

RESEARCH ARTICLE

Expression of TS, RRM1, ERCC1, TUBB3 and STMN1 Genes in Tissues of Non-small Cell Lung Cancer and its Significance in Guiding Postoperative Adjuvant Chemotherapy

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Abstract

Background: To explore the expression of TS, RRM, ERCC1, TUBB3 and STMN1 genes in the tissues of patients with non-small cell lung cancer (NSCLC) and its significance in guiding the postoperative adjuvant chemotherapy. **Materials and Methods:** Real time polymerase chain reaction (PCR) was applied to detect the expression of TS, RRM, ERCC1, TUBB3 and STMN1 genes in the tissues of NSCLC patients so as to analyze the relationship between the expression of each gene and the clinical characteristics and to guide the postoperative individualized chemotherapy according to the detection results of NSCLC patients. **Results:** Expression of TS gene was evidently higher in patients with adenocarcinoma than those with non-adenocarcinoma ($P=0.013$) and so was the expression of ERCC1 ($P=0.003$). The expression of TUBB3 gene was obviously higher in NSCLC patients in phases I/II and IV than those in phase III ($P_1=0.021$; $P_2=0.004$), and it was also markedly higher in patients without lymph node metastasis than those with ($P=0.008$). The expression of STMN1 gene was apparently higher in patients in phase I/II than those in phase IV ($P=0.002$). There was no significant difference between the rest gene expression and the clinical characteristics of NSCLC patients ($P>0.05$). Additionally, the disease-free survival (DFS) was significantly longer in patients receiving gene detections than those without ($P=0.021$). **Conclusions:** The selection of chemotherapeutic protocols based singly on patients' clinical characteristics has certain blindness. However, the detection of tumor-susceptible genes can guide the postoperative adjuvant chemotherapy and prolong the DFS of NSCLC patients.

Keywords: Non-small cell lung cancer - real time polymerase chain reaction - target gene - disease-free survival

Asian Pac J Cancer Prev, 16 (8), 3189-3194

Introduction

It has been reported in many literatures that the expression of TS, RRM1, ERCC1, TUBB3 and STMN1 genes is closely associated with the sensitivity of patients with non-small cell lung cancer (NSCLC) to fluorouracil (Fu), gemcitabine, platinum, paclitaxel and navelbine, respectively (Chen et al., 2011; Fang et al., 2014; Gao et al., 2014; Li et al., 2014; Nie et al., 2015). This study applied real time polymerase chain reaction (RT-PCR) to detect the expression of TS, RRM1, ERCC1, TUBB3 and STMN1 genes in the tumor tissues surgically excised from NSCLC patients so as to analyze the expression of above genes in the tissues of patients with lung adenocarcinoma and non-adenocarcinoma and to explore its relationship with the clinical efficacy of postoperative adjuvant chemotherapy, hoping to provide guidance for the postoperative adjuvant chemotherapy and improve the postoperative disease-free survival (DFS) of NSCLC patients.

Materials and Methods

Cases and samples

Samples from 126 patients with lung cancers who were admitted in Jinan Military General Hospital from 2011 to 2013 were selected. Inclusion criteria: *i*) All samples were obtained from surgically excised or biopsy cancerous tissues that pathologically diagnosed as lung cancers, and the pathological tissue typing referred to World Health Organization (WHO) histological classification standards (2004th version); *ii*) The tumor stages referred to the Union for International Cancer Control (UICC) criteria for TNM stage of lung cancer (7th version) established in 2009; *iii*) All samples received no radiochemotherapy or target therapy before the inclusion. Of the included objects, there were 83 males and 43 females; 60 had smoking history and 66 did not; 71 were with adenocarcinoma, 45 with squamous carcinoma, 3 with squamous adenocarcinoma, 1 with large cell carcinoma, 2 with malignant fibrous histiocytoma, 1 with atypical carcinoid, 2 with sarcomatoid carcinoma and 1 with primitive neuroectodermal tumor (PENT); 89

received complete surgical excision, 10 had local excision and 27 were in advanced stage; 67 were in TNM stage I/II, 53 in III/IV and 6 were unknown; and 123 were with TS gene detection, 126 with RRM1 gene detection, 126 with ERCC1 gene detection, 126 with TUBB3 gene detection and 114 with STMN gene detection.

