Experimental Verification of Gene Expression Related to Lung Cancer in Nasal Epitthelia

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Abstract. Genes expression related to lung cancer are observed in nasal epitthelia, to identify their similarities and differences and provide the basis for possible application. There are three groups:non-lung cancer group (NLC), lung cancer group (LC) and postoperative lung cancer group (PLC).The genes expression in nasal epitthelia were observed by PCR, including the HCK, NCF1, TLR8, EMR3,CSF2RB,DYSF, SPEF2, ANKFN1, HYDIN, DNAH5, C12orf55 and CCDC113. Their expression levels were obtained and statistically compared. Results showed that all the related genes in LC and PLC groups were highly expressed. There are significant difference in HCK, NCF1, TLR8, EMR3, CSF2RB and C12orf55 gene expression between the LC or PLC and NLC, and in EMR3 and C12orf55 between LC and PLC. Conclusions are HCK, NCF1, TLR8, EMR3, CSF2RB, C12orf55 can be used for lung cancer screening, while EMR3 and C12orf55 for the review of post-operative lung cancer.

1 Introduction

Early diagnosis of lung cancer has important clinical significance. Previous studies have found that genes expressions in nasal epitthelia have a certain correlation with lung cancer, that may used to the basis of finding a non-invasive, simple and inexpensive technology to screen early lung cancer, it is with a good prospect of application [1]. In this study, the genes expressions related to lung cancer were observed by PCR technology to verify them.

2 Experimental materials and methods

2.1 Experimental principle

Fluorescence quantitative polymerase chain reaction (PCR) technique is used to track the PCR products by fluorescent dyes or fluorescent labeled probes and monitor the reaction process in real time. With the development of PCR reaction, the reaction product accumulates and the fluorescence signal intensity increases. After each cycle, the fluorescence intensity signal is collected once, so the quantity of the product can be monitored by the change of the fluorescence intensity. Combining with the corresponding software analysis, the fluorescence amplification curve can be obtained and the quantity of the initial template of the sample to be tested can be calculated. When the internal reference gene was introduced into the fluorescence quantitative PCR detection system and the target gene was detected simultaneously, the results were based on 2-ΔΔCt. The expression of the target nucleic acid sequence in a tested sample and the same sequence in the corrected sample can be calculated by the method.

2.2 Main reagents and instruments

2.2.1 Reagent

1. Primer Synthesis: bioengineering (Shanghai) Co., Ltd.;
2. Taq HS enzyme: TAKARA Bioengineering Company;
3. PCR buffer: TAKARA Bioengineering;
4. dBA: TAKARA Bioengineering Co., Ltd.;
5. SYBR Green I: Xiamen Zhishan Biotechnology Co., Ltd.
6. Anhydrous ethanol: Xilong Science Co., Ltd.
7. RNA extraction kit: Kangwei Century Biotechnology Co., Ltd.
8. Reverse transcription kit: first Strand cDNA Synthesis Kit Thermo Scientific

2.2.2 Instrument

1. Thermo Scientific NanoDrop 2000 (Thermo Fish(;
2. BC-subMIDI Electroswimometer (Beijing 61 instrument Factory);
3. Gel imaging system Kodak Gel Logic 200m, H1650-W desktop high speed centrifuge (Hunan Xiangyi centrifuge instrument Co., Ltd.); Hand type centrifuge (Shanghai biotech; Vortex Mixer (Shanghai);
4. Pipette (Eppendorf company), Super clean workbench (Suzhou purification equipment Co., Ltd.);

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Boji 96 hole PCR instrument (Hangzhou Boji Science and Technology Co., Ltd.);

5. Fluorescence quantitative PCR instrument: Bio-Rad CFX384.

2.3 Experimental methods

The SYBR GREEN I method was used to quantify the expression of 12 related genes in 80 cases of nasal swabs, including three groups: 30 cases in normal contrast group, 32 in lung cancer group and 18 in a postoperative group of lung cancer. Fluorescent quantitative PCR technology is used to mark and track the PCR products with fluorescent dyes or fluorescently labeled probes, and monitor the reaction process in real time. With the progress of PCR reaction, the reaction products continue to accumulate, and the intensity of fluorescence signal also increases. After a cycle, a fluorescence intensity signal is collected. In this way, the changes in the amount of the product can be monitored through the change of fluorescence intensity.

The fluorescence amplification curve can be obtained by the corresponding software analysis. The amount of the initial template for the sample to be measured can be calculated. When the internal reference gene was introduced and the target gene was detected in the fluorescence quantitative PCR detection system, the relative change of the same sequence in the same sequence was calculated by the 2- Delta Delta Ct method. The experimental data were used for variance analysis. Welch correction and Tamhane's T2 test were used for statistical analysis and comparison. The expression of genes in nasal mucosa of lung cancer was determined by experimental results.

