SUSD2 down-regulation is correlated with the occurrence and development of lung cancer

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Research article

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Abstract

Background: Molecularly targeted drugs yielded significant therapeutic advances in oncogene-driven non-small cell lung cancer (NSCLC), but the majority of patients develop acquired resistance. It is important to identify new target sites that mediate lung cancer progression for new targeted drugs development. Methods: SUSD2 expression and methylation in 25 paired lung cancer and adjacent normal lung samples were investigated. In addition, the association between SUSD2 expression and prognosis was evaluated. Results: SUSD2 gene expression was down-regulated in 21 of 25 lung cancer samples. Similarly, SUSD2 protein expression was down-regulated in 20 of 25 lung cancer samples. Furthermore, 7 of 25 lung cancer samples showed a higher methylation compared to adjacent normal lung samples, suggesting that SUSD2 methylation was closely associated with silencing of the gene expression. Except for the pathology type and TNM status ($P<0.05$), no correlation was observed between SUSD2 expression and other clinicopathological characteristics. Conclusions: Our findings indicated that SUSD2 expression was down-regulated in lung cancer, and SUSD2 methylation might not be involved in SUSD2 gene expression, suggesting that SUSD2 could be a novel candidate gene for future mechanism research of lung cancer.

Background

Lung cancer is the world's leading cause of cancer-associated mortality. Approximately 85% of all lung cancer cases are NSCLC, which include adenocarcinoma, squamous cell carcinoma and large cell carcinoma [1, 2]. Several oncogenic driver mutations, including *KRAS* (32.2%), *EGFR* (11.3%), *ALK* (3.9%), *MET* exon 14 (4.2%), *BRAF* (7%), *PIK3CA* (2%), *ROS1* (2%), *HER2* (2%) and *RET* (1%), and amplification of *MET* (2.2%) and *HER2* (0.9%), have been discovered in NSCLC, particularly in adenocarcinoma [3, 4]. Except for conventional therapy, targeted drugs for these driving gene mutations have been approved for lung cancer fist-line therapy by the Food and Drug Administration and China Food and Drug Administration [5-7]. Despite molecular targeted therapy partly improved survival, the majority of patients eventually develop acquired resistance [8]. Therefore, more study regarding the screening of effective molecular targeted sites for personalized and precision treatment of lung cancer is necessary.

SUSD2 was identified by a cDNA library enriched for genes which are highly expressed in cancer [9]. SUSD2 is an 822 amino-acid type I membrane protein which is located on chromosome 22, containing several domains including Somatomedin B, AMOP domain, von Willebrand factor type D domain, Sushi domain and a transmembrane region [10]. Overexpression of the mouse SUSD2 homologous in HT1080 fibrosarcoma cells and HeLa cervical carcinoma cells inhibits clonogenicity, anchorage-independent growth, migration, and invasion through matrigel [10, 11]. Watson et al. reported that the interaction of SUSD2 with Gal1 increases the invasion of breast cancer cells and this interaction may play a significant role in inhibiting the body's immune response [12]. A further study on breast cancer demonstrated that SUSD2 promotes tumor-associated macrophage recruitment by increasing MCP-1 levels in breast cancer [13]. In contrast, SUSD2 has tumor suppressive properties in high-grade serous ovarian carcinoma cell, and SUSD2 expression in high-grade serous ovarian cancer correlates with increased patient survival, suggesting that the function of SUSD2 may be tumor cell-type specific maker for prognosis [14, 15].

However, a recent study shows that SUSD2 promotes cancer metastasis in high grade serous ovarian cancer, suggesting that high SUSD2 expression is associated with poor prognosis of ovarian cancer patients [16]. In addition, SUSD2 is frequently down-regulated and functions as a tumor suppressor in renal cell carcinoma and lung cancer, and reduced expression of SUSD2 is associated with progression of non-small cell lung cancer [17, 18]. These studies indicate that SUSD2 has complex functions in different cancer types. However, *SUSD2* expression and methylation and its correlation with lung cancer clinicopathological characteristics have not been investigated yet.