To further explore the guiding significance of the individualized target gene detection on the postoperative adjuvant chemotherapy of NSCLC patients, a total of 65 NSCLC patients who were given postoperative adjuvant chemotherapy according to the target detection after surgery from April 24th, 2011 to March 4th, 2013 served as experiment group while another 65 treated with postoperative chemotherapy according to the pathological examination results after surgery in the corresponding period as control group. There was no significant difference in ages, genders, smoking history, pathological patterns and TNM stages between two groups ($P>0.05$). The detailed data were shown in Table 1.

Therapeutic methods

Establishment of chemotherapeutic protocols: *i*) The chemotherapeutic protocols of experiment group were established based primarily on the gene detection results, for which the chemotherapeutic agents corresponding to the low-expression genes were selected, whereas those for control group were made according to the patients' pathological patterns and the clinical physicians' experiments; *ii*) Agents and their dosages: docetaxel 75 mg/m², d1 plus nedaplatin 80~100 mg/m², d2; gemcitabine 1 250 mg/m², d1 and 8 plus nedaplatin 80~100 mg/m², d2; and pemetrexed disodium 500 mg/m², d1 plus nedaplatin 80~100 mg/m², d2; *iii*) All patients received postoperative adjuvant chemotherapy within 1 month, 1 cycle/3 weeks, for totally >2 cycles, with the median chemotherapeutic cycle being 3.69.

RNA extraction and cDNA establishment

RNA extraction: A total of 126 fresh tissue samples

Table 1. Relationship of Clinical Characteristics Between Two Groups

Programs	Experiment group (n=65)	Control group (n=65)	P value
Ages (years)			
≥60	30	29	0.860
<60	35	36	
Genders			
Male	45	39	0.271
Female	20	26	
Smoking history			
Yes	36	40	0.477
No	29	25	
Pathological patterns			
Adenocarcinoma	31	30	0.860
Non-adenocarcinoma	34	35	
TNM stages			
I	28	30	0.939
II	20	19	
III	17	16	

were collected in this study, which were immediately put into sample solution containing RNase inhibitor for storage. RNAiso Plus (TaKaRa, Dalian, China) was applied for the samples and the extraction was strictly conducted according to the instructions (Sun et al., 2014). The extracted RNA concentration was calculated using spectrophotometer, which was considered to be available if the concentration was >100 ng/μL and OD260/OD280 was 1.8~2.2, otherwise it should be re-extracted. The selected RNA could be applied for the sequential real time-Polymerase Chain Reaction (RT-PCR).

cDNA establishment: 1 000 ng RNA was collected and reversely translated by Oligo (DT) 18 and Random Primer (6mer) (Sangon Biotech Co., Ltd, Shanghai) and M-MLV Reverse Transcriptase (promega, USA) to synthesize cDNA single strand, which was performed according to the instructions of M-MLV Reverse Transcriptase system.

Design of primers

Primer 5 software was applied to design the primers of each gene, and the detailed sequences were shown in Table 2. Note: In each gene, the sense corresponded to the upstream primer (Forward primer) and antisense to downstream primer (Reverse primer).

Extraction of samples by CT-PCR

Extraction methods: After cDNA was synthesized, RT-PCR was used to detect the expression levels of each target gene-corresponded reference gene. The average CT value of each gene was collected to calculate the expression differences among the sample target genes by $2^{-\Delta\Delta Ct}$ method (comparison method of CT values).

ΔCt (calibrated sample) 1=Target gene CT value 1 - Reference gene CT value 1

ΔCt (samples to be determined) 2=Target gene CT value 2 - Reference gene CT value 2

$\Delta\Delta Ct = \Delta Ct$ (samples to be determined) 2 - ΔCt (calibrated sample) 1

Expression difference was $2^{-\Delta\Delta Ct}$.

