

## RESEARCH COMMUNICATION

# Up-regulation of GTSE1 Lacks a Relationship with Clinical Data in Lung Cancer

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### Abstract

Lung cancer is one of the most common cancer types that lead to death in cancer patients across the world. Clarification of differentially expressed genes is essential for lung cancer diagnosis and treatment. According to the public cDNA microarray database, we found that GTSE1 is overexpressed dramatically in lung cancer patients' tissues. To further verify the microarray data, quantitative real-time PCR and immunohistochemical staining were undertaken to test GTSE1 expression both at mRNA and protein levels. GTSE1 is up-regulated in lung cancer tissues compared to the adjacent normal tissues, especially in adenocarcinoma and squamous cell carcinoma. The expression profile of GTSE1 was investigated, and the clinical significance and the association between GTSE1 and the overall survival were assessed in 246 lung cancer patients. No statistically significant correlation, however, was observed between GTSE1, the clinical features and survival. Thus, GTSE1 may not be a prognostic marker in lung cancers.

**Keywords:** GTSE1 - lung cancer - immunohistochemical staining

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### Introduction

Lung cancer is one of the most prevalent malignancies in the world, and it ranks at the leading edge in cancer death these years. According to the report of world health organization in 2009, lung cancer kills 1.2 million annually. Among varied kinds of lung cancers, non-small cell lung carcinoma (NSCLC) accounts for 80%~85%, which can be subdivided into adenocarcinoma, squamous cell carcinoma and large cell carcinoma. 5 year survival rate of NSCLC patients after surgery is below 15% in China. Because there are limited effective methods for early diagnosis and its poor prognosis, the morbidity and mortality of NSCLC is shooting up in last 30 years. Therefore, it is of great importance to explore the underlying molecular mechanisms and suitable markers associated with the lung cancer progression, clinicopathological features and follow-up study.

Through analyzing the public microarray database of differentially expressed genes in human tumor, we characterized several genes that are dramatically up-regulated in cancer tissues compared to their corresponding adjacent normal tissues. Among these, G2 and S expressed protein-1 (GTSE1) attracted our attention. Gtse1, once named B99, was first reported to be cloned as a wt-p53 inducible gene in a murine cell line in 1998 (Utrera et al., 1998). hGTSE1, the human homolog of the murine

Gtse1, which is located in chromosome 22q13.2-q13.3, is composed of 11 introns and 10 exons and spans about 33kb on the human genome. Similar with the murine Gtse1, GTSE1 is thought to localize on the microtubules, specifically express in G2 and S stage of the cell cycle and is regulated both by cell-cycle mechanisms and p53 (Collavin et al., 2000; Monte et al., 2000). Moreover, it is demonstrated that the C-terminal region of GTSE1 can physiologically interact with the C-terminal region of p53, negatively modulating the transactivity of p53 in dependent on the cell cycle progression as well as sensitizing cell to p53 induced apoptosis (Collavin et al., 2000; Monte et al., 2003). Further study suggested that overexpression of GTSE1 can intensify the cytoplasmic localization of p53. Thereby, GTSE1 can shuttle p53 into the cytoplasm and regulate p53 stability in the nucleus depending on PLK1 phosphorylation of GTSE1 at Ser 435 (Monte et al., 2004; Liu et al., 2010). Besides, a recent report indicated that GTSE1 can prevent p21 from proteasome degradation and increase the cell resistance to the paclitaxel induced apoptosis (Bublik et al., 2010). However, to date, the function of GTSE1 in cancer progression remains unexplored. In this research, the expression pattern of GTSE1 and its further correlation analysis with clinical features were investigated in lung cancers. This evidence will shed light on the role of GTSE1 in lung cancer progression.

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## Materials and Methods

### *Analysis of microarray datasets*

4 microarray datasets (Squamous cell carcinoma: a, GSE7339, b, GSE3268, Adenocarcinoma: c, GSE7670, d, GSE10072) of pairwise lung cancer tumor tissues and adjacent normal tissues were retrieved from GEO to investigate the mRNA expression level of GTSE1. The expression level of GTSE1 from only 1 sample of GSE7339 was detected. While in each of the other 3 datasets there were 6 groups of probe replicates of GTSE1 for each sample, we used the t test p value to estimate the significance of GTSE1 expression level on the mean value of the replicates.