2.4 Extraction of total RNA

2.4.1 Sample processing

The part of the swab with cotton swabs was broken in the 1.5ml centrifuge tube, adding 1 ml Buffer RLT. the cells on the swabs could fall off in liquid as much as possible. The centrifuge tube was placed at -20 ℃ for about 1 hour. 200L chloroform was added, 15s was violently concussion, and 2 min was placed at room temperature.

2.4.2 The samples were divided into three layer

the red organic phase, the intermediate layer and the upper colorless water phase mainly in the upper water phase, and the upper water phase was transferred to a new centrifuge tube. The same volume of 70% ethanol was added to the aqueous solution, and the mixture was reversed. The solution obtained from the previous step was added to the adsorption column Spin Column RM which had been packed into the collection tube for 30 s, and the waste liquid from the collection tube was poured out, and the adsorption column was put back into the collection tube. Adding 700 μ l Buffer RW1H 12000 rpm to the adsorption column for 30 s, the waste liquid from the collecting tube was poured out, and the adsorption column was put back into the collection tube.

Adding 500 μ l Buffer RW2 + 12000 rpm to the adsorption column for 30 s, the waste liquid from the collecting tube was poured out, and the adsorption column was put back into the collection tube. Repeat previous step;12000 rpm was centrifuged for 2 mins and the waste liquid was poured out. The adsorption column is placed at room temperature for several minutes to ensure that the residual rinsing solution is thoroughly dried. The adsorption column was put into a new RNase-free centrifuge tube, 35 μ l RNase-free aseptic water was added to the center of the adsorbed column, and 1 mint 12000 rpm centrifuge was placed at room temperature to collect the RNA solution. RNA sample quality detection and control: the concentration and purity of RNA were measured by Thermo Scientific NanoDrop 2000, and the samples of RNA were preserved at -20 ℃.

2.5 Primer design

In the Genebank database (http:// www. ncbi. nlm. nih. gov/ genbank), According to the relevant information of the 12 target genes, such as login number and Gene ID, the corresponding mRNA sequences can be downloaded. Blast.Determine the corresponding conserved section of each locus,Design the corresponding primer and use the UCSC website)http://genome.ucsc.edu/(, the specificity of primer was determined.The primer design table is as follows (Table):

Table 1. Primer design

Oligo name	Direction	Oligo Sequence	Product Size
HCK	F	TCTGCATCCCTGGTGTGTAA	194bp
	R	CCGTAGGTGACGATCTCCAT	
NCF1	F	GCCTACACTGCTGTGGAG	109bp
	R	TGACGTCGTCTTTCCTGATG	
TLR8	F	TGTGATGGTGGTGCTTCAAT	187bp
	R	ATGCCCCAGAGGCTATTTCT	
EMR3	F	CCAGTCTGTGCCATTTTCTCT	124bp
	R	AAGCCAGCATCCTTGTGTTC	
CSF2RB	F	GCGCTGCTACAACGACTACA	144bp
	R	ATGTCATCACTGAGGTCACAGG	
DYSF	F	TTGAGCTTCGGTGTGAACAG	158bp
	R	CTCTGGTGCAGGAAGGAGAC	
SPEF2	F	CCGTGAGTCCCAAGTCATTT	156bp
	R	GTGTTGGCTCCAAGCGAGA	
ANKFN1	F	GGGACTCTACATAGCCGTTA	141bp
	R	CAAGACAGCTTCGTGAACCA	
HYDIN	F	CCCAGGGATTTGCTGCTAT	136bp
	R	GGTACAACACACCTTGCTG	
DNAH5	F	GCTCTACAAACTGTGTGAGG	137bp
	R	TGACAATCGTGGACTCCGTA	
C12orf55	F	TGCTCCATTTACTTCAGAGGTT	131bp
	R	CGTCAGCTGGTATCGCTTC	
CCDC113	F	ATACATTGAGGACATGAACCG	170bp
	R	CGTTCTCTATCTTCAACTGCTG	
β-actin	F	TGGCACCCAGCACAATGAA	186bp
	R	CTAAGTCATAGTCCGCCTAGAAGCA	

2.6 PCR reaction system and its parameters

SYBR GREEN I- fluorescence quantitative PCR reaction system is as follows, the reaction parameters such as below. In Bio-Rad CFX384, using Melt Protocol program. The reaction product SYBR GREEN I real-time RT-PCR, from 60 degrees slowly and evenly heated to 95 DEG C,

change curves of the fluorescence signal and the derivative mapping analysis of the data.

