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Original Article

Serum SQSTM1 is a potential predictor for chemotherapeutic efficacy against non-small cell lung cancer

Xiaohui Miao*, Zhifang Zhuang*, Yao Zhou, Yanjuan Zhou, Jiao Xu, Yafang Liu

*Department of Respiratory, Affiliated Wujin Hospital of Jiangsu University, Changzhou 213017, Jiangsu, PR China. *Equal contributors and co-first authors.*

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Abstract: This study was aimed to evaluate the predictive value of serum SQSTM1 for routine chemotherapy against non-small cell lung cancer (NSCLC). Lung tumor tissues and the paired normal lung tissues from 40 NSCLC patients undergoing primary surgical, and then SQSTM1 expressions in tumor tissues or normal lung tissues were detected by using immunohistochemistry (IHC). Furthermore, serum levels of SQSTM1 were investigated in 80 patients with advanced NSCLC receiving chemotherapy, and other 40 patients with benign pulmonary diseases (BPD) were served as the control. Our results indicated that SQSTM1 is significantly up-regulated in NSCLC tumor tissues, and however, it has no correlation to the clinical outcomes of NSCLC. The serum level of SQSTM1 elevated in 78.75% of the patients prior treatment, and in such individuals were less likely to obtain complete remission (CR) or partial remission (PR) than patients with normal or moderately elevated serum SQSTM1 ($p < 0.05$). After two chemotherapy cycles, 38 of the 47 (80.85%) patients with CR or PR had a SQSTM1 serum level below the cut-off point versus, and there are only four of the 33 (12.12%) patients in the group with stable disease (SD) and progressive disease (PD), and the decreases of SQSTM1 were statistically significant differences in serum between the effective group and ineffective group ($p < 0.01$). These results were validated by western blotting analysis. Overall, the SQSTM1 could be served as a useful diagnosis factor for NSCLC, and our results also support the link between serum level of SQSTM1 and chemotherapeutic efficacy against non-small cell lung cancer.

Keywords: Serum SQSTM1, non-small cell lung cancer, predictor, chemotherapeutic efficacy

Introduction

Currently, cancer is the leading cause of death around the world, accounting for about 27% of all cancer-related deaths [1]. Especially in China, the incidence of lung cancer leads individual cancers in male [2]. Among the two main histological types of lung cancer, non-small cell lung cancer (NSCLC) accounts for approximately 85% and mostly has been locally advanced or metastatic at the time of diagnosis [3, 4].

With the appeal of precision medicine, lung cancer immunotherapy and targeted therapy are thriving fields, especially in NSCLC, but chemotherapy still occupies an important position in all tumor treatments, and more and more attention to precision chemotherapy [5]. Liquid biopsy is defined as a non-invasive procedure for obtaining the plasma or serum [6], which may significantly improve the cancer diagnosis, prognosis and therapeutic efficacy evaluation. For instance, CECs based counts can predict prognosis [7], and CTCs has high specificity in detecting the EGFR mutation status in NSCLC patients [8]. Therefore, serum markers are also suitable for predicting and monitoring the curative effects, and there is an urgent need to seek more effective serum markers.

SQSTM1 (also known as sequestosome-1) was initially isolated as an interacting partner of atypical protein kinase C (aPKC) [9]. Increasing reports have demonstrated that SQSTM1 is enriched in protein-interacting regions, which provides the basis for its essential role as a signaling hub rather than a mere scaffold [10]. SQSTM1 plays multiple roles in cell death and

survival, and SQSTM1 participates in cell autophagy through specifically associates with mTOR [11]. The loss of SQSTM1 leads to an increase in cell apoptosis through the accumulation of Ras-induced ROS [12], and SQSTM1 regulates mitosis via de-gradating Cyclin B1 protein [13]. With respect to cancer, SQSTM1 is also related to tumorigenesis signaling pathways. A large number of studies have shown that SQSTM1 is over-expressed in breast cancer, gastrointestinal tumor and genital neoplasms [14-17]. Of course, SQSTM1 is also associated with the occurrence and development of lung cancer [18]. Most of the researches only focused on the expressions of SQSTM1 in tumor tissues, and it's a pity that the serum level of SQSTM1 has rarely been studied so far.