### *Lung cancer patients' tissue and clinical data collection*

A total of 246 pairs of lung cancer tissues and their adjacent tissues were obtained immediately after surgery from the Tangdu Hospital, the Fourth Military Medical University (Xi'an, China) between June 2009 and April 2011, and preserved in liquid nitrogen. There were no age, sex, or disease stage restrictions for case recruitment. Demographic and personal data were collected through in-person interview using a standardized epidemiological questionnaire, including age, sex, ethnicity, residential region, smoking status, alcohol use, education status, body mass index (BMI) and family history of cancer. Detailed clinical information was collected through medical chart review or consultation with treating physicians, including time of diagnosis, time of surgery, time of recurrence and/or death, tumor stage, differentiation and treatment protocol. A standard follow-up was performed by a trained clinical specialist through on-site interview, direct calling, or medical chart review. The latest follow-up data in this analysis was obtained in April 2011, and 22 (8.9%) patients were lost during follow-up. Tissue samples in this study were approved by patients by signing the informed consent.

### *RNA exaction and cDNA synthesis*

Total RNA of 35 pairs of patients tissues were extracted from frozen lung cancer tissues by E.A.N.A. total RNA kit (omega Bio-Tek, USA) following the protocols. Reverse transcription was processed using the PrimeScript RT reagent DRR037A (Takara, Japan) at 42°C for 20 minutes followed by 70°C for 10 minutes. The total RNA and synthesized cDNA were all kept at -80°C.

### *Quantative Real-time reverse transcription PCR*

Real-time PCR was utilized to measure GTSE1 gene expression in 35 lung cancer and its paired tissues using a MX3005P real-time PCR system (Agilent, USA) and SYBR Premix Ex Taq IIDRR081A (Takara, Japan). We performed the reaction in duplicate and set no-template controls (NTC), following the 3-step method (95°C 30 seconds, 58°C 30 seconds and 72°C 20 seconds) for 40 cycles. POLR2A and ESD were used as internal controls. The primers were designed and synthesized in the Genecore company (Shanghai, China). The sequences of primers for the qRT-PCR are listed below. The Ct method was utilized to analyze the data of qRT-PCR results as

previously described (Livak and Schmittgen, 2001; Kato et al., 2011). GTSE1:F: CACCGATGACCCCAAAAC, R: TCTCCCTTGTTGGTTTCAGTTCTC; POLR2A:F: AGCCGCAGATGTTTGGTGAA, R: CTGGGCTGTCGCTTTCTTCT; ESD:F: TGATCAAGGGAAGATGACCA, R: AACCTCTTGCAATCGAAAA

### *Immunohistochemical staining*

All lung cancer tissues were assessed by pathologists according to HE staining to assure that the rate of tumor cells was more than 90%. The immunostaining was processed following the protocol of Histostain-Plus Bulk Kit (Invitrogen, USA) using rabbit anti-hGTSE1 antibody with the Cat.number bs-2516R (BoAoSen, China).

### *Evaluation of immunohistochemical staining results*

The results of immunohistochemical staining were evaluated for extent and intensity independently. According to the percentage of positive cells in each tumor specimen slide, we defined five grades: <10%(0), 10-25%(1), 26-50%(2), 51-75%(3), and >75%(4). Besides, the staining intensity is divided into 4 grades(intensity score): negative(0), light brown(1), brown(2), dark brown(3). For each patient's tissue slide, pictures of 5 different visual fields under 400x magnification were taken, then percentage scores and intensity scores were estimated according to the standards described in previous studies. Score for each slide was calculated by multiplying the percentage score and intensity score. Final score was the mean value of five visual fields (Lee et al., 2009; Kong et al., 2010; Kato et al., 2011).

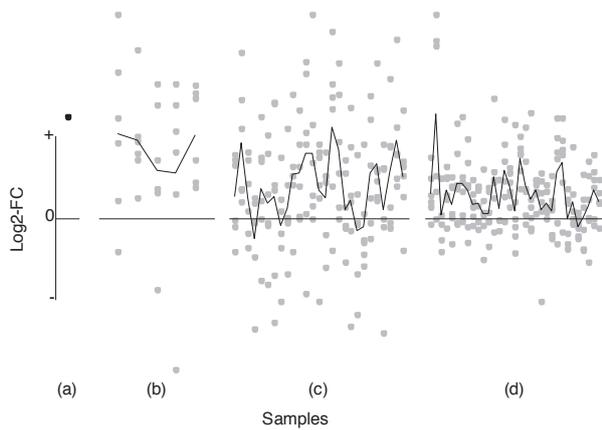
### *Statistical analysis*

All statistical analyses were performed using the SPSS 16.0 statistical package (SPSS, Chicago, IL). Student's t test and Pearson  $\chi^2$  were used to test the association of GTSE1 expression with clinical characteristics, including age, sex, smoking state, tumor stage, tumor histology, tumor diameter, volume, subcellular location, recurrence, death and event. Hazard ratios (HRs) and 95% confidence interval (95% CI) were estimated with a multivariate Cox proportional hazards model, adjusting to age, sex, smoking state, tumor diameter and volume where appropriate. Kaplan-Meier curve and log-rank test were used to assess the differences of overall survival. All P values were based on 2-sided tests. A probability level of 0.05 was used as the criterion for statistical significance. The endpoint was overall patient survival, which was defined as the time from initial treatment to death from any cause (Kim et al., 2008; Yi et al., 2010).