2.7 Calculation of relative gene expression

Three parallel experiments were carried out for each sample. The Ct data in the reaction were collected by setting the corrected threshold. The β-actin gene was used as the internal reference gene by real-time fluorescence quantitative PCR, and the relative quantification was performed by 2-ΔΔCt method.

3 Experimental results and analysis

3.1 Genes expression related lung cancer

There are high expressions of genes related lung cancer on the patients with lung cancer and postoperative lung cancer, of which TLR8, CSF2RB, EMR3, NCF and HCK-1 are the higher than normal group (Figure 1 and Table 3).

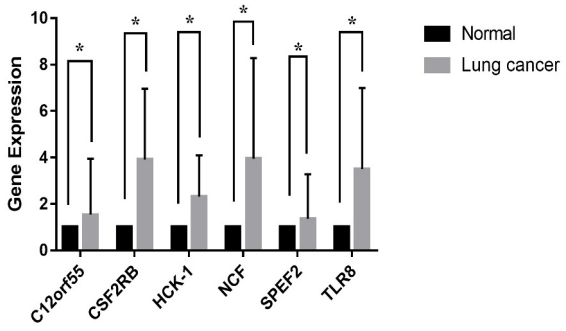

Figure 1. The Hub genes of C12orf55, CSF2RB, HCK-1, NCF, SPEF2 and TLR8 were analyzed by standard qRT-PCR approach and show the higher than normal group.

3.2 Statistical comparison of gene expression

There is a significant difference between two groups: #: Gene expression of lung cancer and control group, Tamhane's T2 /P Values of HCK, NCF1, TLR8, EMR3, CSF2RB and DYSF were 1.313/0.001, 2.937/0.001, 2.496/0.001, 3.051/0.000, 2.910/0.000, 2.578/0.009; ##: Comparison of two methods of postoperative lung cancer with control group, Tamhane's T2 and P value of HCK, CSF2RB, SPEF2, C12orf55 were 1.442/0.012, 2.280/0.34, 1.345/0.031, 2.384/0.008; b: Comparison of Tamhane's T2 and P between lung cancer and postoperative lung cancer, Tamhane's T2 / P value of EMR3 and C12orf55 is 2.021 / 0.022 / 1.850 / 0.036, respectively (Table 2).

4 Discussion

4.1 Research significance

Lung cancer is one of the most common malignant tumors in the world. The early stage lung cancer involves the expression changes of genes related to lung cancer. The research on genetic susceptibility of lung cancer mainly focuses on the gene polymorphism of metabolic enzymes, sensitivity to mutagens, DNA repair ability and mutation or deletion of genes and so on. Tumors are classified into many different types and subtypes in histomorphology. Accurate diagnosis of tumor subtypes is of great significance to their clinical treatment.

Table 2. Comparison of expression related genes in nasal epitthelia

Groups	Nomal (28)	Cancer (32)	P-cancer (10)	F	P
HCK	1.008±0.006	2.321±1.771$^{\#}$	2.450±1.335$^{\#\#}$	7.039	0.002*
NCF1	1.005±0.007	3.942±4.341$^{\#}$	2.554±1.170	6.176	0.004*
TLR8	1.004±0.003	3.500±3.493$^{\#}$	1.959±1.690	6.890	0.002*
EMR3	1.006±0.004	4.057±2.985$^{\#b}$	2.006±0.889b	15.085	0.000*
CSF2RB	1.006±0.004	3.916±3.045$^{\#}$	3.303±2.082$^{\#\#}$	8.947	0.000*
DYSF	1.004±0.003	2.853±2.312$^{\#}$	2.305±1.855	5.709	0.016*
SPEF2	1.008±0.006	1.366±1.909	2.352±2.164$^{\#\#}$	2.452	0.095
ANKFN1	1.005±0.005	1.333±2.029	2.187±2.294	1.679	0.196
HYDIN	1.004±0.004	1.053±1.573	1.873±2.390	1.348	0.268
DNAH5	1.004±0.004	1.683±2.833	2.098±2.036	1.073	0.349
C12orf55	1.003±0.003	1.537±2.412^{b}	3.387±3.053$^{\#\#b}$	3.750	0.029*
CCDC223	1.006±0.005	1.202±1.255	1.516±1.424	0.928	0.401
ACTIN	1.007±0.005	1.007±0.005	1.004±0.005	1.194	0.309

Note: *P<0.05. Due to the variance of the data, using welch correction and Tamhane's T2 test.