In order to better implement the principle of precise chemotherapy, we designed this study to evaluate the potential roles of serum SQSTM1 in the development of NSCLC, and our investigation also explored the correlation between SQSTM1 and chemotherapeutic efficacy.

Materials and methods

Clinical samples collection

Tumor tissue and their matched normal lung tissues were obtained from 40 underwent surgical resection early stage NSCLC patients without any preoperative chemotherapy in the period 2014.01-2015.09. All resected tissues were evaluated by a pathologist to confirm the diagnosis and were graded using the 7th Edition TNM classification criteria (International Union Against Cancer).

Eighty patients with advanced NSCLC receiving sequential chemotherapy were studied between January 2014 and September 2016. The age, sex, pathological type and the basic values of carcinoembryonic antigen (CEA) were recorded. All patients had a performance score of 0, 1 or 2 (ECOG), and would receive up to at least three 21-day cycles of cisplatinbased chemotherapy (cisplatin [75 mg/m^2, day 1] + either docetaxel [75 mg/m^2, day 1] or gemcitabine [1250 mg/m^2, days 1, 8], or pemetrexed [500 mg/m^2, day 1; nonsquamous NSCLC only]. Before the first and the third cycle of chemotherapy, general assessment was evaluated with RECIST1.1 standard, the complete remission (CR) and partial remission (PR) were regarded as the effective group, while the stable disease (SD) and progressive disease (PD) were classified as invalid group. Serum samples of these participants were taken to detect SQSTM1 levels. Additionally, 40 serum samples of patients with BPD were acquired as controls.

All tumor tissues and serum samples acquisition were carried out in accordance with the institutional guidelines, and all the samples were collected from the Wujin People's Hospital (Changzhou, China). The present study was approved by the ethics board of the Wujin People's Hospital (No. 2014 EB-021a). All participants signed written informed consent, and these consent procedures were approved by this ethics board of the Wujin People's Hospital (Changzhou, China).

Immunohistochemistry analysis

All the tissues samples were de-paraffinized in xylene and de-hydrated through graded ethanol after formalin-fixed and paraffin embedded, and then antigen retrieval was accomplished by using the boiling citric acid buffer (pH = 6.0) in a water bath for 20 min; Next sections were incubated with 3% hydrogen peroxide to block endogenous peroxidase activity, and rinsed with 5% bovine serum albumin (BSA) to prevent non-specific straining; Again, the slides were incubated with rabbit anti SQSTM1 (PM045, 1:1000; MBL, Nagoya, Japan) overnight at 4°C, followed by three washes with phosphate-buffered saline (PBS); Thereafter, the sections were incubated with biotin-labeled anti-rabbit secondary antibodies at room temperature for 30 min followed by three washes with PBS, and detecting with 3,3-diaminobenzidine (DAB). Finally, the slides were de-hydrated and mounted with coverslipsafter counterstaining with hematoxylin, and subsequently examined using light microscopy.

Immunohistochemical determination

The intensity for SQSTM1 immunostaining was judged on a scale as follows: 0) negative (0%); 1) weak positive (< 25%); 2) moderate positive (< 75%); 3) strong positive (≥ 75%). For simple statistical analysis, the patients were divided into a low immune-reactive group (grade 0 and 1) and a high immune-reactive group (grade 2

Table 1. Clinical parameters and SQSTM1 expression by IHC

Clinical parameters	Cases	SQSTM1 expression		*P* value
		High	Low	
Types				
Tumour tissue	40	35	5	< 0.001
Matched tissue	40	2	38	
Gender*				
Male	22	18	4	0.355
Female	18	17	1	
Age (years)*				
< 60	24	22	2	0.372
≥ 60	16	13	3	
Stage*				
I/II	32	29	3	0.257
III	8	6	2	
Pathology*				
AD	30	27	3	0.584
SCC	10	8	2	

AD-adenocarcinoma; SCC-squamous cell carcinoma; *Represents the data of NSCLC patients; P < 0.05 was considered statistically significant.

and 3). All of the immune-staining results were evaluated by two senior pathologists and interpreted by the chief physician of pathology department if there was ambiguity.

