Original Article

Quantifying the expression of tumor marker genes in lung squamous cell cancer with RNA sequencing

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Background: We measured the expression of some commonly used tumor markers with RNA sequencing (RNA-Seq) to identify any that might be useful for the evaluation of squamous cell lung cancer and identify possible correlations between these tumor markers and any clinical characteristics.

Methods: RNA-Seq was performed on five pairs of squamous-cell lung cancer and normal tissues and another 39 squamous-cell lung cancer tissues obtained by our department between September and December, 2012. The expression of 13 commonly used tumor markers was determined.

Results: All of the patients in our study were male. The expressions of CA125, CYFRA21-1, NSE and SCC increased in tumor samples and there were statistically significant differences between squamous cell lung cancer and normal tissues (P=0.008, P<0.001, P<0.001, P=0.001). The expression of β2M and CA15-3 was reduced in squamous cell carcinoma relative to normal tissues and there was no significant difference in the expression of other tumor markers, including AFP, AFU, CT, FER and HE4.

Conclusions: CA125, CYFRA21-1, NSE and SCC may be appropriate tumor markers for squamous cell lung cancer.

Keywords: RNA sequencing (RNA-Seq); tumor marker; squamous cell lung cancer

Submitted May 22, 2014. Accepted for publication Aug 07, 2014.
doi: 10.3978/j.issn.2072-1439.2014.08.27
View this article at: http://dx.doi.org/10.3978/j.issn.2072-1439.2014.08.27

Introduction

Lung cancer is the most commonly diagnosed cancer as well as the leading cause of cancer-related death. This disease accounted for 13% (1.6 million) of total cancer cases and 18% (1.4 million) of deaths caused by cancer in 2008 (1). Squamous-cell lung cancer, one of the most common types of lung cancer, typically originates in the large airways and represents approximately 20-30% of lung cancer cases in recent years (2). The overall survival of early stage patients is quite good, but most patients are diagnosed at an advanced stage when there are fewer curative opportunities. Therefore, early diagnosis is the key to improve patient outcomes for squamous-cell lung cancer.

Tumor markers have been widely applied in cancer diagnosis over the last two decades due to convenience, low cost, reproducibility and non-invasiveness. Most previous studies have focused on tumor marker levels in the serum. Here, we studied the expression of several commonly used tumor-marker genes in tumor and normal tissues. Squamous-cell lung cancer and adenocarcinoma are so different from each other (3). Therefore, we measured the expression of some commonly used tumor markers in squamous-cell lung cancer tissues using RNA sequencing (RNA-Seq) to screen for genes that may be suitable tumor markers in squamous cell lung cancer. We then investigated possible correlations between these tumor markers and clinical characteristics.

Materials and methods

Ethics statement

This study was approved by the Ethics Committee of Zhongshan Hospital, Fudan University {Approved No. 2011-219[2]}. Written informed consent was obtained from each patient participating in this study.

Tissue samples and clinicopathological characteristics

Samples were obtained from patients with squamous-cell lung cancer who underwent surgical resection between September and December, 2012 at Zhongshan Hospital, Fudan University, Shanghai, China. Clinicopathological characteristics were recorded for all patients. Normal lung specimens were resected at least 3 centimeters away from the tumor margin, while tumor samples were carefully extracted from the center of squamous-cell carcinoma. All samples were flash frozen in liquid nitrogen after removal and then saved at –80 ℃. Part of each sample was paraffin embedded, HE stained and checked by an experienced pathologist to make ensure that no cancer cell existed in the normal tissues and more than 80% of cells in every tumor sample were squamous carcinoma. Finally, five matched pairs of normal tissue samples and lung squamous-cell carcinoma tissues and another 39 lung squamous-cell carcinoma tissues were obtained. These samples were then used in RNA-Seq.

RNA preparation

Total RNA from each sample was extracted with Trizol (Invitrogen, Carlsbad, CA, USA), re-dissolved in DEPC-treated water and quantified using NanoVue Plus spectrophotometry (GE Healthcare, Fairfield, CT, USA). RNA integrity was evaluated using agarose gel electrophoresis, and DNA contamination was eliminated using gDNA Eraser (Takara, Tokyo, Japan) according to the manufacturer's guidelines.