Results statistics: The database was from Shanghai

Table 2. Primer Sequences of Each Gene

Genes	Primer sequences
GAPDH	
Sense	5'-GCCACATCGCTCAGACACC-3'
Antisense	5'-GATGGCAACAATATCCACTTTACC-3'
ERCC1	
Sense	5'-GGCTATCCTCTCAGAGTGACATTTTA-3'
Antisense	5'-GCGGAGGCTGAGGAACAG-3'
RRM1	
Sense	5'-GCGGAGGCTGAGGAACAG-3'
Antisense	5'-AGCCGCTGGTCTTGTCTTA-3'
STMN1	
Sense	5'-AAGGATCTTCCCTGGAGGA-3'
Antisense	5'-TGTGCCTCTCGGTTCTCTTT-3'
TS	
Sense	5'-CGCCCTGCTCACGTACATG-3'
Antisense	5'-GTGTGTATAAAGTCACCTGGCTTCAG-3'
TUBB3	
Sense	5'-AGTCGCCCACGTAGTTGC-3'
Antisense	5'-CGCCAGTATGAGGGGAGAT-3'

Biotecan Medical Diagnostics Co., Ltd. According to the normal distribution of each target gene, low expression: gene expression<25%; middle expression: gene expression was 25%~75%; and high expression: gene expression>75% (Pentheroudakis et al., 2011).

Follow up

Phone call and outpatient or hospitalized re-examination was the primary form of follow up. The

follow up was ended on December 31th, 2013. The follow-up period was 6~32 months, with the median one being 18 months. All patients had complete clinical follow-up data. The disease-free survival (DFS) was defined as the period from the day of giving surgery to disease recurrence conformed objectively or the terminal follow-up day.

Statistical data analysis

SPSS19.0 software was applied for all data analysis. The relationship between clinical characteristics and the expression of target genes were detected with χ^2 test and Fisher exact probability method while comparison of average value between groups and survival with one-Way ANOVA and life table method, respectively. $P<0.05$ was considered to be statistically significant.

Results

Amplification curve of each gene compared to reference genes

Figure 1 was showing the amplification curves of each gene compared to the reference genes.

Relationship between the expression level of each gene with patients' clinical characteristics

A total of 126 samples from NSCLC patients were included in this study. When the relationship between the expression of each gene and the patients' clinical characteristics was detected, some clinical characteristics of partial patients could not be diagnosed due to the retrospective study, so the data of these patients were excluded from the statistics on the consideration of the varacity of the experimental results. Therefore, the total numbers of each item after the terminal statistics were

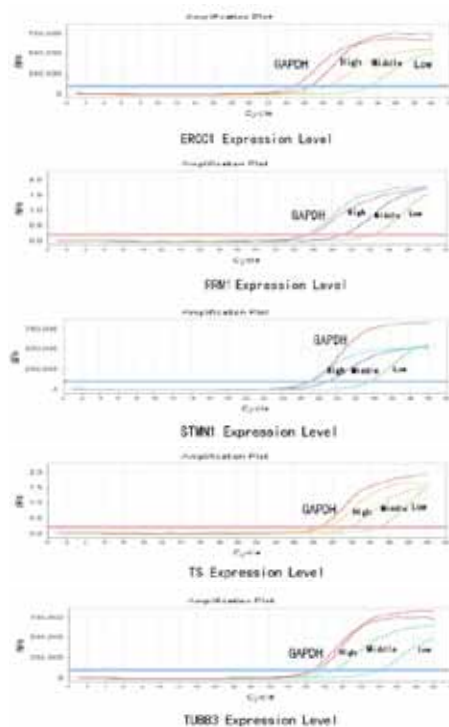


Figure 1. Amplification Curve of Each Gene Compared to Reference Genes

Table 3A. Relationships between Expression of TS, RRM1, ERCC1, TUBB3 and STMN1 with Clinical Characteristics of NSCLC Patients [n(%)]

