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Original Article

Sestrin2 expression is a favorable prognostic factor in patients with non-small cell lung cancer

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Abstract: The purpose of this study was to evaluate the prognostic value of Sestirn2 in patients with non-small cell lung cancer (NSCLC). Quantitative real-time (RT-PCR) and western blot were performed to investigate the mRNA and protein expression of Sestirn2 in NSCLC and corresponding non-cancerous tissues. Immunohistochemistry was used to detect the expression of Sestirn2 in 210 NSCLC tissue samples. Overall survival was calculated by the Kaplan-Meier method and analyzed by the log-rank test between different groups. The results indicated that the Sestirn2 expression was significantly lower in NSCLC tissues than the corresponding non-cancerous lung tissues. Low Sestirn2 expression was related to poor tumor differentiation, advanced TNM stage, and lymph node metastasis. Patients with high Sestirn2 expression had longer overall survival than those with low expression levels, which was consistent with the results of the subgroup analysis. Multivariate analysis showed that high Sestirn2 expression was a favorable prognostic factor for NSCLC patients. Our study indicated that Sestirn2 could play an important role in the observation of prognosis in NSCLC and might be a valuable marker for predicting the treatment outcome in patients with NSCLC.

Keywords: Sestrin2, NSCLC, prognosis, survival

Introduction

Lung cancer is the first leading cause of cancer-related death worldwide. Non-small cell lung cancer (NSCLC) constitutes approximately 80% of total lung malignancies [1]. Despite significant advances in multidisciplinary treatment modalities, such as surgery, chemotherapy and radiotherapy, the 5-year survival rate of lung cancer is about 20% [2, 3]. It is hard to detect this disease at an early stage, and the cancer shows resistance to almost all available chemotherapy drugs and radiation regimens. Chemoradiotherapy is the standard treatment approach for advanced NSCLC; however, drug and/or radiation resistance is a major factor influencing the clinical outcome of patients [4]. Therefore, it make important sense to investigate novel therapeutic targets and prognostic markers to improve the treatment response and the prognosis of patients with NSCLC.

Sestrins are a family of highly conserved, stress-inducible genes that can defend the cell against oxidative damage and oncogenic signaling [5, 6]. Recently, two members of this family, Sestrin1/2, have been found to play an important role in suppressing mTOR in response to genotoxic challenge through the regulation of AMPK signaling [7]. Sestirn2 (Sesn2) was identified as the product of hypoxia-inducible gene 95 (Hi95), whose expression was induced by genotoxic or oxidative stress as well as by prolonged hypoxia [8]. In addition, Sestrin2 has been considered as a tumor suppressor that can inhibit angiogenesis and autophagy [9], underscoring the importance of elucidating the molecular mechanism by which Sestrin2 regulates pathways of metabolism and survival. It is uncertain to date whether Sestrin2 has clinical significance in NSCLC. Therefore, the present study was conducted to investigate whether Sestrin2 expression might be a molecular biomarker for predicting the prognosis of NSCLC patients.

Materials and methods

Patients and tissue samples

This study was approved by the Institutional Review Board of our institute and written

informed consent was obtained from all patients. A total of 210 primary lung cancer specimens and corresponding non-cancerous lung tissues were collected from 210 patients with NSCLC undergoing surgery at our institute between January 2006 and January 2014. Patients were staged according to the 7th edition of the International System of Staging for Lung Cancer. Patient information including baseline demographics, clinicopathological characteristics and survival were recorded. None of the patients received radiotherapy or chemotherapy before surgery. The present study was carried out in accordance with the basic principles for all medical research, the Helsinki Declaration.

Quantitative RT-PCR

Total RNA was extracted using TRIZOL reagent (Invitrogen, USA) from tissues according to the protocol. An equal amount of RNA (10 μg) was reversely transcribed into cDNA by Reverse Transcriptase (Invitrogen, USA) according to the manufacturer's instructions. Sestrin2 and GAPDH were then amplified by quantitative real-time PCR using the following primers: Sestrin2: forward: 5'-GCGAGATCAACAAGTTGCTGG-3', reverse: 5'-ACAGCCAAACACGAAGGAGG-3'; GAPDH: forward: 5'-TGAAGGTCGGAGTCAACGG-3', reverse: 5'-CTGGAAGATGGTGATGGGATT-3'. Gene amplification was performed in an ABI 7900HT real-time PCR system using SYBR Green master mix (Invitrogen, USA). The mixture was preheated for 10 min at 95°C and followed by 40 cycles of amplification (30 s at 95°C and 1 min at 58°C, respectively). The C_t value of each sample was calculated, and the relative expression of Sestrin2 mRNA was normalized to the GAPDH (C_t method).