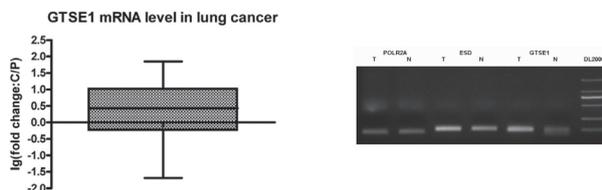
## Results

### *Up-regulation of GTSE1 expression in human lung cancer microarray database*

In order to verify the GTSE1 expression level of mRNA, we analyzed the public microarray database (Squamous cell carcinoma: a, GSE7339, b, GSE3268, Adenocarcinoma: c, GSE7670, d, GSE10072). The log<sub>2</sub> transformed fold change of GTSE1 RNA level in tumor



**Figure 1. GTSE1 Log2 Transformed Expression Level.** Fold change of each sample between tumor tissues and non-tumorous tissues in microarray from GEO in squamous cell carcinoma (a, GSE7339, b, GSE3268) and adenocarcinoma (c, GSE7670, d, GSE10072). The black line was the mean value of the probe replicates in b, c and d. To show the results intuitively without losing any information, we rescaled the Log-FC value for each datasets and omitted the true value on y coordinate



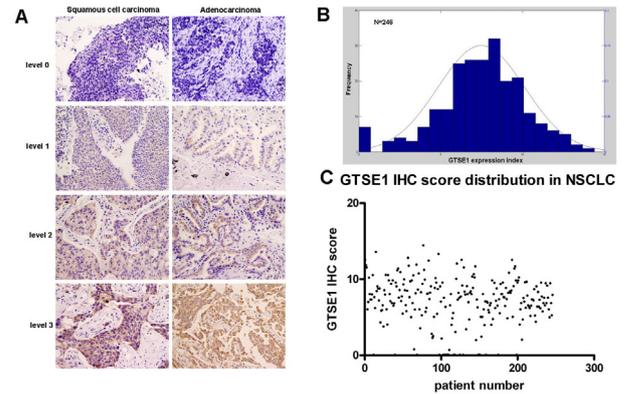
**Figure 2. A). Quantitation of GTSE1 mRNA Level by Realtime RT-PCR in Lung Cancer Tissues (N=35); B) Specific Products of Primers of GTSE1 Gene and Internal Control Genes used in Real-time RT-PCR**

tissue versus non-tumorous tissue RNA level was shown in Figure 1. GTSE1 was up-regulated in all these 4 databases both in squamous cell carcinoma and adenocarcinoma with the t-test p-value (b): 0.0014, (c): 5.6e-6, (d): 5.9e-7. Quantitative real-time RT PCR further confirmed GTSE1 up-regulation in human lung cancer patients

We measured the GTSE1 transcript levels by quantitative real-time PCR to address the expression status of GTSE1 in lung cancer tissues. 35 lung cancer patients' samples (15 squamous cell carcinoma, 15 adenocarcinoma and 5 other types of lung cancer) including the cancer tissues and its paired adjacent tissues were detected. According to the results, we found that in 74.2% patients (26/35) GTSE1 mRNA level was apparently much higher in lung cancer tissues than in its adjacent benign tissues. Figure 2A demonstrated the lg transformed fold change of GTSE1 RNA level in tumor tissues versus non-tumorous tissues. Gene POLR2A and ESD were used as internal controls. The electrophoretic analysis of quantitative real-time RT PCR products from one patient's tissue was shown in Figure 2B, which also indicated the specificity of all PCR primers (Wang and Fang, 2011; Nagahara et al., 2011; Li et al., 2011).