Enzyme-linked immunosorbent assay (ELISA)

Recombinant IMP2/SQSTM1, which is same to the IGF2BP2 isoform, has been expressed from a clone isolated from cDNA expression library by immunoscreening with serum antibody from a patient with HCC [18]. Purified recombinant protein was diluted in PBS to a final concentration of 0.5 μg/mL, and 100 μl were added into each well to coat microtitre plates (Fisher Scientific, Houston, TX, USA) overnight at 4°C. Plates were blocked with gelatin post-coating solution for 2 h at room temperature. The human serum samples were diluted at 1:200, incubated with the antigen-coated wells at 37°C for 90 min followed by washing with PBS containing 0.05% Tween 20 (PBST), then incubated with goat antihuman IgG-HRP (Invitrogen, NY) as a secondary antibody diluted 1:4000 for 90 min followed by washing with PBST. The solution of 2,2'-azino-bis-3-ethylbenzo-thiazoline-6-sulfonic acid (ABTS, Invitrogen) was used as detecting agent. The optical density (OD) value of each well was read at 450 nm. Each sample was tested in duplicate. The cut-off value for determining a positive reaction was designated as the mean OD value of the 40 control human sera plus 2 standard deviations (mean + 2SD) [19].

Western blot analysis

The protein was extracted from tumor tissues and 50 μg total proteins were separated using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% non-fat milk and subsequently incubated with the corresponding primary antibodies overnight at 4°C. After incubation with horseradish peroxidase-conjugated goat anti-mouse secondary antibodies, protein bands were visualized using chemiluminescence peroxidase reagents. β-actin was used to measure protein loading.

Statistical analysis

Statistical analysis was performed using SPSS 20.0, and the experimental data were expressed as Mean ± SD. The paired t test was used to detect the change of serum levels of SQSTM1 before and after treatment, and the Mann-Whitney test was detect the alteration of serum SQSTM1 in different therapeutic effect groups. Furthermore, bivariate correlation analysis was used to analyze the relationship between serum SQSTM1 and carcinoembryonic antigen (CEA). Moreover, count data were compared using the Chi square test or Fisher exact probability method. A value of $p < 0.05$ was considered statistically significant.

Results

The SQSTM1 overexpressed in tumor tissues of NSCLC patients

We collected the general clinical characteristics and analyzed the SQSTM1 protein expressions in 40 NSCLC tumor tissues and paired normal lung tissues by IHC assay. **Table 1** showed the gender, age, surgery staging and histopathologic types. The average age was 54 years (range 40-76 years). High immune-reactive group was more frequently found in NSCLC tumor tissues than in normal lung tissues (**Figure 1A-C**), and the overexpressed SQSTM1

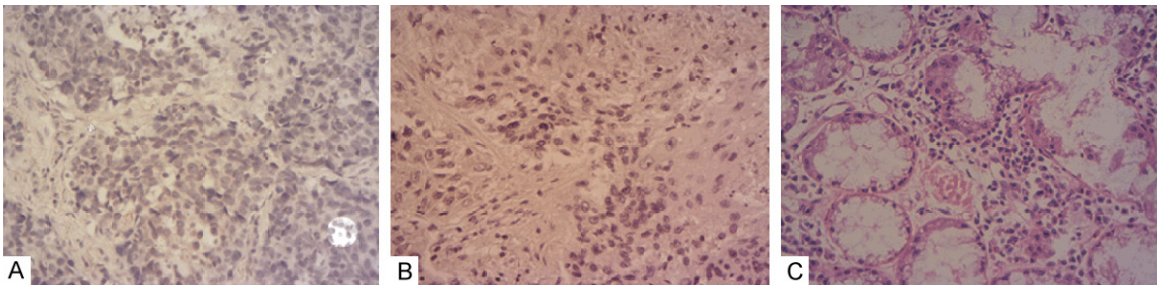

Figure 1. SQSTM1 protein expression in NSCLC tissues and matched normal tissues using IHC. A. Staining in adenocarcinoma tissues; B. Staining in squamous cell carcinoma tissues; C. Staining in paired normal tissues.