RNA sequencing (RNA-Seq)

The mRNA component of total RNA was converted into a library of template molecules suitable for sequencing using TruSeq®RNA Sample Preparation Kit v2 (Illumina, SanDiego, CA, USA) according to the manufacturer's guidelines. mRNA was purified and fragmented and first and second strand cDNA was synthesized. RNA was then subjected to end repair, 3' end adenylation, ligation of adapters, PCR amplification of cDNA libraries procedure, among others. Sequencing was then performed using a Genome Analyzer II (Illumina, SanDiego, CA, USA) according the manufacturer's recommendation. Sequence analysis was performed using Galaxy software (http://galaxyproject.org) to calculate the reads per kilobase of exon model per million mapped reads (RPKM) of every transcript. Then the RPKM of all transcripts from each gene were added up to evaluate that gene's expression. Each sample was sequenced twice and the average of the RPKM value of each gene was adopted to reflect its expression level.

Statistical analysis

The RPKM data derived from RNA-Seq were analyzed using SPSS for windows, version 20 (IBM, Armonk, NY, USA). The mean of the RPKM was used to evaluate the gene expression level. The expression profiles of tumor maker genes in normal samples were compared with paired tumor samples using the paired *t*-test and *t*-test for comparing genes' expressions between normal and all tumor samples. Spearman correlation analysis was used to identify correlations between the expression of tumor marker gene expression and clinicopathological characteristics, and Pearson correlation analysis was used to identify correlations between CA125, CYFRA21-1, NSE and SCC.

Results

Patient clinicopathological characteristics

The clinicopathological characteristics of all 44 patients are listed in *Table 1*. All of the patients in our study were male, because most squamous-cell lung cancers occur in older male patients. Female patients accounted for only 7.8% (104/1,320) of squamous-cell lung cancer in our department from 2005-1-1 to 2011-12-31. In our study, none of the patients were at N3 or M1 stage, because these patients were not suitable for radical surgical resection. No patients were in stage IV for the same reason.

Expression profiles of commonly used tumor markers

We evaluated 13 commonly used tumor markers, including β2M, AFP, AFU, CA125, CA15-3, CEA, CT, CYFRA21-1, FER, HE4, NSE, PSA and SCC. Their full names, encoding genes and functions are presented in *Table 2*. We listed all encoding genes and expression profiles for tumor

Table 1 The clinicopathological characteristics of all 44 patients

Clinicopathological characteristics	No.
No.	44
Gender	
Male	44
Female	0
Age	
≥60	26
<60	18
Smoking status	
Yes	23
No	21
Primary tumor	
T1	15
T2	19
T3	6
T4	4
Regional lymph nodes	
N0	29
N1	8
N2	7
N3	0
Metastasis	
M0	44
M1	0
Differentiation	
Well differentiated	8
Moderately differentiated	18
Poorly differentiated	18
TNM stage	
I	23
II	12
III	9
IV	0

markers that are encoded by more than one gene, with the exception of CEA. CEA is encoded by a group of genes, including at least 12 carcinoembryonic antigen-related cell adhesion molecule genes and 11 pregnancy-specific beta-1-glycoprotein genes, so we listed the four most highly expressed genes. The expression of PSA was too low to be detected in most of our samples, so it is not listed in *Table 3*.

The expression of CA125, CYFRA21-1, NSE and SCC increased in tumor samples, and there was statistical significance in the difference between squamous cell lung cancer and normal tissues for these genes. While CEA was produced by the co-expression of multiple genes, only expression from some of these genes produced statistical significance. The expression of β2M and CA15-3 was lower in squamous cell carcinoma relative to normal tissues, and there was statistical significance in these differences. There is no statistical significance in the expression of the other tumor markers tested, including AFP, AFU, CT, FER and HE4 (*Table 3*).

Correlation analyses between the expression of four tumor marker genes and patient clinicopathological characteristics

We calculated the correlation between the expression of four tumor marker genes (CA125, CYFRA21-1, NSE and SCC) and patient clinicopathological characteristics, including age, smoking status, size of primary tumor, condition of regional lymph nodes, differentiation and TNM stage. Statistical significance only existed for differences in NSE across different T stages.

The expression levels of NSE and SCC tended to increase with increasing TNM stage, but changes did not reach statistical significance (*Table 4*). Increasing sample numbers may achieve statistical significance, however. There were no obvious correlations in the expression profiles of CA125, CYFRA21-1, NSE and SCC (*Table 5*).

