ORIGINAL ARTICLE

Expression and significance of squalene epoxidase in squamous lung cancerous tissues and pericarcinoma tissues

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Abstract

Background: A high expression of squalene epoxidase (SQLE) is related to tumor occurrence, development, and prognosis in a variety of cancers. In this study, the expression and significance of SQLE was analyzed in patients with squamous lung cancer and pericarcinoma tissues.

Methods: The SQLE mRNA and protein expression were separately examined by semi-quantitative reverse transcription polymerase chain reaction, fluorescence quantitative polymerase chain reaction and Western blot in 65 cases of squamous cell lung carcinoma tissues and adjacent non-cancerous lung tissues.

Results: The expression of SQLE mRNA and protein in lung squamous cancerous tissues was significantly higher than in pericarcinoma tissues (63.07% vs. 44.61%, $P = 0.0348$; 67.69% vs. 38.46%, $P = 0.0008$). The positive expression rate of SQLE mRNA was not associated with gender, age, smoking, or tumor size ($P > 0.05$). The expression of SQLE mRNA was closely correlated with poor differentiation, clinical stages, and lymphatic metastasis ($P < 0.05$). The expression of SQLE mRNA was negatively associated with overall survival rate ($P < 0.05$).

Conclusion: A high expression of SQLE was found in squamous lung cancer tissues. The high expression of the SQLE gene may be closely related to the occurrence and development of squamous cell lung carcinoma. SQLE expression predicts a poor prognosis and may serve as a novel lung carcinoma molecule marker.

Introduction

Lung cancer, a malignant tumor, has become a serious global health problem in the 21st century. In China, the number of lung cancer patients is expected to reach one million by 2025, which will be the highest in the world. Squamous cell lung carcinoma, one of the most common pathological types of lung cancer, accounts for 30%–50% of the total number of lung cancer cases globally.¹ Surgery will determine the main treatment of lung squamous cell carcinoma. As obvious symptoms are lacking in the early stage, most lung cancer patients are diagnosed in advanced stage. The five-year survival rate is lower than 10% because of the lack of effective treatment. It is important to diagnose early, improve prognosis, and prolong survival of lung cancer patients, therefore, determining the molecular markers of lung cancer with high sensitivity and specificity is an urgent task.² In the early phase of our study, with the serum proteomic method, we obtained the protein spectrum of squalene epoxidase (SQLE), which can produce autoantibodies, and succeeded in constructing

the phage display library of the lung cancer tissue style. SQLE has been determined as one of the five lung cancer associated antigens by high-throughput screening technology.

SQLE, in the endoplasmic reticulum microsome, is one of the key rate-limiting enzymes in the first step of cholesterol biosynthesis. It can catalyze squalene (SQ) to generate 2,3-oxidosqualene, which can synthesise more important materials, for example, sterol and cholesterol,³ and reduce cholesterol levels or inhibit the growth of pathogenic fungi.^{4,5} Several studies have reported that SQLE, which is expressed in many malignant tumors, such as breast, pancreatic, colon, and prostate cancers, is required for the occurrence, development, and metastasis of the disease.^{6,7} Domestic studies reported that SQLE is over-expressed in squamous cell lung carcinoma patients.⁸ SQLE genes were separately examined by fluorescence quantitative polymerase chain reaction (PCR) and Western blot in the squamous cell lung carcinoma tissues and adjacent non-cancerous lung tissues. We investigated the role of SQLE in the occurrence, development, clinical features, and prognosis of lung cancer, so that we would

have more valuable information in the prediction of these factors.

Patients and methods

Patients

The postoperative tissue samples of 65 squamous lung cancer patients (46 male, 19 female, mean age 57 ± 11 years) were enrolled in our study. Inclusion criteria: tissues were surgically removed and diagnosed by pathology between December 2006 and December 2011 at the Qingdao Medical College Affiliated Hospital; and patients were untreated before surgery. Written informed consent was obtained from all patients prior to surgery.

Of the 65 total patients, 10 were poorly differentiated or undifferentiated, 12 were differentiated, and 43 were high differentiated. According to the standards of the 2002 Union for International Cancer Control (UICC), nine cases were stage I (Ia + Ib), 20 cases were stage II (IIa + IIb), and 36 cases were stage III (IIIa + IIIb). Thirty-five cases were lymph node metastasis, and 48 cases had a smoking history.

The control group was made up of the pericarcinoma tissues of the same patients (>5 cm tumor edge).

Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR)

RNAs were extracted from the lung squamous cell carcinoma tissues (50 mg) and adjacent non-cancerous lung tissues by the traditional Trizol method. The RNAs were then used for reverse transcription with the reverse-transcription kit. The acquired cDNA had to be stored at -20°C . All primers were designed and synthesized by the Shanghai Biological Engineering Co., Ltd. The primers used for SQLE amplification were F-5'-TTAGAGGAGAAATGCCAAGGAA-3' and R-5'-CACTGATGAAGGAGGAAGGAAG-3'. Fifty μl : 5 μl Buffer, 1 μl dNTP, 2 μl primer, 1.25 u Taqplus enzyme were added to each quantitative PCR. Reverse transcription was conducted by incubating for three minutes at 95°C , followed by 40 cycles of 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 10 minutes. According to requirements, the acquired cDNA is 152 bp. Reaction products were then added on the 2% agarose gel for electrophoresis with constant voltage 120 V for 30 minutes. Staining by an ethyl bromide organism took place for 30 minutes and photos were recorded under an ultraviolet analyzer.

Real-time fluorescent quantitative PCR

Serial ten-fold dilutions of cDNA were stored at -20°C until required. The reaction mixture prepared for real-time fluorescent quantitative PCR contained 10 μl SYBRP remix the

Ex Taq (TaKaRa), 1.6 μl PCR primers, 2 μl template cDNA, and 6.4 μl dH₂O. The cycling was performed at 95°C for four minutes, followed by 40 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds. After amplification, a melting curve was generated by a gradual increase of heating temperature at $0.2^{\circ}\text{C}/\text{second}$ from 70°C to 90°C with continuous in fluorescence measurement. The amplification products were calibrated with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and analyzed by the $2^{-\Delta\Delta\text{Ct}}$ ratio method.

Western blot

All of the frozen specimens were ground in liquid nitrogen and homogenized in lysis buffer. The lysate was centrifuged, and the supernatant was prepared. Fifty μg of each protein sample was separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membrane. After blocking in 5% skimmed milk/Tris-buffered saline (5 x Tris-buffered-saline with Tween [TBST], 0.1% Tween-20) for two hours, the membranes were incubated with rabbit anti-SQLE (1:1000) in 4°C overnight. During TBST flushing, the proteins were in secondary antibody incubation for one hour and visualized by the enhanced chemiluminescence (ECL) system. The immunoblots were quantitated using Gel-pro Analyzer software.

Follow-up visit

All 65 patients were followed up from between five to 60 months and their survival times were recorded. Two patients were lost to follow-up because of telephone number changes and home relocation.

Statistical analysis

Statistical analyses were conducted using SPSS for windows, version 17.0. The influence of the expression of SQLE on the clinical stage and prognosis was verified using the χ^2 test or Fisher method. Kaplan-Meier and Cox multivariate analysis were used to calculate survival with a 95% confidence interval.

Results

Expression of squalene epoxidase (SQLE) mRNA in squamous lung cancer tissues

The results of semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) (Fig 1) and real-time fluorescent quantitative PCR (Fig 2) were as follows: the mRNA expression of SQLE ($\chi^2 = 4.4571$, $P = 0.0348$) was found to be

Figure 1 The expression of squalene epoxidase (SQLE) mRNA and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA detected by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). M, Marker; T1-8, squamous lung carcinoma tissues; N1-8: pericarcinoma tissues.

significantly over-expressed in the squamous lung carcinoma group 63.07% (41/65), compared with the pericarcinoma tissues 44.61% (29/65).

Expression of SQLE protein in squamous lung cancer tissues

The results of Western blot analysis were as follows: the protein expression of SQLE ($\chi^2 = 11.1499$, $P = 0.0008$) was found to be significantly over-expressed in the squamous lung carcinoma group 67.69% (44/65), compared with the pericarcinoma tissues 38.46% (21/65) (Fig 3).

Influence of clinical features on the expression of SQLE mRNA in cancerous tissues

The expression of SQLE mRNA was associated with tumor stage, differentiation degree, and lymph node metastasis ($P < 0.05$). The differentiation degree was different in squamous lung carcinoma. The expressions of SQLE mRNA in high and medium differentiation tissues were significantly lower than

that in low differentiation ($P = 0.005$). The expressions of SQLE mRNA in stage I and II groups were lower than stage III ($P = 0.025$). The expressions of SQLE mRNA were higher than the lymphatic metastasis positive group compared with the negative group ($P = 0.002$). However, there was no obvious relationship between SQLE gene expression and gender, age, smoking history, or tumor size ($P > 0.05$). (Table 1).