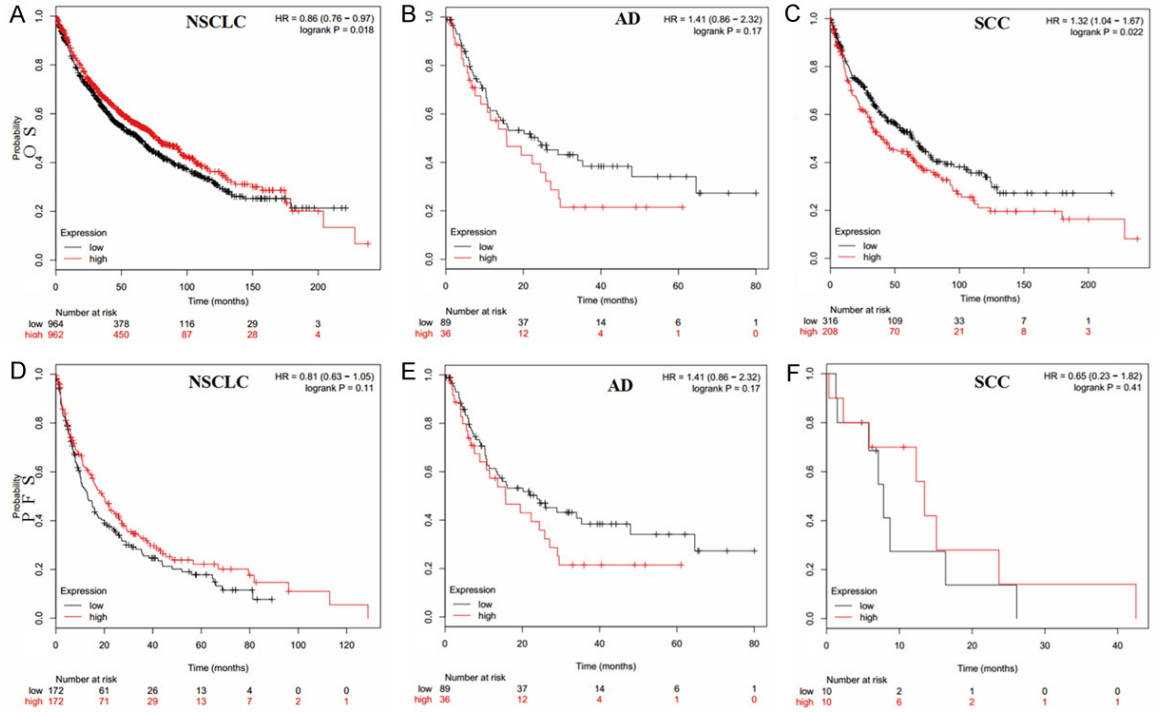

Figure 2. The expression of SQSTM1 and the survival curves in NSCLC patients from the Kaplan-Meier plot database. A. SQSTM1 low expression is associated with worse OS in NSCLC; B. SQSTM1 expression did not affect OS in AD; C. SQSTM1 high expression is associated with worse OS in SCC; D-F. The expression of SQSTM1 was not correlated with PFS in NSCLC, AD or SCC. Red line: the high SQSTM1 expression subgroup; Black line: the low expression subgroup. OS: overall survival; PFS: progression-free survival. NSCLC: non-small cell lung cancer; AD: adenocarcinoma; SCC: squamous cell carcinoma.

had no relation with age, sex and stage in NSCLC (**Table 1**).

The high-expressed SQSTM1 in tumor tissues was not associated with prognosis

In order to validate the relationship between SQSTM1 expression and prognosis of NSCLC, we mined the data from the publicly available Kaplan-Meier plot (http://kmplot.com/analysis/index. php?p=background) database. The browser indicated that the patients with SQSTM1 mRNA high-expression subgroup (red line) had favorable overall survival (OS) in NSCLC; in contrast, the SQSTM1 mRNA high-expression was associated with poor prognosis of squamous cell carcinoma (SCC) while its high-expression was not related to the prognosis of adenocarcinoma (AD). In addition, as shown in **Figure 2**, there was no correlation between SQSTM1 mRNA expression and progression-free survival (PFS) in patients with NSCLC, AD or SCC.

Table 2. Serum levels of SQSTM1 in NSCLC patients and BPD patients

Groups	Cases	Serum SQSTM1 (ng/L)		t value	*P* value
		> 118.83	< 118.83		
NSCLC	80	63	17	58.422	< 0.001
BPD	40	2	38		

NSCLC-non-small cell lung cancer; BPD-benign pulmonary diseases; P < 0.05 was considered statistically significant.