Discussion

Lung cancer is the leading cancer site in males and accounts for 11% of total female cancer deaths in developing countries (1). Squamous-cell lung cancer is one of the most common subtypes of lung cancer. Most squamous-cell lung cancer occurs in male patients who smoke, and this disease is often diagnosed at an advanced and inoperable stage (4). Although considerable progress has been made in the early diagnosis and treatment of lung cancer, outcomes are typically not satisfactory. Because most squamous-cell lung cancers originate in the main bronchus, they are difficult to detect with imageological examination. The evaluation of serum tumor markers could provide an important supplementary examination method for diagnosis when disease manifestations are not obvious and imageological examination is negative (5,6). Therefore, there is significant clinical significance for the research of serum tumor markers in squamous cell

Table 2 The full names, code genes and functions of common tumor makers

Name	Full name	Gene	Function
β2M	β2-microglobulin	*B2M*	The heavy chain of the major histocompatibility complex (MHC) class I
AFP	α-fetoprotein	*AFP*	The fetal counterpart of serum albumin
AFU	α-L-fucosidase	*FUCA1 and FUCA2*	A enzyme involved in the degradation of fucose-containing glycoproteins and glycolipids
CA125	Carbohydrate antigen 125	*MUC16*	A glycoprotein located in cell membrane
CA15-3	Carbohydrate antigen 15-3	*MUC1*	A glycoprotein located in cell membrane
CEA	Carcinoembryonic antigen	*A group of CEACAs and PSGs*	A glycoprotein functioned as immunoglobulin and cell adhesion molecule
CT	Calcitonin	*CALCA*	A hormone involved in the regulation of calcium and phosphorus
CYFRA21-1	Cytokeratin 19 fragments	*KRT19*	Fragments of cytokeratins 19
FER	Ferritin	*FTL and FTH1*	An intracellular iron storage protein
HE4	Human epididymis protein 4	*WFDC2*	A protein possibly involved in sperm maturation
NSE	Neuron-specific enolase	*ENO2*	An isoenzyme of enolase involved in glycolysis and gluconeogenesis
PSA	Prostate specific antigen	*KLK3*	A protease present in seminal plasma
SCC	Squamous cell carcinoma antigen	*SERPINB3*	A member of the serine protease inhibitors

Table 3 The expression profiles of tumor maker genes in normal samples, paired tumor samples and all tumor samples

Name	Gene	Normal samples	Paired tumor samples	P value	All tumor samples	P value
β2M	*B2M*	3,941±1,259	1,640±604.5	0.015	2,140±1,246	0.004
AFP	*AFP*	0.151±0.178	0.013±0.013	0.172	0.153±0.439	0.987
AFU	*FUCA1*	31.46±6.864	17.92±10.12	0.017	20.42±12.87	0.067
	FUCA2	20.85±2.216	14.54±2.294	0.013	21.57±8.795	0.857
CA125	*MUC16*	0.562±0.434	1.216±0.714	0.088	2.284±3.915	0.008
CA15-3	*MUC1*	475.3±140.7	195.5±334.7	0.200	158.3±194.5	0.001
CEA	*CEACAM1*	2.138±0.936	5.282±3.849	0.175	7.047±8.263	0.001
	CEACAM5	5.942±4.120	5.804±9.735	0.977	52.15±108.9	0.008
	CEACAM6	116.2±36.66	10.91±14.61	0.007	68.40±119.4	0.382
	CEACAM19	5.622±1.708	8.163±5.586	0.251	7.337±5.705	0.511
CT	*CALCA*	0.204±0.058	0.187±0.178	0.848	1.621±7.314	0.670
CYFRA21-1	*KRT19*	353.0±127.4	1,524±1,158	0.122	2,193±1,883	<0.001
FER	*FTL*	6,233±1,493	5,866±4,284	0.868	4,555±2,659	0.175
	FTH1	3,923±1,042	3,979±1,371	0.957	3,498±1,769	0.603
HE4	*WFDC2*	167.4±73.47	136.2±198.2	0.752	282.7±406.1	0.533
NSE	*ENO2*	16.73±1.366	36.84±30.54	0.223	39.96±35.33	<0.001
SCC	*SERPINB3*	0.795±0.844	16.68±15.51	0.076	26.59±47.62	0.001