Clinical characteristics		TS			RRM1		
		High	Middle	Low	High	Middle	Low
Gender	Male	31(37.8)	29(35.4)	22(26.8)	21(25.3)	32(38.6)	30(36.1)
	Female	22(53.7)	12(29.3)	7(17.1)	17(39.5)	19(44.2)	7(16.3)
P value		0.223			0.052		
Age (years)	≥60	28(50.0)	14(25.0)	14(25.0)	19(33.3)	23(40.4)	15(26.3)
	<60	25(37.3)	27(40.3)	15(22.4)	19(27.5)	28(40.6)	22(31.9)
P value		0.185			0.713		
Smoking history	Yes	20(33.9)	23(39.0)	16(27.1)	14(23.7)	25(42.4)	20(33.9)
	No	33(51.6)	18(28.1)	13(20.3)	24(35.8)	26(38.8)	17(25.4)
P value		0.141			0.302		
Pathological patterns	Adenocarcinoma	36(52.9)	20(29.4)	12(17.6)	25(35.2)	30(42.3)	16(22.5)
	Non-adenocarcinoma	17(30.9)	21(38.2)	17(30.9)	13(23.6)	21(38.2)	21(38.2)
P value		0.041			0.13		
Differentiation degrees	High/Middle	30(42.9)	23(32.9)	17(24.3)	24(33.3)	28(38.9)	20(27.8)
	Low	8(36.4)	8(36.4)	6(27.3)	6(25.0)	8(33.3)	10(41.7)
P value		0.864			0.436		
TNM stages	I/II	27(39.7)	25(36.8)	16(23.5)	24(34.8)	26(37.7)	19(27.5)
	III	5(22.7)	9(40.9)	8(36.4)	5(20.0)	10(40.0)	10(40.0)
	IV	17(60.7)	6(21.4)	5(17.9)	8(27.6)	14(48.3)	7(24.1)
P value		0.093			0.51		
Lymph node metastasis	Yes	13(37.1)	12(34.3)	10(28.6)	11(29.7)	15(40.5)	11(29.7)
	No	20(35.7)	22(39.1)	12(21.2)	10(17.5)	14(25.0)	10(17.5)

Table 3B. Relationships between Expression of TS, RRM1, ERCC1, TUBB3 and STMN1 with Clinical Characteristics of NSCLC Patients [n(%)]

Clinical characteristics	ERCC1			TUBB3			STMN1		
	High	Middle	Low	High	Middle	Low	High	Middle	Low
Gender									
Male	13(15.7)	25(30.1)	45(54.2)	18(21.7)	15(18.1)	50(60.2)	25(32.5)	35(45.5)	17(22.1)
Female	11(25.6)	18(41.9)	14(32.6)	7(16.3)	13(30.2)	23(53.5)	13(35.1)	14(37.8)	10(27.0)
P value		0.066			0.283			0.723	
Age (years)									
≥60	10(17.5)	19(33.3)	28(49.1)	15(26.3)	14(24.6)	28(49.1)	19(35.8)	19(35.8)	15(28.3)
<60	14(20.3)	24(34.8)	31(44.9)	10(14.5)	14(20.3)	45(65.2)	19(31.1)	30(49.2)	12(19.7)
P value		0.878			0.146			0.324	
Smoking history									
Yes	6(10.0)	22(36.7)	32(53.3)	9(15.0)	14(23.3)	37(61.7)	21(36.8)	25(43.9)	11(19.3)
No	18(27.3)	21(31.8)	27(40.9)	16(24.2)	14(21.2)	36(54.5)	17(29.8)	24(42.1)	16(28.1)
P value		0.046			0.429			0.505	
Pathological patterns									
Adenocarcinoma	20(27.8)	25(34.7)	27(37.5)	15(21.1)	19(26.8)	37(52.1)	22(34.9)	26(41.3)	15(23.8)
Non-adenocarcinoma	4(7.40)	18(33.3)	32(59.3)	10(18.2)	9(16.4)	36(65.5)	16(31.4)	23(45.1)	12(23.5)
P value		0.007			0.273			0.903	
Differentiation degrees									
High/Middle	14(19.4)	31(43.1)	27(37.5)	13(18.1)	16(22.2)	43(59.7)	22(34.4)	27(42.2)	15(23.4)
Low	7(29.2)	4(16.7)	13(54.2)	5(20.8)	6(25.0)	13(54.2)	9(40.9)	9(40.9)	4(18.2)
P value		0.067			0.891			0.817	
TNM stages									
I/II	15(21.7)	24(34.8)	30(43.5)	18(26.1)	12(17.4)	39(56.5)	27(42.2)	26(40.6)	11(17.2)
III	3(12.0)	11(44.0)	11(44.0)	1(4.00)	4(16.0)	20(80.0)	7(31.8)	10(45.5)	5(22.7)
IV	6(20.7)	6(20.7)	17(58.6)	6(20.7)	11(37.9)	12(41.4)	3(12.0)	11(44.0)	11(44.0)
P value		0.371			0.015			0.034	
Lymph node metastasis									
Yes	4(10.8)	17(45.9)	16(43.2)	3(8.10)	5(13.5)	29(78.4)	11(36.7)	13(43.3)	6(20.0)
No	14(24.6)	18(31.6)	25(43.9)	16(28.1)	11(19.3)	30(52.6)	23(41.1)	23(41.1)	10(17.9)
P value		0.177			0.027			0.919	