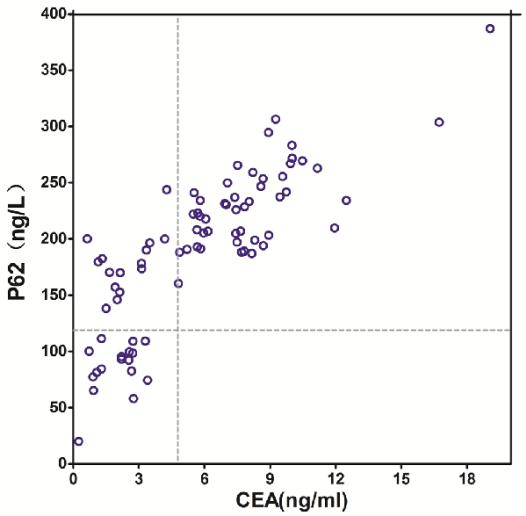

Figure 3. Correlation between baseline SQSTM1 and CEA levels in 80 patients. The dashed lines represent the cut-off values of 118.83 ng/L for SQSTM1 and 5 ng/mL for CEA.

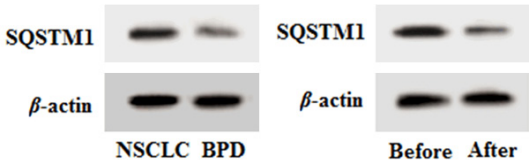

Figure 4. SQSTM1 protein expression in NSCLC tissues and matched normal tissues using western blotting analysis. NSCLC-non-small cell lung cancer; BPD-benign pulmonary diseases; Before-before chemotherapy; After-after two chemotherapy cycles.

Serum SQSTM1 levels in NSCLC patients were elevated

The mean value of serum SQSTM1 of 40 patients with BPD was 89.23 ± 14.8 ng/L, therefore the cutoff value was 118.83 ng/L. Under the cutoff standard, serum SQSTM1 levels were elevated in 63 of the 80 (78.75%) NSCLC patients before treatment, but were within the normal range in 38 of the 40 BPD patients. There was significant difference between the two groups (**Table 2**). The western blotting also showed the SQSTM1 expression in the NSCLC patients was higher than that of BDP patients (**Figure 4**).

Serum SQSTM1 levels declined significantly after two treatment cycles

A significant decline from the initial serum SQSTM1 concentrations was seen after two chemotherapy cycles. The average of serum SQSTM1 in 80 NSCLC patients decreased from 191.08 ng/L (19.93-387.04 ng/L) to 124.91 ng/L (14.84-300.98 ng/L), the SD changed from 68.34 ng/L to 59.96 ng/L. There was significant statistical difference, $p < 0.001$ (**Table 3**). The results of western blotting analysis also indicated SQSTM1 expression was decreased after the treatments (**Figure 4**).

Therapeutic effects were evaluated after two treatment cycles, 47 of the 80 (58.75%) patients were effective (CR + PR), the remaining 33 (41.25%) patients were invalid (SD + PD). Further subgroup analysis, the decreased amplitude of the effective group was significantly higher than that of the invalid group, and the mean values of decline were 87.94 ng/L and 35.18 ng/L in two subgroup, respectively. The result of the Mann-Whitney test showed that the *p* value was less than 0.001 (**Table 4**).

Initial SQSTM1 serum concentration could predict the chemotherapeutic efficacy

Serum SQSTM1 values were divided into normal levels (< 118.83 ng/L) and high levels (> 118.83 ng/L). Using this classification, a favorable outcome (CR or PR) was established in the patients with normal SQSTM1 levels, but not the high levels group ($p = 0.026$). Besides, the initial SQSTM1 serum concentration had no relationship with the age and gender (**Table 5**).