Western blot analysis

All proteins were separated by 12% SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. The membranes were incubated with primary antibodies at 4°C overnight, including monoclonal antibodies against Sestrin2 (dilution 1:100, Santa Cruz Biotechnology), monoclonal antibodies against GAPDH (dilution 1:4000, Santa Cruz Biotechnology). After three washes with TBST, the membranes were incubated at room temperature for 2 h with HRP-conjugated goat anti-mouse or goat anti-rabbit IgG antibodies (Santa Cruz Biotechnology). Molecular Images ChemiDoc XRS$^+$ imager with image lab software (Bio-Rad) was used to visualize the intensity of bands according to manufacturer's instructions.

Immunohistochemistry

Expression of Sestrin2 in NSCLC and non-cancerous lung tissues were examined via IHC. A polyclonal anti- Sestrin2 antibody was from Santa Cruz Biotechnology (working dilution 1:100). All procedures were implemented according to the manufacturer's instructions. For negative controls, sections were treated with 0.01 mol/L phosphate-buffered saline instead of primary antibodies. Two hundred cells from two selected representative fields of each section were counted by two independent observers for the determination of their immunostaining intensity.

Sestrin2 expression was evaluated according to the ratio of positive cells per specimen and staining intensity. The ratio of positive cells per specimen was evaluated quantitatively and scored as follows: 0, staining of ≤ 1%; 1, staining of 2-25%; 2, staining of 26-50%; 3, staining of 51-75%; and 4, staining of > 75% of examined cells. Staining intensity was divided into four groups: 0, no signal; 1, weak staining; 2, moderate staining; and 3, strong staining. A total score of 0-12 was determined as follows: total score = ratio of positively staining cells (score) × intensity of immunoreactivity (score). Scores were graded as negative (-; score: 0-1), weak (+; score: 2-4), moderate (++; score: 5-8) or strong (+++; score: 9-12).

Statistical analysis

Levels of Sestrin2 were expressed as mean ± standard deviation. Statistical analyses were performed with SPSS 19.0 software (SPSS, Chicago, IL). Cumulative survival time was calculated by the Kaplan-Meier method and analyzed by the log-rank test. Cox's proportional hazards regression model was used to analyze the independent prognostic factors. For the comparison of individual variables, t tests and χ^2 tests were carried out as appropriate. Statistical significance was determined for two-tailed tests at $p<0.05$.

Results

Sestrin2 mRNA expression in NSCLC tissues

We tested the mRNA expression of Sestrin2 on the fresh surgical NSCLC tissue samples and