Table 4 The correlation analyses between the expressions of tumor marker genes and the clinicopathological characteristics

Characteristics	No.	CA125	P value	CYFRA21-1	P value	NSE	P value	SCC	P value
Gene		*MUC16*		*KRT19*		*ENO2*		*SERPINB3*	
Age			0.176		0.210		0.375		0.213
≥60	26	1.624±3.015		1,898±1,734		43.90±33.51		19.18±42.76	
<60	18	3.237±4.775		2,619±2,004		34.27±37.07		37.29±52.04	
Smoking status			0.314		0.520		0.700		0.140
Yes	23	2.852±4.119		2,018±1,756		41.93±29.06		36.65±51.38	
No	21	1.662±3.577		2,385±1,995		37.80±41.01		15.57±40.35	
Primary tumor			0.368		0.274		0.003		0.577
T1	15	1.091±2.662		2,127±1,627		28.36±27.48		15.25±33.28	
T2	19	3.039±4.473		2,206±2,186		42.64±34.62		37.33±60.62	
T3	6	2.958±3.496		2,157±1,487		46.96±33.21		16.97±27.52	
T4	4	2.218±4.438		2,433±1,748		60.23±50.28		32.53±31.55	
Regional lymph nodes			0.897		0.792		0.288		0.196
N0	29	1.940±3.147		1,960±1,952		37.06±30.68		24.55±41.68	
N1	8	1.607±1.717		2,886±1,517		57.54±43.53		20.57±42.28	
N2	7	4.486±6.788		2,367±1,750		31.90±36.42		41.93±68.70	
Differentiation			0.708		0.207		0.765		0.361
Well differentiated	8	2.588±3.340		1,967±1,664		35.97±28.64		20.97±32.79	
Moderately differentiated	18	2.414±4.726		1,992±1,583		34.58±30.47		33.57±60.98	
Poorly differentiated	18	2.019±3.162		2,492±2,186		47.11±40.88		22.11±33.55	
TNM stage			0.389		0.757		0.172		0.082
I	23	2.165±2.975		2,186±2,035		34.79±29.56		21.98±40.26	
II	12	2.337±3.117		2,432±1,578		44.03±40.92		26.47±40.16	
III	9	2.517±6.270		1,892±1,807		47.75±38.54		38.53±67.80	

Table 5 The correlation analyses between CA125, CYFRA21-1, NSE and SCC

Name	Gene	Value	CA125	CYFRA21-1	NSE	SCC
CA125	*MUC16*	R	1	–0.212	–0.005	0.182
		P		0.167	0.976	0.236
CYFRA21-1	*KRT19*	R	–0.212	1	–0.050	0.134
		P	0.167		0.748	0.386
NSE	*ENO2*	R	–0.005	–0.050	1	–0.110
		P	0.976	0.748		0.475
SCC	*SERPINB3*	R	0.182	0.134	–0.110	1
		P	0.236	0.386	0.475	

lung cancer. An elevated level of serum tumor markers is typically caused by the level of these tumor markers in tumor tissues. In this study we performed RNA-Seq on five matched pairs of normal and squamous-cell lung cancer tissues and another 39 squamous-cell lung cancer tissues. Thirteen commonly used tumor marker genes were tested to screen for appropriate tumor markers. Finally, the genes encoding CA125, CYFRA21-1, NSE and SCC were shown to be expressed at higher levels in tumor than in normal tissues. Therefore, they might be suitable as tumor markers for screen squamous-cell lung cancer.