#### Distribution of patients' characteristics

The characteristics of the study population are listed below (Ambroise et al., 2011; Fernandez et al., 2011; Nagahara et al., 2011; Zhan et al., 2011; Yoshitake et



**Figure 3. Immunohistochemical staining of GTSE1 in lung cancer tissues.** A) 4 different levels of GTSE1 expression intensity scores in lung cancer tissues, including squamous cell carcinoma and adenocarcinoma. B. Histogram illustrating the frequency and range of GTSE1 immunolabeling index of 246 lung cancer patients. C. The immunohistochemistry score distribution of GTSE1 in 246 lung cancer patients

al., 2011). The median age at the time of diagnosis was 59 years (range, 30-84 years) for all the patients. 186 (75.6%) patients were male and 147 (59.8%) patients smoke. The pathological stages were as follows: stage I (n = 81, 32.9%), stage II (n = 50, 20.3%), stage III (n = 101, 41.1%), and 14 patients' (5.7%) pathological stage was unknown. 126 patients (51.2%) had squamous cell carcinoma, 78 patients (31.7%) had adenocarcinoma, others belong to other types. The majority (n=125, 50.8%) of patients had moderately differentiated tumors. 15 patients' cancer tissues showed specific nuclei localization of GTSE1 protein and others all demonstrated cytoplasm localization. During the median follow-up of 15 months (ranging from 3 to 22 months), 71 patients (28.9%) died.

#### Overexpression of GTSE1 protein in human lung cancer by immunohistochemical staining

In 246 paraffin-embedded lung cancer tissue specimens, we detected the expression level and subcellular localization of GTSE1 protein by immunohistochemistry. GTSE1 expression is upregulated dramatically in 226 (including 71 adenocarcinomas, and 118 squamous cell carcinomas) of the 246 tumor specimens (including 78 adenocarcinomas and 126 squamous cell carcinomas) with a 91.8% positive staining rate. While the degree of positive staining varied significantly among different specimens according to the immunohistochemical staining of GTSE1, in order to evaluate GTSE1 protein expression level more precisely we divided the expression intensity score of GTSE1 into 4 different levels (Figure 3A). 20 samples were considered negative for GTSE1 staining, which were set as level 0. Light brown, brown and dark brown were set as level 1, 2 and 3 respectively. For each specimen, we calculated the histologic score following the method described before (Lee et al., 2009; Kong et al., 2010). The distribution of histologic score of GTSE1 staining in 246 NSCLC patients was demonstrated in figure 3 B and C (Hwang et al., 2011; Wang and Fang, 2011; Nagahara et al., 2011; Fernandez et al., 2011; Ambroise et al., 2011). Expression of GTSE1 protein was mostly present in

**Table 1. Distributions of GTSE Level with Clinical Characteristics**

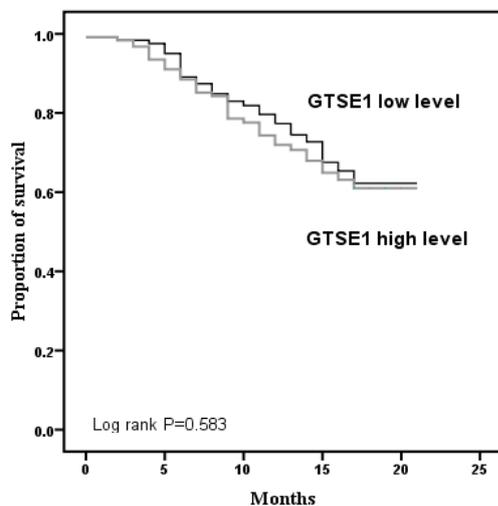
Variables		Low	High	P value
Age	≤59	66	62	0.610
	>59	57	61	
Gender	Male	91	95	0.553
	Female	32	28	
Smoking	Yes	78	69	0.242
	No	45	54	
TNM	I	42	39	0.698
	II	24	26	
	III	46	55	
Histology	SCC	66	60	0.005
	AC	31	47	
	SCLC	12	1	
	Other	14	15	
Tumor diameter	≤4.7	57	55	0.798
	>4.7	66	68	
Tumor volume	≤72	64	60	0.610
	>72	59	63	
Location	Nucleus	8	7	0.790
	Cytoplasm	115	116	
Death	Yes	33	38	0.482
	No	90	85	

SCC, Squamous cell carcinoma; AC, Adenocarcinoma; SCLC, Small cell lung cancer

the cytoplasm. The scores were given by the intensity and percentage of each specimen. Low-level of GTSE1 expression was described as 0-2, medial-level was from 3-7 and 8-12 was regarded as high-level. Information lost samples, unrepresentative samples with too few tumor cells, inappropriate staining- samples were not utilized in the data summary.