However, the classification of tumors has been difficult in the clinic. Many tumors with similar morphology need different treatment methods. From the point of view of molecular biology, tumors are a kind of complex gene diseases, which are caused by DNA damage on some chromosomes and abnormal gene expression in cells, leading to uncontrolled growth, lack of differentiation and abnormal proliferation. Because of the complexity of cancer development mechanism, the researches have been more than 100 years and have not uncovered the mystery. With the development of molecular biology, it has become the hot-spots to study the expression of tumour genes, to search for tumour-related genes and to discover the expression characteristics of tumour genes.

4.2 Research methods

If we hope to study the relationship between the gene expression and tumorigenesis, even if the gene has been overexpressed in tumors, it is necessary to observe the differences effects of gene overexpression and inhibition on the growth and death of tumors, in order to further confirm the role of the gene in tumorigenesis and development from both positive and negative aspects. Because tumorigenesis is a process of multi-step and multi-gene alteration, and the genes function in vivo is a interrelated and interacted gene network. The abnormal expression of a single gene may be the most fundamental and direct cause of tumorigenesis, or it may only be an accompanying phenomenon in vivo, so it is necessary to amplify the gene to study the relationship between gene

function and tumorigenesis. Abnormal expression (too high/too low) is a routine and necessary method. Polymerase chain reaction (PCR) is a molecular biological technique that simulates the natural replication process of DNA in vivo. It is mainly used to amplify DNA segments between two known sequences in vitro. After denaturation, annealing and extension of two oligonucleotide primers on both sides of the DNA fragment to be amplified, the DNA amplification was 2 fold [1]. It has good detection and observation of gene expression, early diagnosis of lung cancer, research of targeted drugs and formulation of treatment plan.

4.3 Genes related lung cancer and characteristic

4.3.1 EMR3 gene

The mucin like hormone receptor, EMR3 is one of the members of the epidermal growth factor 7 transmembrane protein family (EGF-TM7). The family also includes CD97, EMR1, EMR2 and EMR4, which are mainly expressed in the immune system cells. The exact function of EMR3 is unclear as well as its ligand or downstream signal [2]. Previous studies have showed that its expression was localized in mature granulocytes, other members of the family mediate cell migration and leukocyte migration [3]. Ari and Kane found that EMR3 is expressed in glioblastoma cells, and can mediate cell migration and invasion. EMR3 had the highest level of neutrophils, monocytes and macrophages in the peripheral blood of Crohn patients [4]. It might play a role in myeloid interaction in the immune and inflammatory reactions, and it was the work of the inflammatory bowel disease (IBD).

4.3.2 CSF2RB gene

Granulocyte colony stimulating factor is a hematopoietic growth factor that directionally acts on granulocyte progenitor cells. It can specifically stimulate and regulate the proliferation, differentiation, survival and activation of granulocyte system. The protein encoded by the colony stimulating factor 2 receptor beta (CSF2RB) is a common beta chain of high affinity receptor IL-3, IL-5 and CSF. CSF2RB locates in the region-22q12.3. of chain between bipolar disorder and schizophrenia, and is expressed in most cells. Research results show that CSF2RA or CSF2RB mutation can cause hereditary pulmonary alveolar proteinosis (PAP) and CSF2RB is a risky factor to schizophrenia and depression in Chinese Han population [5,6].

4.3.3 TLR8 gene

As the main category of pattern recognition receptors, Toll like receptors, TLRs play a crucial role in the invasion of pathogens. TLRs belongs to a subfamily of Toll-like receptors composed of TLR7, TLR8 and TLR9, which are larger than other TLRs molecules. The extracellular domain of TLR7, TLR8 and TLR9 contains about 800 amino acids and 26 LRR modules. Compared with other TLRs, TLR8 are particularly effective in the polarization in monocyte and myeloid dendritic cells. Therefore, ligand of TLR8, as an independent immunotherapy drug or adjuvant, has become an interesting target in tumor immunotherapy. More and more evidences show that TLRs is an important regulator of tumor biology besides infection. However, their role in the cancer cells is still unknown. Activation of TLRs results in antagonistic effects: anti-tumor and tumor-inducing. On the one hand, the agonists of TLR can induce dendritic cells (DC) to increase the function of CO stimulators (CD80, CD86 and CD40), enhance DC processing and antigen presentation and promote the production of Thl type immune response inflammatory cytokines (TNF-a and IL-12), and these has an immune stimulating effect. On the other hand, the agonists of TLR exert immunosuppressive effects by inducing immunosuppressive factors including IL-10, TGF-P and regulatory T cells. Therefore, the interpretation of the exact function of each TLR in tumor is very important for the type of TLRs and the agonist of TLR used in tumor therapy. In addition, for specific tumor types, the administration dose and route TLR agonist are also important factors that determine the survival [7].

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