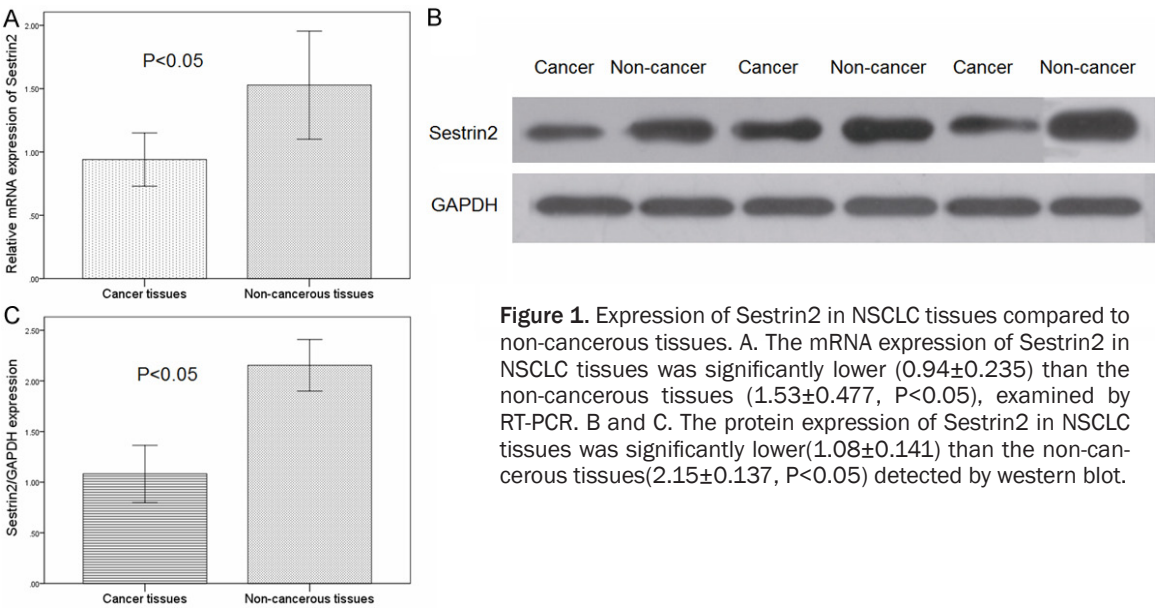

Figure 1. Expression of Sestrin2 in NSCLC tissues compared to non-cancerous tissues. A. The mRNA expression of Sestrin2 in NSCLC tissues was significantly lower (0.94±0.235) than the non-cancerous tissues (1.53±0.477, P<0.05), examined by RT-PCR. B and C. The protein expression of Sestrin2 in NSCLC tissues was significantly lower(1.08±0.141) than the non-cancerous tissues(2.15±0.137, P<0.05) detected by western blot.

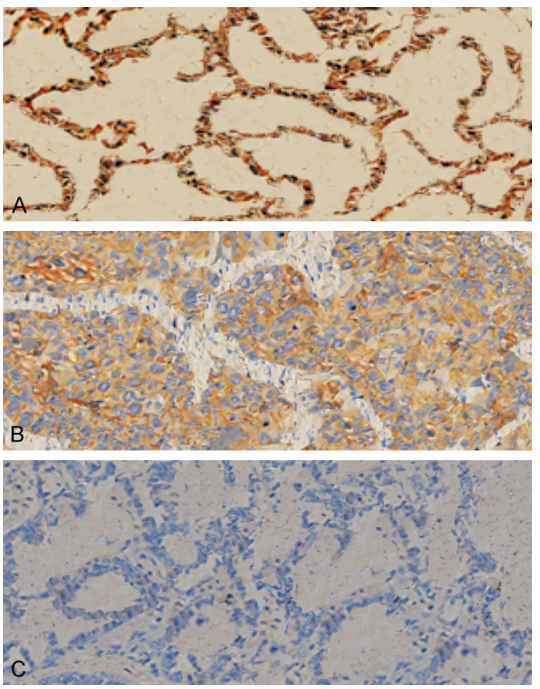

Figure 2. Immunohistochemical analysis of Sestrin2 in NSCLC patients. A. Strong expression of Sestrin2 in normal lung tissues. B. Moderate expression of Sestrin2 in squamous cell carcinomas. C. Negative expression of Sestrin2 in adenocarcinomas. Original magnification × 200.

the paired adjacent non-cancerous tissues from the same patients by using qRT-PCR. The mRNA expression of Sestrin2 was significantly lower in NSCLC tissues than the corresponding adjacent non-cancerous lung tissues (**Figure 1A**, P<0.05). All the paired tissues were tested in 3 times.

Protein expression of Sestrin2 in NSCLC tissues

We further examined the protein expression of Sestrin2 by western blot on the same paired NSCLC tissues and adjacent non-cancerous tissues. Consistent with the mRNA expression of Sestrin2, western blot analyses showed that the protein expression of Sestrin2 was markedly decreased in NSCLC tissues compared with non-cancerous tissues (**Figure 1B** and **1C**, P<0.05).

Relationship of Sestrin2 expression with clinicopathological variables in NSCLC

Immunohistochemical staining showed that Sestrin2 was mainly localized within the cytoplasm (**Figure 2**). In NSCLC tissues, 45.71% (96/210) of samples had high Sestrin2 expression. In adjacent non-cancerous, percent of samples with high Sestrin2 expression was significantly higher [67.62% (142/210), P< 0.001]. The relationship between Sestrin2 expression and clinicopathological features was shown in **Table 1**. The low (low: negative and low, IHC scores; high: strong and moderate, IHC scores) expression of Sestrin2 in NSCLC was significantly correlated with poor tumor differentiation (P = 0.030), more advanced TNM

Table 1. Association between Sestrin2 expression and various clinicopathological factors of NSCLC patients