#### Relationship between GTSE1 and the clinicopathological variables of lung cancer patients

As shown in Table 1, we compared the GTSE1 histologic score stratified by host characteristics. We first dichotomized the GTSE1 expression level into high and low groups using the median (50th percentile) value as the cutoff point. The clinicopathological features include age (≤59 versus >59), sex (male versus female), smoking status (yes versus no), TNM stage (I, II, III), histological



**Figure 4. Kaplan-Meier Plots of Overall Survival Analysis of GTSE1 in Lung Cancer Patients**

category, tumor diameter, tumor volume, the GTSE1 sub-cellular location (nuclei versus cytoplasm) and the death state (yes versus no). Statistical analysis results showed that GTSE1 expression was only associated with the histological types of the lung cancer ( $p=0.005$ ). No correlation was observed between GTSE1 overexpression and age ( $P=0.610$ ), gender ( $P=0.553$ ), smoking status ( $P=0.242$ ), TNM stage (0.698) and Tumor volume (0.610). Although the histological scores of GTSE1 expression becomes larger when the tumor's malignancy is more severe, the P value did not show significant difference between patients with varied TNM stages. That is, the expression of GTSE1 may have no relationship with the clinical features of the lung cancers (Yoshitake et al., 2011; Zhan et al., 2011).

#### GTSE1 expression and survival analysis

We next performed logistic regression analysis to assess the association between GTSE1 expression level and overall survival of patients. No significant difference in survival between lung cancer patients with high and low level of GTSE1 expression (adjusted HR, 1.12; 95% CI, 0.70-1.79). Kaplan-Meier analysis also showed that there was no statistical significance in both subgroups ( $P = 0.583$ , Figure 4). These results suggested that there might be no modifying effect of GTSE1 on the prognostic significance in lung cancers (Kim et al., 2008; Yi et al., 2010; Zhan et al., 2011)

## Discussion

Since the lung cancer is a high-risk tumor, and its development and progression is such a complicated process with multi-stages, it is desirable to identify the underlying molecular mechanism to develop new approaches for diagnosis and therapy. Clarifying genes and proteins that are differentially expressed between lung cancer tissues and their adjacent normal tissues is critical to elucidate the complexity of lung cancer progression and may be an ideal way to identify suitable biomarkers for diagnosis. Public microarray database has laid the basis for analyzing thousands of differentially expressed genes in cancer research. The GEO repository has accumulated massive gene expression data and facilitated further research on these genes.

In our previous study, we analyzed GTSE1 expression profiles of lung cancer tissues in GEO database. In this research, real-time PCR and immunohistochemical staining were conducted separately to evaluate the mRNA and protein expression levels of GTSE1 in lung cancer. The results demonstrated that consistent with the microarray data, GTSE1 is up-regulated compared to its adjacent tissue counterpart in most lung cancer tissues, mainly in the adenocarcinoma and squamous cell carcinoma. To find out the relationship between GTSE1 expression and the clinicopathologic features of lung cancers, the clinical data of these lung cancer patients were studied then, including age, gender, smoking status, pathological type, and TNM stage and so on. The statistical analysis revealed that none of them showed a significant correlation with high expression of GTSE1 protein. Since it is suspected

that the subcellular localization of GTSE1 may play an important role in the development of lung cancer, we intended to analyze the significance of localization of GTSE1 in these specimens, but were restrained by the limited number of specimens (only 15 patients' samples have the GTSE1 nuclear staining). Besides, the overall survival analysis also indicated no significance. We clarified the up-regulation of GTSE1 in lung cancer for the first time, however, our research didn't demonstrate that GTSE1 is a prognostic factor in lung cancer. According to previous studies, GTSE1 is specifically expressed in G2 and S stage of the cell cycle, and is modulated by cell cycle regulatory mechanisms (Collavin et al., 2000). It can bind p53 and shuttle p53 from nucleus to cytoplasm to enhance its cytoplasmic localization and regulate p53 protein level (Monte et al., 2004). Furthermore, GTSE1 can regulate p21 stability by protecting it from proteasome-dependent degradation (Bublik et al., 2010). By oppositely regulating p53 and p21 as former studies demonstrated, GTSE1 protein may display a combined role in promoting cell survival by shifting the equilibrium of p53 response from apoptosis to survival. Because of the important function of cell cycle in cancer occurrence and progression (Schwartz and Shah, 2005), it is highly probable that GTSE1 also play an important role in lung cancer development through regulating the cell cycle or interacting with other key factors, such as p53, p21, PLK1, CDKs and CDKIs. Thus far, no related research has been reported and the mechanism of GTSE1 in lung cancers remains to be elucidated. More detailed work on the functional analysis needs to be done and the combined expression analysis of GTSE1 with those key factors, like p53, p21, PLK1 and mdm2 may imply more diagnostic and prognostic significance in lung cancer, which shows promise for obtaining new approaches for cancer therapy.

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