In order to elucidate the relationship between *SUSD2* expression and methylation and lung cancer, *SUSD2* expression was measured by qRT-PCR and western blot, and *SUSD2* methylation was detected by bisulfite sequencing in 25 paired lung cancer and adjacent normal lung samples. In addition, the potential association between *SUSD2* expression and methylation and lung cancer clinicopathological characteristics were analyzed.

Materials And Methods

Patients and tissue samples

In the present study, 25 paired NSCLC and adjacent normal lung tissue samples were collected from patients with pathologically diagnosed lung cancer, who were not subjected to chemotherapy or radiotherapy prior to surgery, between 2011 and 2015 at the Haidian Section of Peking University Third Hospital. The age of the 25 patients ranged from 35 to 86 years. Clinicopathological characteristics of the patients, including gender, age, histological grade, clinical stage, pathology type, pTNM status and tumor size were collected. Cancer stages were classified according to the American Joint Committee on Cancer criteria. Written informed consent was obtained from each recruited patient according to institutional guidelines. The present study was approved by the Human Research Ethical Committee of Haidian Section of Peking University Third Hospital, China.

qRT-PCR

SUSD2 expression was determined by qRT-PCR. Total RNA was extracted according to the manufacturer instructions (Stratagene). Reverse transcription was carried out on total RNA (5 μg) from tissues, and cDNA was generated using the random hexamer primer. SUSD2 forward primer (5-CTCCAATGACTGCCGCAACTA-3) and reverse primer (5- GAACATTCCTTTCAGGTCCATCC-3) and GAPDH forward primer (5-AGCCTCGCCTTTGCCGA-3) and reverse primer (5-CTGGTGCCTGGGGCG-3) were synthesized by Integrated DNA Technologies. qRT-PCR protocol was as follows: 94 ℃ for 3 min, 94 ℃ for 1 min (30 cycles), 65 ℃ for 1 min, and 72 ℃ for 1 min, with a final 5 min extension at 72 ℃. *SUSD2* expression was calculated based on the following equation: $F = 2^{-\Delta Ct}$, where ΔCt = Ct (*SUSD2*) – Ct (*GAPDH*).

Western blot

Total proteins from 25 pairs of fresh lung cancer and adjacent normal tissues were extracted using radioimmunoprecipitation assay buffer containing 1 mM phenylmethylsulfonyl fluoride (Beyotime Institute of Biotechnology, Haimen, China). Western blot was performed as follows: proteins were separated on SDS-PAGE gels and then transferred to PVDF membranes by electrophoresis (Sangon Biotech, China). The membranes were then incubated with a rabbit polyclonal anti-SUSD2 antibody, 1:1,000 dilution, for 12 h at 4 ℃ (Abcam, UK). Subsequently, horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Multisciences, China) was applied at a dilution of 1:3,000 for 1 h at room temperature. Signals were visualized using an enhanced chemiluminescence detection kit (GE Healthcare Life Sciences, UK).

Bisulfite sequencing

A search for the CpG island of the *SUSD2* gene was carried out using GENETYX-Mac Ver.12.2.0 software. The CpG island was determined using MethPrimer online software. The criteria used were the following: island size > 200 bp, GC % > 50.0, and observed/expected CpG ratio > 0.6.