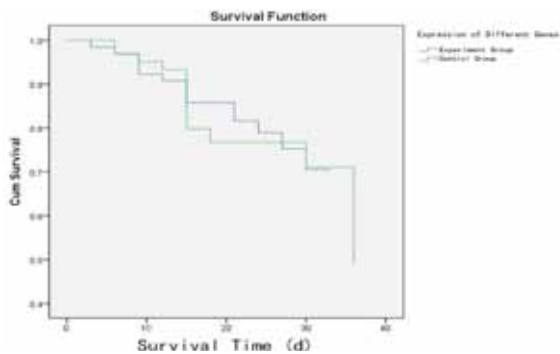


Figure 2. Comparison of DFS between Two Groups

not completely the same. The relationship between the expression of each target gene and clinical characteristics of NSCLC patients were shown in Table 3A,3B.

Comparison of DFS between two groups

The follow-up results demonstrated that DFS in experiment group and control group were (20.78±7.60) months and (17.25±9.57) months, respectively, and there was significant difference according to the one-Way ANOVA method, indicating that the postoperative adjuvant chemotherapy based on target gene detection results could significantly prolong the DFS of patients with NSCLC. However, the long-term clinical efficacy needed to be further studied because of the short-term follow up in this study.

Discussion

NSCLC accounts for >80% of patients with lung cancers, whose common therapeutic model is the comprehensive therapies (Ji et al., 2014; Cui et al., 2014). However, studies found that no matter how radical the surgery was, the postoperative 5-year survival rate was still unsatisfactory (31.8%~42.4%), and the primary failure reasons were local recurrence and distant metastasis (Winton et al., 2005; Pignon et al., 2008; Strauss et al., 2008). Meanwhile, other researches proved that postoperative adjuvant chemotherapy containing platinum could provide great benefits to the survival time of NSCLC patients (about 5%), but the outcomes were still unsatisfactory, which was predicated to be associated with the randomness and blindness of chemotherapeutic agent selection (Arriagada et al., 2004; Kato et al., 2004; Hamada et al., 2005; Douillard et al., 2006).

In recent years, research on the genes sensitive to chemotherapeutic agents for NSCLC patients has become a hot topic in clinic, which can accurately predicate the sensitivity of specific agents for special patients via detecting the expression condition of relevant genes, thus providing precise theoretical guidance for individualized chemotherapy. Studies demonstrated that ERCC1 took part in the repair of tumor cell DNA induced by cisplatin chemotherapeutic agents, whose mRNA expression level was in negative association with the clinical efficacy and