SCC has been implicated in tumor growth, and it also inhibits the apoptosis of tumor cells (7,8). As a commonly used tumor marker, SCC is valuable for the detection of many types of squamous cell carcinoma, including esophageal, head and neck and lung squamous cell carcinoma (9,10). SCC resides in the cytosol of squamous cells and is released into the circulation during squamous cell carcinoma (11). NSE is widely used in the screening in small cell lung cancer (12-14), but its function in squamous cell lung cancer has not been clearly studied. NSE as an enzyme active in glycolysis, and the rate of glycolysis is extremely elevated in tumor proliferation, a phenomenon called the "Warburg effect" (15). We also detected that the level of NSE was significantly higher in tumors than in normal tissues. CYFRA 21-1 is the serum dissolution fragment of cytokeratin 19, which is expressed exclusively in epithelial cells and tumors of epithelial origin (16). Previous studies have suggested that CK 19 played a part in the aggressive behavior of tumor cells and was connected with the differentiation and invasion of tumor cells (17). Increased serum CYFRA 21-1 is the result not only of cytokeratin release as a consequence of cell lysis or necrosis, but also of the degradation of cytokeratin filaments by activated proteases in tumor cells (18,19). The high level of CYFRA21-1 in patients with squamous cell lung cancer makes this protein the most sensitive of all of the currently studied tumor markers (20). CA125 is also found at a high level in ovarian carcinomas and lung cancer (21,22). Although previous studies have suggested that tumor markers such as CT, Ferritin and HE4 were present at high levels in lung cancer (23-25), they were not found to be elevated in the tumor tissues from our study.

Most previous studies have focused on the detection of tumor markers in the serum. However, few studies have investigated tumor markers in tissue and, in particular, the expression of their encoding genes. To address this shortcoming, we measured the expression of tumor markers in tissues in our study. In the 13 tumor markers we tested, CA125, CYFRA21-1, NSE and SCC have been commonly used in clinical practice for diagnosis or prognosis of NSCLC (21,26-28). These tumor markers also express at a higher level in tumor samples than in normal samples in our study. Our study also supports their use in clinical practice. Several studies have demonstrated the use of these tumor markers in TNM staging (29-31). We also evaluated any correlations between the expression of the encoding genes of these tumor markers and patient clinicopathological characteristics, but we identified no statistically significant differences. We evaluated whether serum levels of these tumor markers would increase with increasing tumor volume, this being the reason why serum levels for these tumor markers can reflect TNM stages. The outcome of RNA-seq is an expression of unit volume, however, so it may not change with tumor growth.

Squamous cell lung cancer and adenocarcinoma are the two most common histologic subtypes of non-small cell lung cancer. However, these two subtypes are quite different in host susceptibility, clonal evolution, molecular evolution and molecular profiling (3). Previous studies have suggested that the elevated serum SCC percentage is highest in squamous-cell lung cancer, while this percentage is substantially lower in other types (32). NSE expression is higher in squamous-cell lung cancer with neuroendocrine differentiation. The serum CYFRA 21-1 level has been shown to be particularly elevated in squamous cell cancers (28). CA125 has been shown to be substantially expressed in large cell carcinoma and adenocarcinoma, but this protein could not be detected in a squamous lung cell line (33). Therefore, it is not clear whether or not our study can be directly applied to other types of lung cancer, such as adenocarcinoma or large cell lung cancer.

RNA-Seq has emerged as a popular high-throughput technology in recent years. In this technique, transcript levels are quantified in RPKM, which reflects the molar concentration of a transcript normalized by the total read number of the measurement. This normalization avoids common experimental deviations and also facilitates comparisons between multiple genes and samples. As such, RNA-Seq is an ideal method for global gene expression analysis.

Conclusions

Encoding-gene expression for CA125, CYFRA21-1, NSE and SCC was elevating in tumor tissues of squamous-cell lung cancer, while β2M and CA15-3 were expressed

at a lower level in squamous cell lung cancer tissues. PSA could not be detected in most samples, and there was no significant difference between tumor and normal samples for the others tumor markers evaluated in this study, AFP, AFU, CEA, CT, FER and HE4.

Acknowledgements

Funding: This work was supported by Doctoral Program Foundation of Institutions of Higher Education of China (No. 20110071120066).

Authors' contribution: Qun Wang supervised this study, designed experiments and edited the paper with Yu Shi. Lin Wang, Cheng Zhan and Yongxing Zhang designed and carried out experiments, collected and analyzed data and co-wrote the paper. Jun Ma collected tissues and clinical characteristics of the patients with Junjie Xi and Wei Jiang, they also performed experiments with Yu Shi.

Disclosure: The authors declare no conflict of interest.

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Cite this article as: Wang L, Zhan C, Zhang Y, Ma J, Xi J, Jiang W, Shi Y, Wang Q. Quantifying the expression of tumor marker genes in lung squamous cell cancer with RNA sequencing. J Thorac Dis 2014;6(10):1380-1387. doi: 10.3978/j.issn.2072-1439.2014.08.27