Genomic DNA was extracted from lung cancer tissues and adjacent normal lung tissues using a QIAamp Fast DNA Tissue Kit (Qiagen, USA). After purification, the genomic DNA was treated with sodium bisulfite using a DNA Methylation Kit (Kangwei, China). The target region in the SUSD2 gene was amplified from the genomic DNA by PCR using a PCR Mix Kit (Kangwei, China) and primers were designed as follows: forward 1: 5-GTTGGGATTTAGGGTTTGAG-3; forward 2: GTTTGTTAAAGGTTTTGTGGT; reverse: 5-CCTATATCTACAACCAACAATAC-3. PCR was performed under the following conditions: 95 °C for 2 min; 40 cycles at 95 °C for 30 s, 53 °C for 30 s, 72 °C for 1 min and finally 72 °C for 5 min. To perform hot start PCR, the reverse primer was added to the reaction mix when the reaction mix first reached 95 °C. After being purified, the PCR products were cloned into a pGEM-T vector. Approximately each of the 5 clones obtained from three independent experiments was sequenced. CpG methylation status was analyzed by the web-based tool QUMA.

Statistical analysis

A database was established and SPSS 11.5 software was used for statistical analysis. Student's *t*-test was used to analyze the statistical differences between two groups. The relationship between the expression of SUSD2 and clinicopathological characteristics was analyzed by the chi-squared test. Data were shown as mean ± standard deviation, and $P < 0.05$ was considered statistically significant.

Results

SUSD2 expression in lung tumor tissue

SUSD2 gene expression in the 25 paired lung cancer and adjacent normal lung tissues were detected by qRT-PCR. The results showed that its expression was down-regulated in 21 of 25 (84%) tumor samples

compared with normal tissue, in which this gene was down-regulated in 21/25 (84%), and it was up-regulated in 4 of 25 (16%) tumor samples (Figure 1).

SUSD2 protein expression was detected by western blot. The results showed that SUSD2 protein was down-regulated in 20 of 25 (80%) tumor samples (Figure 2).

Methylation of the *SUSD2* gene

To determine the methylation status of the *SUSD2* gene in paired lung cancer and adjacent normal lung tissues, the methylation of the CpG region of the *SUSD2* gene was detected by bisulfite sequencing PCR. The results revealed that the methylation of the *SUSD2* gene in 7 of 25 tumor samples was higher than normal samples (Table 1).

Correlation between SUSD2 expression and clinicopathological characteristics of patients with lung cancer

In order to reveal the clinical significance of SUSD2 protein expression in lung cancer tissues, the correlation between SUSD2 protein expression in cancer tissues and various clinicopathological characteristics was analyzed (Table 2). The results showed that except for the pathology type and TNM status ($P<0.05$), no correlation was observed between SUSD2 protein expression and other clinicopathological characteristics, including gender, age, histology, and tumor size.

The progression free survival (PFS) between SUSD2 down-regulated group and up-regulated group was also analyzed (Figure 3). *P*-values were calculated using log-rank values, which weight values at the end of the curve more heavily, and Wilcoxon's transformed values, which weight value at the beginning of the curve more heavily; $P = 0.260$ and 0.443, respectively.

Discussion

Targeted therapy has been successful used in many malignancies, such as lung cancer, lymphomas, gastrointestinal stromal tumors, and chronic myeloid leukemia. [19-23]. However, the majority of patients eventually develop acquired resistance. Therefore, efforts are required to provide more targeted therapy sites and targeted drugs for lung cancer patients.

In this study, we performed a comprehensive analysis of *SUSD2* expression and methylation in paired lung cancer and adjacent normal lung tissues. The decreased expression of *SUSD2* in lung cancer tissues was confirmed by qRT-PCR and western blot, indicating that SUSD2 might play an inhibitory role in lung cancer tumorigenesis and progression. On the contrary, SUSD2 expression is increased in breast cancer tissues and ovarian cancer, where it can promote the invasion of breast cancer cells [24, 25]. Therefore, we speculated that SUSD2 might exert different effects in different cancers.

In our study, Methylation has been detected only in a subset of samples with downregulated SUSD2. Even though perturbation in methylation levels is known to occur during cancer development, this change does

not explain the downregulation of SUSD2 in most samples [26-28]. It seems that other epigenetic mechanism(s) may occur to perturbate SUSD2 expression.