Pretreatment CEA level was positively correlated with the SQSTM1 level

The normal range of CEA level was 0-5 ng/mL in our hospital. There was a positive correlation between initial SQSTM1 serum concentration and pretreatment CEA level ($r = 0.819$, $p < 0.001$, **Table 5** & **Figure 3**). A simpler classification, in which CEA levels were divided into > 5 ng/mL and ≤ 5 ng/ml. CEA levels could not predict the efficacy of chemotherapy ($p = 0.352$, **Table 6**), while combining SQSTM1 data would

Table 3. The concentration of serum SQSTM1 before and after treatment in NSCLC patients

Groups	Cases	Serum SQSTM1 (ng/L)		t value	*P* value
		Mean	Range		
Before	80	191.08	19.93~387.04	11.791	< 0.001
After	80	124.91	14.84~300.98		

Before-before chemotherapy; After-after two chemotherapy cycles; P < 0.05 was considered statistically significant.

Table 4. The decreased amplitude of serum SQSTM1 in different efficacy subgroups

Groups	Case	ΔSerum SQSTM1 (ng/L)		U value	*P* value
		Mean	Range		
CR + PR	47	87.94	-3.55~97.03	307	< 0.001
SD + PD	33	35.18	5.09~178.28		

CR-complete remission; PR-partial remission; SD-stable disease; PD-progressive disease; Δsymbols indicates the decreased amplitude; P < 0.05 was considered statistically significant.

Table 5. Relationship of initial SQSTM1 levels with clinical characteristics and outcome after therapy in NSCLC patients

Clinical parameters	Cases	Serum SQSTM1 (ng/L)		*P* value
		> 118.83	< 118.83	
Gender				
Male	21	16	5	0.738
Female	59	47	12	
Age (years)				
< 60	28	21	7	0.547
≥ 60	52	42	10	
Response				
CR + PR	47	33	14	0.026
SD + PD	33	30	3	
CEA (ng/mL)				
> 5	46	46	0	< 0.001
≤ 5	34	17	17	

CR-complete remission; PR-partial remission; SD-stable disease; PD-progressive disease; P < 0.05 was considered statistically significant.

increase the power to predict chemotherapeutic efficacy ($p < 0.001$, **Table 6**).

Discussion

Our study identifies SQSTM1 is expressed in NSCLC tumor tissues, but not in adjacent non-malignant cells. This is consistent with the previous report [20], who showed that high-expressed SQSTM1 was detected in 49.03% (51 of 104) of NSCLC tumor tissues, and the SQSTM1 expression was correlated significantly with TNM stage and lymph node metastasis, suggesting that SQSTM1 expression is of significance in diagnosing NSCLC.

Previous studies have showed that SQSTM1 was an independent risk factor related to OS for NSCLC patients, and the prognostic impact of SQSTM1 status was pronounced in adenocarcinoma patients [20-22]; Furthermore, Anna *et al* pointed out that general SQSTM1 high-expression was significantly associated with aggressive tumor behavior [22]. But, according to the previously published Kaplan-Meier plot data, the ability of SQSTM1 to predict prognosis is different in various pathological types (**Figure 2**). In 2016, German scholars suggested that the prognostic power of cytoplasmic SQSTM1 depends on the mutation status of KRAS in colorectal cancer [23]. Based on diverse results, the following reasons are considered: first, tumor heterogeneity might be cause this phenomenon; then, racial disparities in participants and different sample size are inevitable existence; finally, mRNA levels and protein levels are not completely consistent.

SQSTM1 is a multifunctional adapter protein implicated in selective autophagy [24], and the overexpression of SQSTM1 in tumor tissues is not in doubt. High levels of SQSTM1 autoantibody in several cancer patients' sera had also been confirmed in recent 5 years [19, 25, 26]. Our data support the working hypothesis that autoantibody against SQSTM1 may be a useful serum biomarker for early stage NSCLC cancer screening, and SQSTM1 can induce relatively higher frequency of autoantibody responses in patients with NSCLC (78.75%). Liquid markers have also been detected in NSCLC [27-29]. Besides, commonly used serum markers of lung cancer that are currently recommended by multiple guidelines on the diagnosis of lung cancer [30], include CEA, neuron-specific enolase (NSE), cytokeratin fragment (Cyfra21-1), pro-gastrin-releasing peptide (ProGRP) and squamous cell carcinoma antigen (SCC). However, their specificity and sensitivity are relatively scarce, and thus the combined detection of them was usually performed in clinical prac-

Table 6. Outcome after therapy according to initial CEA and SQSTM1 levels

SQSTM1 Levels	CEA Levels	Outcome		t value	*P* value
		CR + PR	SD + PD		
	> 5	25	21	0.866	0.352
	≤ 5	22	12		
> 118.83	> 5	25	21	5.773	< 0.001
	≤ 5	8	9		
≤ 118.83	> 5	0	0		
	≤ 5	14	3		

CR-complete remission; PR-partial remission; SD-stable disease; PD-progressive disease; P < 0.05 was considered statistically significant.

tice. In such circumstances, SQSTM1 appears add brilliance to the diagnosis of lung cancer.