Variables	Sestrin2 protein expression (n = 210)		*P* value
	Low (n = 114)	High (n = 96)	
Gender			0.937
Male	60	50	
Female	54	46	
Age			0.651
<60	44	40	
≥60	70	56	
Histological type			0.691
Squamous cell carcinoma	61	54	
Adenocarcinoma	53	42	
Smoking history			0.635
No	69	55	
Yes	45	41	
Tumor differentiation			0.030
Well-moderate	25	34	
Poor	89	62	
TNM stage			0.028
I-II	29	38	
III-IV	85	58	
Tumor size			0.220
T1+T2	37	39	
T3+T4	77	57	
Lymph node metastasis			0.019
Absent	30	40	
Present	84	56	

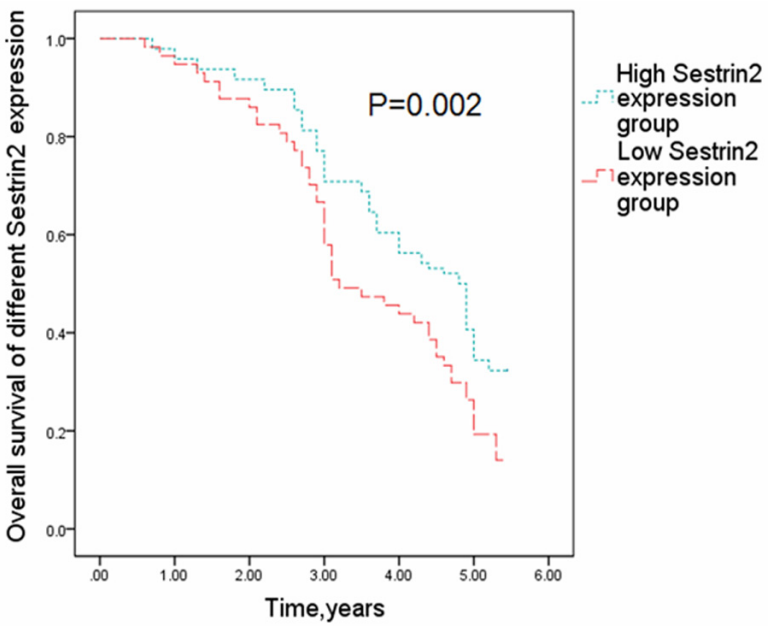

Figure 3. Overall survival of NSCLC patients estimated according to the Sestrin2 expression level in NSCLC tissue samples (Kaplan-Meier method) with immunohistochemical staining (P = 0.002).

staging (P = 0.028) and lymph node metastasis (P = 0.019).

Low Sestrin2 expression correlates with poor prognosis

Patients with low Sestrin2 expression had worse overall survival compared with those with high Sestrin2 expression (P = 0.002, **Figure 3**). In subgroup analyses, both in squamous cell carcinoma group and adenocarcinoma group, patients with high Sestrin2 expression had better overall survival compared with those with low Sestrin2 expression (P = 0.001, 0.014, separately; **Figure 4**). Similarly, both in well-moderate differentiation group and poor differentiation group, patients with high Sestrin2 expression had longer overall survival compared with those with low Sestrin2 expression. (P = 0.027, 0.007, respectively; **Figure 5**). Univariate analysis indicated that tumor differentiation, TNM stage, lymph node metastasis, and Sestrin2 protein expression were significantly associated with overall survival of NSCLC patients (P <0.001, <0.001, <0.001 and 0.004, separately; **Table 2**). On multivariate analysis, the results showed that Sestrin2 protein expression is an independent prognostic factor for overall survival, in addition to TNM stage, tumor differentiation and lymph node metastasis (P = 0.021, 0.006, <0.001 and 0.012, respectively; **Table 2**).