In order to reveal the prognostic value of SUSD2 protein expression in lung cancer tissues, the correlation between SUSD2 expression and clinicopathological characteristics was analyzed. Only pathology type and TNM stage, rather than gender, age, tumor grade, histology and tumor size, were significantly associated with SUSD2 expression. Similarly, it has been reported that SUSD2 is correlated with poor histological grade, advanced clinical stage, higher T status and higher N status [17]. Further survival analysis demonstrated no significant correlation between SUSD2 protein expression and patient survival. Thus, a large lung cancer patient cohort is necessary to determine whether these results could be used for clinical application.

In conclusion, *SUSD2* mRNA and protein were down-regulated in lung cancer tissues, and DNA methylation might be not involved in *SUSD2* gene expression. Our findings highlighted that the abnormal expression of *SUSD2* was associated with the occurrence and development of lung cancer. Therefore, a study proposing a mechanism of action of SUSD2 in lung cancer may need to be verified in the future study.

Abbreviations

NSCLC: non-small cell lung cancer

Declarations

Ethics approval and consent to participate

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and analyzed in the current study would be available from the corresponding author upon request.

Competing interests

The authors declare that they have no competing interests.

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Authors'contributions

Guarantor of integrity of the entire study: Qiang Liu; study concepts: Qiang Liu, Jun Wang; study design: Qiang Liu; literature research: Qiang Liu, Shuai Wang, Guotian Pei, Yingshun Yang; experimental studies: Qiang Liu, Xianjun Min; data acquisition: Qiang Liu, Yuqing Huang; data analysis: Qiang Liu, Jun Liu, Fan Yang; manuscript preparation: Qiang Liu; manuscript review: Qiang Liu, Jun Wang.

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Figure Legends

Figure 1 qRT-PCR analysis of *SUSD2* gene expression in paired lung cancer and adjacent normal lung tissue. N: adjacent normal lung tissues, T: tumor tissue. Data were shown as mean ± standard deviation of three separate experiments. *= $P < 0.05$ in lung cancer tissue compared to adjacent normal lung tissue.

Figure 2 Western blot analysis of SUSD2 protein expression in paired lung cancer and adjacent normal lung tissues. N: adjacent normal lung tissues, T: tumor tissue. The optical density of SUSD2 bands was determined from western blot and each band was normalized to the β-actin band (A). The normalized density is shown in the SUSD2 histogram (B).

Figure 3 Progression-free survival (PFS) of lung cancer patients with SUSD2 down-regulation (n = 16), and SUSD2 up-regulation (n = 9). Patients with SUSD2 down-regulation had a PFS of 24.2 months after initial diagnosis. Patients with SUSD2 up-regulation had a PFS of 50.6 months after initial diagnosis. *P*-values were calculated using log-rank values, which weight values at the end of the curve more heavily, and Wilcoxon's transformed values, which weight value at the beginning of the curve more heavily; $P = 0.260$ and 0.443, respectively.

Tables

Due to technical limitations, tables 1 and 2 are only available as a download in the supplemental files section.

