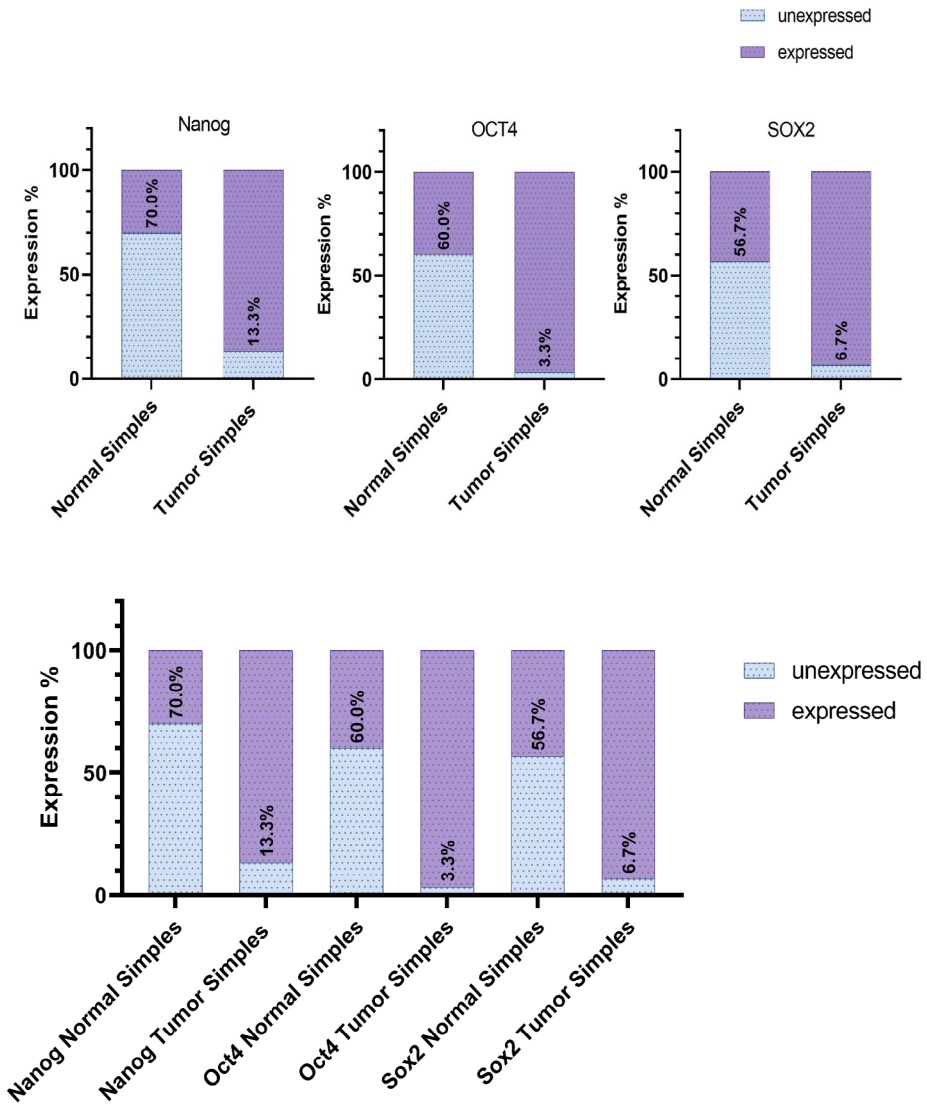

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Figure 3. Expression of Nanog, Oct4, and Sox2 in tumor and their adjacent paracancerous tissues in 30 patients with NSCLC.

likely shows that the process of carcinogenesis and epigenetic changes related to cancer cells has already begun in some of these normal cells. It is worth noting that the expression of these genes in adjacent normal tissues was observed in patients with higher tumor stages; it was associated with lymph node involvement. As a result, these cells cannot be considered reliable normal tissue due to their heterogeneous mode.

According to a study by Shinya Nirasawa in 2009, the Nanog gene plays a vital role in renewing embryonic cells by maintaining non-differentiated embryonic stem cells and accelerating the cell cycle [35]. In his study, Nanog gene expression was assessed by IHC and qRT-PCR in lung cancer tissues, non-cancerous lung tissue, and adjacent tumor cells. Nanog gene expression was detected in 84.8% of lung cancer tissues, which is in close concordance with the results of this study (86.7%). In addition, comparing the mRNA levels of tumor tissues and adjacent tumor cells in each patient showed an increasing level of Nanog mRNA in the cancer tissues in all patients. The Nanog gene also showed high expression levels even at the early stage of lung cancer [36].

In a study conducted in 2013, Nanog gene expression and its correlation with clinicopathological characteristics were evaluated in the lung cancer tissues of 163 individuals. RT-PCR, immunofluorescence, and IHC

measured Nanog gene expression in the lung cancer tissues. The results indicated that the Nanog gene had higher mRNA and protein expression levels in the lung cancer tissues than in the adjacent tumor tissue. It was also found that the Nanog gene expression is associated with less differentiation of tumors and higher stages of the disease (P=0.001) [37]. As the results revealed in the present investigation, there was a significant relationship between the expression of the Nanog gene in the tumor samples and higher stages of cancer (P=0.002).

In another study on 147 patients with NSLCS, Sox2 gene amplification and Sox2 protein expression were investigated by fluorescence in situ hybridization (FISH) and IHC, respectively. This investigation revealed that the protein expression of Sox2 was detected in 79% of squamous cell carcinoma of pathological subtypes compared to 18% in adenocarcinoma of the lung (P=0.0001). Similarly, Sox2 gene amplification in SCC (72%) was more common than ADC (8%) (P=0.0001) [37]. In the present study, 12 patients were diagnosed with SCC, and all expressed the Sox2 gene. This finding could be a confirmation of the correlation between Sox2 expression and squamous cell carcinoma of the lung.

5. Conclusion

Overall, the expression of Sox2, Nanog, and Oct4 reprogramming genes was investigated in NSCLC patients, and it was observed that most cases were positive for these markers. The high percentage of the expression of these markers among NSCLC patients was in line with previous studies; it shows the potential role of these markers for diagnosis, choosing targeted therapy, and prognosis. The expression of Nanog increases the self-renewal capacity of tumor cells, which in turn results in poorer clinical outcomes for patients with NSCLCs.

Ethical Considerations

Compliance with ethical guidelines

There were no ethical considerations to be considered in this research.

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Authors' contributions

All authors equally contributed to preparing this article.

Conflicts of interest

The authors declared no conflict of interest.

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