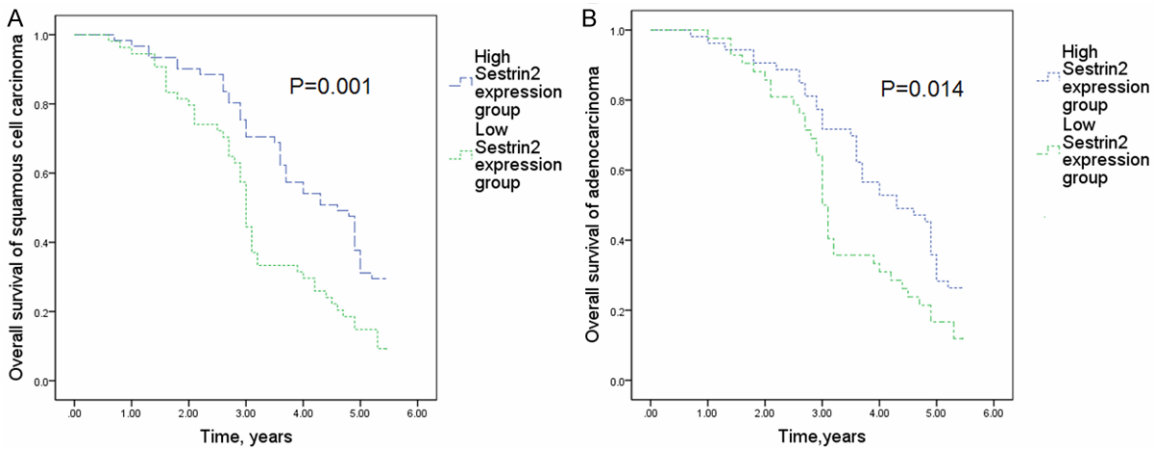

Figure 4. Overall survival of different histological subgroups. A. Overall survival of NSCLC patients with squamous cell carcinoma according to the Sestrin2 expression (P = 0.001). B. Overall survival of NSCLC patients with adenocarcinoma according to the Sestrin2 expression (P = 0.014).

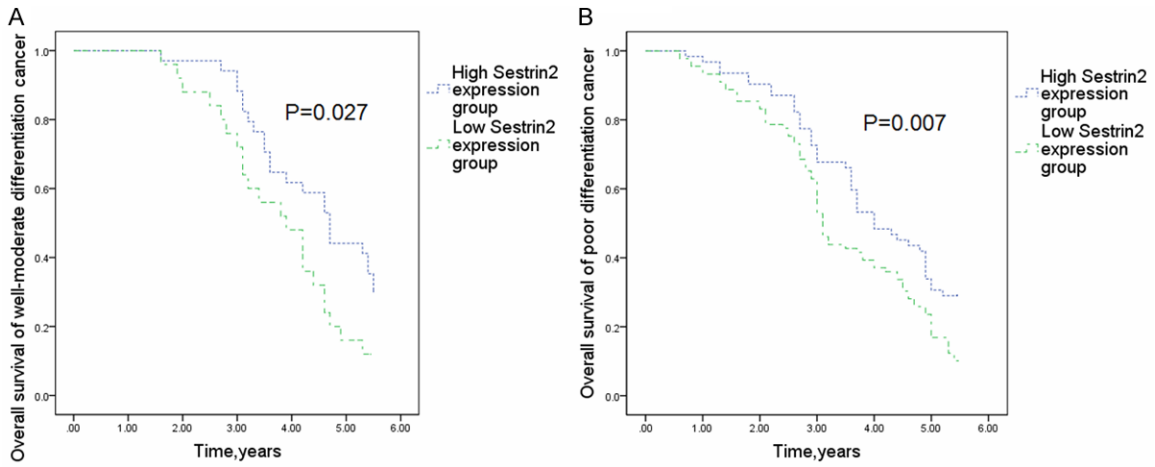

Figure 5. Overall survival of different tumor differentiation subgroups. A. Overall survival of NSCLC patients with well-moderate tumor differentiation according to the Sestrin2 expression (P = 0.027). B. Overall survival of NSCLC patients with poor tumor differentiation according to the Sestrin2 expression (P = 0.007).

Discussion

During the past three decades, because of phenotypical and histological heterogeneity of NSCLC, identification of new and better prognostic markers is very importance for the selection of high risk patients with NSCLC. Some genetic markers can further stratify NSCLC into effective and non-effective treatment subgroups. The prognosis may be improved with a focus on molecular markers of risk that may lead to updated detection and treatment methods [10].