Figures

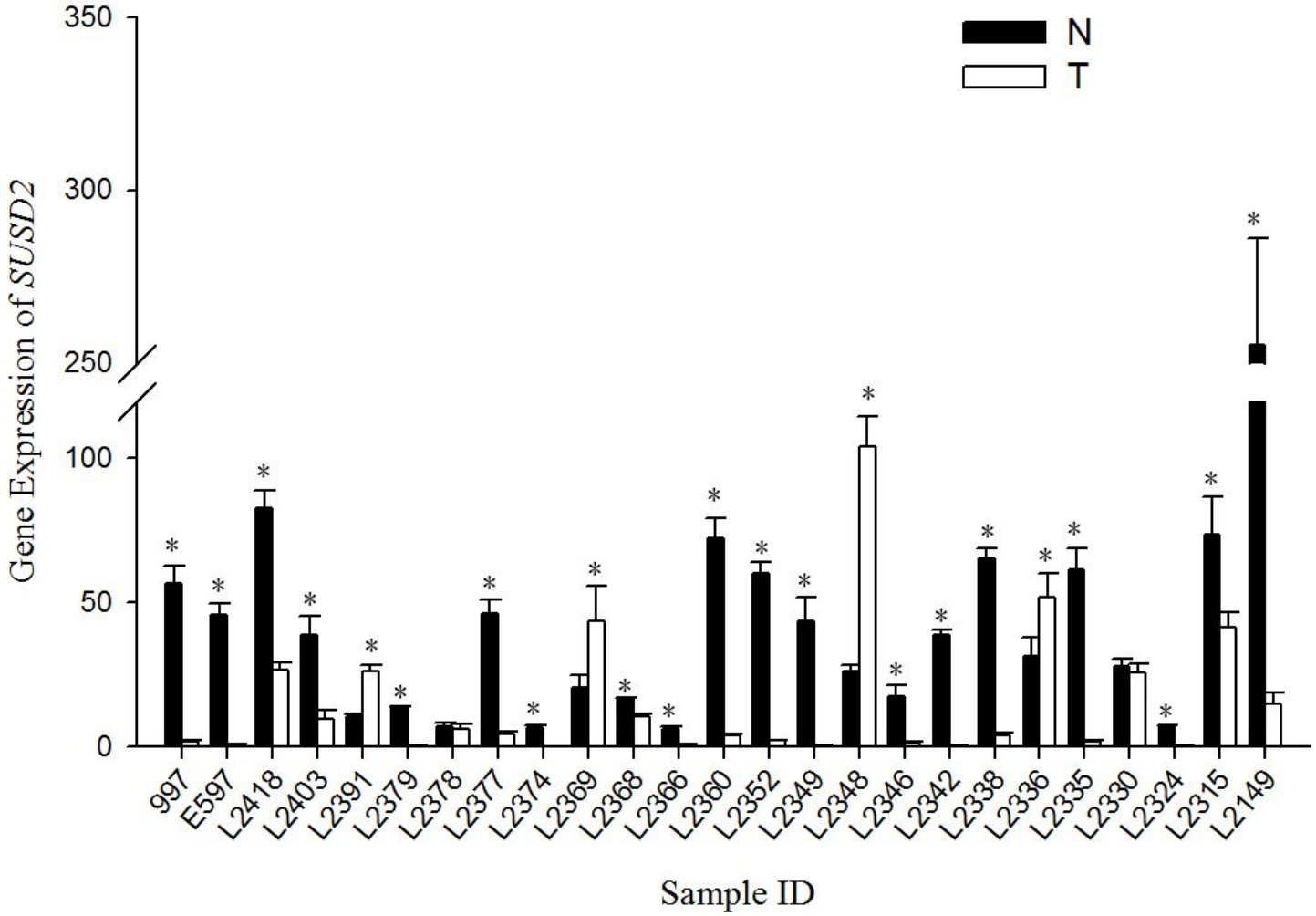

Figure 1

qRT-PCR analysis of SUSD2 gene expression in paired lung cancer and adjacent normal lung tissue. N: adjacent normal lung tissues, T: tumor tissue. Data were shown as mean ± standard deviation of three separate experiments. *= $P < 0.05$ in lung cancer tissue compared to adjacent normal lung tissue.