Influence of the expression of SQLE mRNA in cancerous tissues on prognosis

Follow-up visits commenced on the day of surgery and continued until patient death. We noted significant differences in SQLE mRNA expression between the positive and negative groups (Log Rank = 10.127, $P < 0.01$) (Fig 4).

Cox multivariate analysis was utilized to investigate the following factors in the 65 cases: gender, age, smoking history, tumor size, tumor node metastasis (TNM) stage, differentiation degree, lymphatic metastasis, and expression of SQLE mRNA. The smoking history, tumor stage, lymphatic metastasis, and expression of SQLE mRNA had an influence on survival time alone. The expression of SQLE mRNA, tumor stage and lymphatic metastasis were synergetic as dangerous factors in the prognosis of squamous lung carcinoma. Patients with a high expression of SQLE mRNA experienced a poor prognosis (hazard ratio = 2.517, 95% confidence

Figure 2 The expression of squalene epoxidase (SQLE) mRNA in squamous lung carcinoma tissues was much higher than in normal lung tissues by real-time polymerase chain reaction (PCR) ($\chi^2 = 4.4571$, $P = 0.0348$). The results are normalized to β -actin. T, squamous lung carcinoma; N, pericarcinoma tissues.

Figure 3 The expression of squalene epoxidase (SQLE) protein and β -actin protein detected by Western blot. T1-3, squamous lung carcinoma tissues; N1-3, pericarcinoma tissues.

Table 1 Relationship between expression level of SQLE mRNA and clinical characteristics of patients

Clinical characteristics	Cases (N)	SQLE mRNA expression score		χ^2	P-value
		Negative N (%)	Positive N (%)		
All patients	65	26 (40.0)	39 (60.0)		
Gender				2.095	0.148
Male	46	21 (45.6)	25 (54.3)		
Female	19	5 (26.3)	14 (73.6)		
Age				0.106	0.745
<60	21	9 (42.8)	12 (57.2)		
≥60	44	17 (38.6)	27 (61.4)		
Smoking				0.478	0.489
Non-smoker	17	8 (47.1)	9 (52.9)		
Smoker	48	18 (37.5)	30 (62.5)		
T-stage				0.820	0.365
T1, T2	34	15 (44.1)	19 (55.9)		
T3	31	11 (35.4)	20 (64.5)		
TNM-stage				5.022	0.025
I + II	29	16 (55.1)	13 (44.9)		
III	36	10 (27.7)	26 (72.3)		
Differentiation				7.741	0.005
Well/Mod	22	14 (63.6)	8 (36.4)		
Poor	43	12 (27.9)	31 (72.1)		
Lymph node				9.286	0.002
Negative	30	18 (60.0)	12 (40.0)		
Positive	35	8 (22.8)	27 (77.2)		

SQLE, squalene epoxidase; TNM, tumor node metastasis.

interval = 1.298–4.879, $P = 0.006$). Patients with lymphatic metastasis accompanied by the higher pathological stage had a shorter lifetime than the lymphatic metastasis accompanied by the lower pathological stage. (Table 2).

Figure 4 Relationship between expression level of squalene epoxidase (SQLE) mRNA and overall survival rate of squamous cell lung carcinoma patients (Kaplan-Meier method). (—) SQLE low level, (—) SQLE high level, (—) SQLE low level-censored, (—) SQLE high level-censored.

Discussion

Lung cancer is one of the most malignant tumors with the highest mortality. Methods of diagnosis mainly include radiological examination, exfoliative cell examination of sputum, fiber bronchoscope, thoracoscope, and mediastinoscopy.^{9,10} These techniques are all traumatic and dangerous. Because of its ease, the low degree of trauma associated, and good repeatability, tumor marker detection has become the preferred method of clinical screening and early diagnosis.¹¹

Table 2 Cox multivariate analysis of prognostic factor

Variables	RR	95% CI	P-value
Univariate analysis			
SQLE	2.365	1.334–4.193	0.003
Gender	0.631	0.340–1.174	0.146
Age	1.219	0.672–2.212	0.515
Smoking history	1.763	1.006–3.092	0.048
Differentiation	1.073	0.625–1.843	0.799
Tumor size (T3+T4/T1+T2)	1.046	0.609–1.796	0.871
Clinical stage (III/IV + II)	2.722	1.451–5.106	0.002
Lymph node (±)	3.031	1.565–5.869	0.001
Multivariate analysis			
SQLE	2.517	1.298–4.879	0.006
Smoking history	1.159	0.629–2.134	0.637
Clinical stage (III/IV + II)	2.540	1.323–4.876	0.005
Lymph node (±)	2.510	1.273–4.948	0.008

CI, confidence interval; RR, relative risk; SQLE, squalene epoxidase.