Sestrin2 negatively modulates mTOR signaling and executes this function through the activation of AMPK and TSC2 phosphorylation [5]. Sestrin2 is involved in the cellular response to oxidative stress and DNA damage. It exerts its cytoprotective function by regenerating peroxiredoxins, therefore having an important role in the antioxidant defense of the cells. Sestrin2 is a target of the tumor suppressor gene p53 and has been shown to have an essential link between genotoxic stress, p53 and the mTOR pathway [11]. In a previous study, Zhao et al. published that Sestrin2 promoted AKT activation and survival in response to UVB stress and chemotherapeutics and suggested that Sestrin2 was an oncogene in skin SCC and melanoma [12]. Sanli et al. reported that elevated Sestrin2 inhibited irradiation-induced mTOR

Table 2. Univariate and multivariate analysis of prognostic factors in 210 NSCLC patients

Variables	Univariate analysis		*P* value	Multivariate analysis		*P* value
	HR	95% CI		HR	95% CI	
Age	1.15	0.84-1.58	0.394			
Gender	1.18	0.86-1.61	0.301			
Smoking history	1.25	0.92-1.70	0.154			
Histological type	1.26	0.94-1.69	0.124			
Tumor differentiation	1.31	1.20-1.42	<0.001	1.30	1.17-1.44	<0.001
TNM stage	3.14	2.28-4.33	<0.001	1.59	1.14-2.21	0.006
Tumor size	1.16	0.84-1.60	0.379			
Lymph node metastasis	2.22	1.65-3.00	<0.001	1.78	1.29-2.45	0.012
Sestrin2 expression	0.65	0.48-0.87	0.004	0.70	0.51-0.95	0.021

signaling and sensitized MCF7 cells to irradiation through an AMPK-dependent mechanism [13].

However, the precise role of Sestrin2 in NSCLC remains unclear. To explore the role of Sestrin2 in NSCLC, we investigated the expression patterns of Sestrin2 in human NSCLC tissues, and the relationship between Sestrin2 expression and the clinicopathological features of NSCLC. Our current study indicated that the expression of Sestrin2 was lower in NSCLC tissues compared with adjacent non-cancerous tissues. Survival analyses indicated that patients with low Sestrin2 expression had poor overall survival compared with those with high Sestrin2 expression. Moreover, in subgroup analyses based on different histological type or tumor differentiation, patients with high Sestrin2 expression was associated with better overall survival compared with those with low Sestrin2 expression. Multivariate analysis showed that Sestrin2 expression was an independent prognostic indicator for overall survival of NSCLC patients. These results indicated that low Sestrin2 expression could act as independent biomarkers of prognosis in NSCLC. Sestrin2 also had potential as a novel therapeutic target for the treatment of NSCLC.

A prior study has reported that Sestrin2 is highly expressed in both human skin SCC and melanoma [12]. Sestrin2 promotes AKT activation through a PTEN-dependent mechanism. Sestrin2 deletion or knockdown sensitizes squamous cell carcinoma (SCC) cells to 5-fluorouracil-induced apoptosis and melanoma cells to UVB- and vemurafenib-induced apoptosis [12]. Conversely, Ding et al. has reported that Sestrin2 is a member of a small family of antioxidant proteins and inhibitors of mechanistic target of rapamycin complex 1 (mTORC1) kinase. Down-regulation of Sestrin1/2 leads to genetic instability and accelerates the growth of lung adenocarcinoma xenografts [14].

The current study revealed that Sestrin2 was downregualted in NSCLC tissues and the low Sestrin2 expression was significantly correlated with the characteristics of aggressive NSCLC including advanced TNM stage, lymph node metastasis and poor tumor differentiation. The multivariate analyses indicated that Sestrin2 expression was an independent, favorable prognostic factor for patient outcome. Our results not only suggested a potentially promising use of Sestrin2 as a valuable prognostic indicator, but also implied a possible link between the biological function of Sestrin2 and the pathogenesis of NSCLC [14, 15]. Low Sestrin2 expression was a significant predictor of poor prognosis in NSCLC patients. Thus, our results indicated that Sestrin2 might inhibit the development and progression of human NSCLC, supporting the anti-oncogenic role of Sestrin2 in human NSCLC.

In most cells, Sestrin2 predominantly resides within the cytoplasm and is involved in gene transcription, alternative splicing, and nuclear export [16]. Moreover, Sestrin2 might contribute to anti-neoplastic and anti-autophagic functions through different molecular mechanisms in different cancer types or cellular contexts [17-19].

In conclusion, our study showed that Sestrin2 was an independent prognostic factor for survival in NSCLC patients. Sestrin2 might be valuable for evaluating prognosis, as well as providing potential targets for new therapeutic approaches for NSCLC. However, further studies on the mechanism by which Sestrin2 is involved in the development and progression of NSCLC are required in vivo and in vitro.

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Disclosure of conflict of interest

None.

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