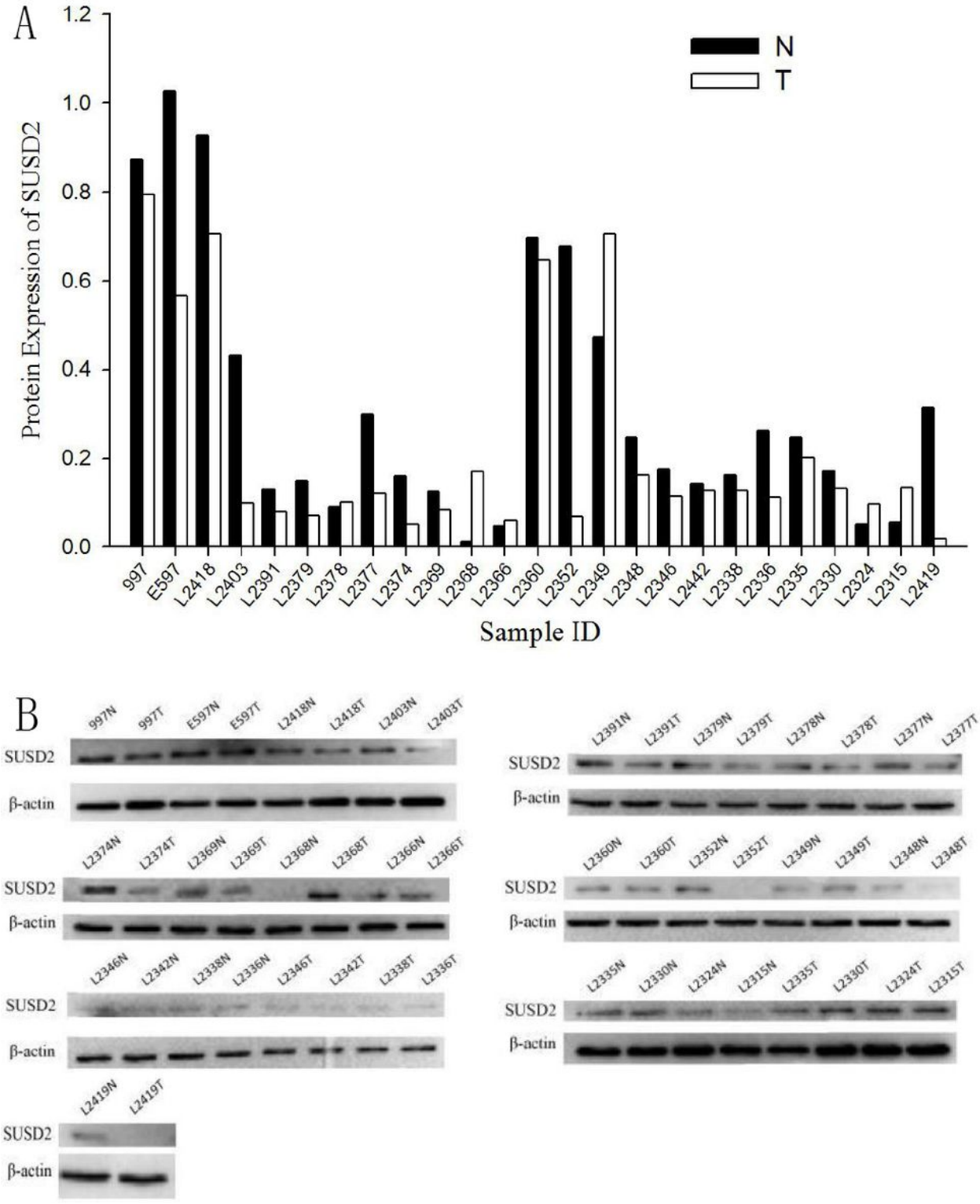

Figure 2

Western blot analysis of SUSD2 protein expression in paired lung cancer and adjacent normal lung tissues. N: adjacent normal lung tissues, T: tumor tissue. The optical density of SUSD2 bands was determined from western blot and each band was normalized to the β-actin band (A). The normalized density is shown in the SUSD2 histogram (B).