In order to improve the diagnostic rate, scientists are looking for tumor molecular markers with high specificity and sensitivity in the field of the chemical characteristics of tumor cells, cytopathology, immune response, and gene expression. They have made rapid progress.¹²

In this study, we investigated SQLE mRNA and protein expressions in 65 squamous cell lung carcinoma patients and correlated them with clinicopathological features and prognoses to determine whether this biomarker could predict disease outcome. SQLE expression in squamous cell lung carcinoma tissue was significantly higher than in the pericarcinoma tissue, as Liu *et al.* found in human squamous cell lung carcinoma. High SQLE expression was significantly correlated with aggressive clinicopathological features. Moreover, the overexpression of SQLE was a statistically significant risk factor affecting the overall survival (OS) of squamous cell lung carcinoma patients, suggesting that SQLE could be a valuable marker of squamous cell lung carcinoma progression and prognosis.

SQLE is a monooxygenase; its gene is located in the long arm area of chromosome 8 (8q 24.1). It is involved in cholesterol biosynthesis, cell metabolism, and regulating organ specificity.¹³ Helms *et al.*¹⁴ confirmed that the SQLE gene can increase the risk of early metastasis in breast cancer with positive estrogen receptors. SQLE gene can play an important role in the progression, metastasis, and inhibition of the differentiation of tumors. Its high amplification area in chromosome 8q can be used as the oncogene of pancreatic cancer. Some studies have found that the SQLE gene has tumor-invasiveness; its low expression can improve the survival rate of colon cancer patients.¹⁵ Zhao *et al.*¹⁶ also showed that the low expression of the SQLE gene has an effect on the morbidity of prostate cancer patients. Moreover, Liu *et al.*⁸ found an expression of SQLE in squamous cell lung carcinoma, but the underlying biological mechanisms that might explain the relationship between SQLE expression and lung cancer are still not known. However, we observed that the expression of SQLE was significantly high in squamous cell lung carcinoma and it was correlated with differentiation, clinical stage, and lymphatic metastasis. These results suggest that SQLE may play an important role in the tumorigenesis and deterioration of tumors.

In our study, the expression of SQLE mRNA and protein were separately examined using fluorescence quantitative PCR and Western blot in 65 cases of squamous lung carcinoma and pericarcinoma tissues. SQLE mRNA and protein expression in squamous lung carcinoma tissues was higher than that in pericarcinoma tissues. Our results also showed that SQLE expression had a role in the clinical features, for example, tumor stage, differentiation degree, and lymphatic metastasis. These results were in line with previous studies of breast cancer;¹³ therefore, we conclude that the SQLE gene may influence the development and invasiveness of squa-

mous lung carcinoma. We further investigated the influence of SQLE in OS on squamous cell lung carcinoma patients. Analyzing follow-up data in the Kaplan–Meier survival analysis, patients with a high expression of SQLE were significantly associated with shorter OS. A high expression of SQLE should be more obviously associated with a poor prognosis in advanced stage patients; however, our results did not provide strong support for this. After careful consideration, we think the low sample size may have influenced these results. Importantly, univariate analyses revealed that increased SQLE expression in squamous cell lung carcinoma tissues is significantly associated with the OS rate. Moreover, on multivariate analysis, we found that the influence of SQLE mRNA expression on tumor stage and lymphatic metastasis could influence survival time: the higher the SQLE mRNA expression, the worse the prognosis. This evidence showed that SQLE expression might be used as an index in the diagnosis and prognosis of squamous lung carcinoma.

Several limitations of our study must be acknowledged. First of all, as a prospective study, the evaluation of clinical response and OS is often imprecise. Secondly, the number of patients in our study was small and, therefore, our results may be generalized to squamous cell lung carcinoma only. A larger study is necessary to validate our data, which suggests that SQLE level may influence OS in squamous cell lung carcinoma.

Conclusion

In conclusion, we found that the SQLE gene expression has a significant relationship to the occurrence, development, and prognosis of squamous lung carcinoma. Because of the complexity of signal transduction pathways, it is apparent that the mechanism of the SQLE gene must be validated in further functional and clinical studies.

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Disclosure

No authors report any conflict of interest.

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