Serum markers can be used not only for diagnosis but also for therapeutic monitoring. A recent Meta-analysis [31] showed that the baseline Cyfra21-1 level is a better factor in predicting treatment benefit, and Cyfra21-1 and CEA exhibited the good performance in forecasting objective response during treatment, and more, the decline of Cyfra21-1 levels during treatment is highly indicative for objective response. Our study identifies serum accumulation of SQSTM1 as a strong predictor of an ineffective treatment behavior of NSCLC, and substantial decline of SQSTM1 in plasma is correlated with effective treatment. In addition, combination of basic CEA level resulted in increased predictive power of the assay suggesting that serum SQSTM1 level could potentially be valuable chemotherapy efficacy markers. Confirmed by experiments, high expression of SQSTM1 could prevent ROS stress-induced apoptosis or ER stress-induced apoptosis by regulating the Keap1-Nrf2-ARE signaling pathway, results in cisplatin resistance in human ovarian cancer cells [32, 33]. The same result, the activation of Nrf2 and SQSTM1 was associated with doxorubicin resistance in breast cancer [34]. Our data also suggested that a high level of SQSTM1 was not sensitive to conventional chemotherapy in NSCLC patients, and it is conceivable that high SQSTM1 level inhibits apoptosis or autophagy.

Autophagy is a cellular degrading process that regulates degradation cytoplasmic macromolecules and organelles by transportating to lysosomes [35]. Autophagy is involved in both tumor formation and tumour suppression [24]. SQSTM1 is an autophagic substrate, and it confers a highly selective between cancer and autophagy through the degradation of important signaling molecules. Although SQSTM1 carcinogenic effects have been demonstrated by most of the relevant experiments (including this study), SQSTM1 also acts as a tumor suppressor through applying to tumor-associated macrophages (TAM) in the tumor microenvironment [36]. SQSTM1 causes cancer not only through autophagy, but also through inflammatory pathways [37]. Distinguishing the role of SQSTM1 in cacner might help in identifying high risk patients who would benefit from autophagy related anticancer drugs. The exciting recent investigations formed SQSTM1 as a potential therapeutic target in some types of cancer. As Chang *et al* said [38], SQSTM1 may be a rational target for bone metastatic in prostate cancer. Furthermore, some traditional herbal medicines have been used in the treatment or prevention of cancer via regulating SQSTM1 mediated signaling pathway [39-42], and SQSTM1 vaccine has proven strong antitumor activity in solid tumors (lung and breast carcinomas, melanoma and sarcoma) in hundreds of animals [43].

Our study was the first research to determine the serum SQSTM1 level in NSCLC patients, and it is the first evidence that SQSTM1 can predict the efficacy of conventional chemotherapy, in particularly after the combination of CEA. But there still has several limitations. First, it belongs to a single center study, insufficient sample size may cause an error that cannot be ignored; thus, multicenter studies are needed. Second, we only concluded that high serum SQSTM1 levels were more likely to occur in the resistance group, further mechanisms need to be explored.

Conclusions

In summary, our findings clearly show that SQSTM1 over-expression has been implicated in NSCLC development, and serum SQSTM1 level is valuable for predicting the chemotherapeutic efficacy. Thus, serum SQSTM1 can be used as an early diagnostic and therapeutic management biomaker.

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Disclosure of conflict of interest

None.

Address correspondence to: Zhifang Zhuang, Department of Respiratory, Affiliated Wujin Hospital of Jiangsu University, No. 2 Yongning North Road, Changzhou 213017, PR China. Tel: +86-0519-85336190; E-mail: zfzhuangwj@126.com

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