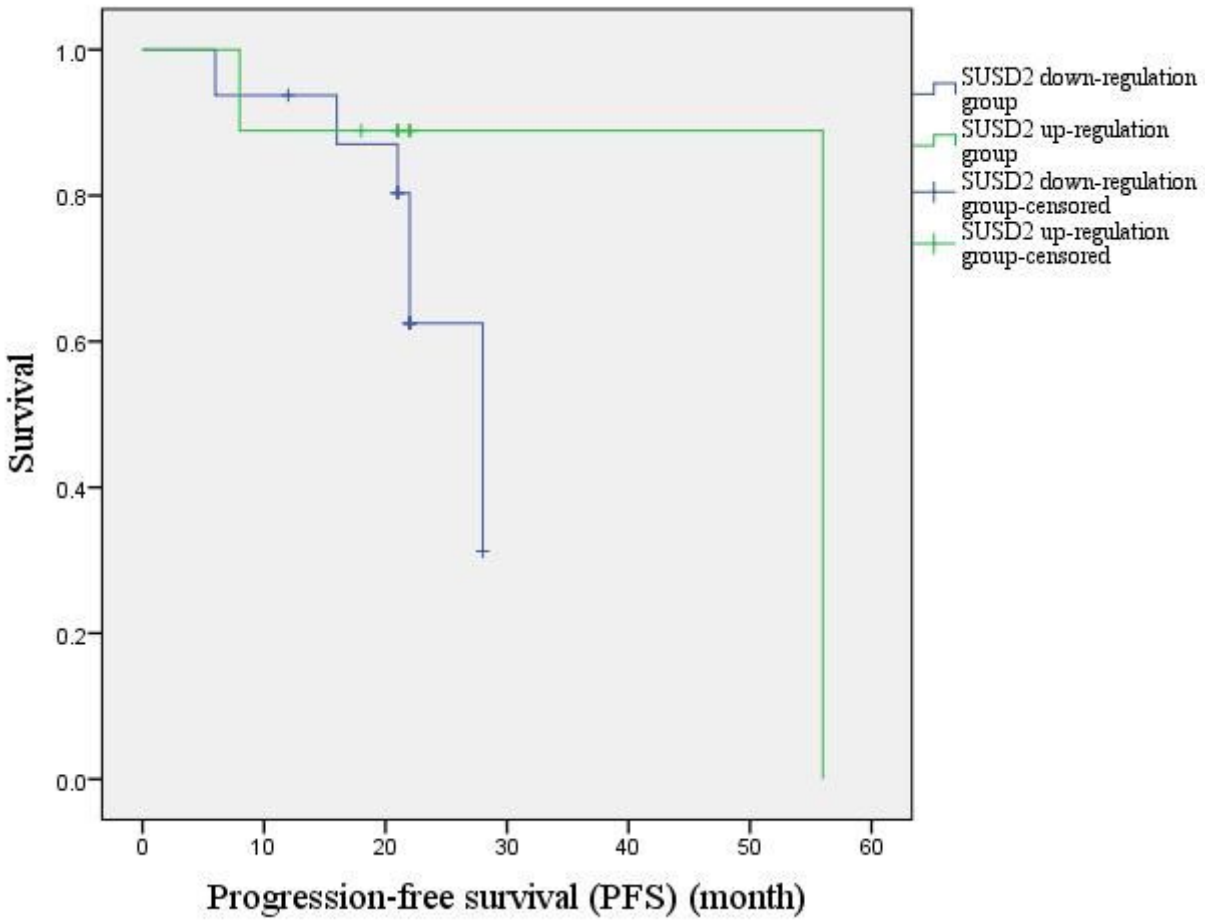

Figure 3

Progression-free survival (PFS) of lung cancer patients with SUSD2 down-regulation (n = 16), and SUSD2 up-regulation (n = 9). Patients with SUSD2 down-regulation had a PFS of 24.2 months after initial diagnosis. Patients with SUSD2 up-regulation had a PFS of 50.6 months after initial diagnosis. P-values were calculated using log-rank values, which weight values at the end of the curve more heavily, and Wilcoxon's transformed values, which weight value at the beginning of the curve more heavily; P = 0.260 and 0.443, respectively.

Supplementary Files

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