survival time of cancer patients undergoing multiple cisplatin chemotherapies, which meant that patients with low-expression ERCC1 was more sensitive to cisplatin (Seyhan et al., 2011; Tseden-Ish et al., 2012). TA gene-coded TA is a limiting velocity enzyme synthesized by pyrimidine nucleotide, an important factor for tumor growth, and a target enzyme for the cytotoxicity of pemetrexed. In addition, TS mRNA is negatively connected with the clinical efficacy of pemetrexed (Zhao et al., 2014). TUBB3-coded β -Tubulin-III (type III β microtubulin) is an important part of cytoskeleton, a basic constructive unit of spindle during mitosis period, and a primary functional target of anti-microtubule agents, and tumors are sensitive to anti-microtubule chemotherapeutic agents when TUBB3 mRNA expression is low enough (Gan et al., 2007; Wu et al., 2014). STMN1-coded STMN1 protein has certain impact on the formation of spindle during mitosis period through improving the depolymerization of microtubule or inhibiting the synthesis of microtubule, so suppressing the expression of STMN1 protein can interfere the mitosis of malignant tumor cells so as to influence the proliferation and apoptosis of tumor cells. Moreover, cancer patients with low expression of STMN1 mRNA will have favorable clinical efficacy and longer median survival time after being treated with vinorelbine/cis-platinum, and the higher the expression of STMN1, the lower the survival rate and the higher the metastatic risk in cancer patients (Rana et al., 2008; Jeon et al., 2010). RRM1 gene-coded ribonucleotide reductase (RNR) M1 subunit, as the inhibitor gene of tumors, can inhibit the metastasis of tumors. It is also the primary functional target of gemcitabine and expresses widely in almost all tumors, which has significant differences in its expression among the tumors. Lung cancer patients with low-expression level of RRM1 mRNA have relevantly higher sensitivity, better clinical efficacy and longer median survival time after being treated with gemcitabine (Su et al., 2011; Han et al., 2013).

As to the relationship between the expression of each target gene and the clinical characteristics of NSCLC patients, there is great difference among reports at home and broad, which is predicated to be associated with the population distribution or sample size. As to TS gene expression, the research results of Miyoshi et al indicated that there was no connection between the expression level of TS gene and the patients' clinical characteristics (Miyoshi et al., 2007). In this study, it was suggested that the high-expression rate of TS gene was evidently higher in adenocarcinoma tissue than that in non-adenocarcinoma tissues; that the expression level of TS gene was in negative correlation with the sensitivity of patients to some chemotherapeutic agents (5-Fu and pemetrexed, etc.) and the higher the expression level of TS gene, the lower the sensitivity of patients to the chemotherapeutic agents. At present, the application of pemetrexed is clinically recommended to patients with adenocarcinoma. However, data analysis showed that of the patients with low expression of TS gene, patients with squamous carcinoma accounted for 52.17%, and of those with high expression of TS gene, patients with adenocarcinoma accounted for 57.58%, illustrating that if agents like pemetrexed was

selected only by the pathological patterns of the tumors, some patients might loss the opportunity of being treated with the optimal chemotherapeutic agents, and meanwhile, another parts of patients might not received corresponding clinical efficacy after being treated with the plausible agents, thus leading to the delayed treatment of disease and the increase of pain and burdens. The low-expression rate of ERCC1 gene was markedly higher in patients without adenocarcinoma and smoking history than those with, and it was also similar to the point mentioned above that the expression of ERCC1 gene was negatively associated with the sensitivity of patients to cisplatin. In addition, data analysis revealed that the high-expression rates of ERCC1 gene in adenocarcinoma and non-adenocarcinoma tissues accounted for 27.80% and 7.40%, respectively. Though the rates were low, it prompted that some patients were less sensitive to cisplatin, and postoperative adjuvant chemotherapy containing cisplatin was not advisable to all NSCLC patients.

Tumor individuated target detection techniques could diagnose the sensitivity of patients to different chemotherapeutic agents by detecting the expression levels of TS, RRM1, ERCC1, TUBB3 and STMN1 genes, so as to establish the optimal chemotherapeutic protocols which were of great significance in further promoting the postoperative chemotherapeutic efficacy and prolonging the survival time of NSCLC patients (Wu et al., 2014). This study also proved that the DFS of patients receiving postoperative adjuvant chemotherapy under the guidance of target detection was superior to control group.

To sum up, there were significant differences among different individuals in gene expression, demonstrating that the chemotherapeutic protocols established only based on the patients' clinical characteristics and the physicians' clinical practices had great blindness, which might trigger chemotherapeutic adverse responses to patients without bringing about satisfactory clinical efficacy. Chemotherapeutic agent sensitivity gene detection for tumors can be used as a reliable basis to guide the chemotherapy of NSCLC patients, which can avoid the blindness of established chemotherapeutic protocols, thus achieving the aims of individualized chemotherapy. In the future experiment, the DFS of each patient will be followed up to further evaluate the long-term benefits of postoperative adjuvant chemotherapy under the guidance of target gene detection to NSCLC patients.

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