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Establishment of preliminary regulatory network of TRPV1 and related cytokines

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Abstract Our purpose was to investigate the regulatory mechanism of TRPV1 and related cytokines on children bronchial asthma. TRPV1 mRNA level and two SNP genotypes of children in case group and control group were detected by real-time quantitative PCR. Western blot and ELISA were used to measure the levels of cytokines like IgE, IL-2, etc. Their correlations were analyzed by Logistic regression and KEGG analysis. Moreover, tertiary structure of protein and miRNA binding sites were also predicted by online tools. Case group was obviously different from control group in TRPV1 mRNA level, the two SNP genotypes distribution and the related cytokines levels. Logistic regression analysis further demonstrated that TRPV1 mRNA level, EOS, IL-4 and IL-5 may be risk factors for children bronchial asthma. And based on that, the preliminary regulatory network of children bronchial asthma was drawn. What's more, mutation of rs4790521 and rs4790522 in TRPV1 gene both induced its corresponding miRNA binding site's change. The preliminary regulatory network of TRPV1 and related cytokines on children bronchial asthma established in this study provides certain theoretical basis for pathogenesis and treatment of children bronchial asthma.
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1. Introduction

Bronchial asthma is a common chronic respiratory disease in childhood. According to the World Health Organization (WHO) statistics, morbidity and mortality of asthma (especially in children) in the United States, Australia, and many developed countries in European have been rising in recent

years, and in China, the prevalence rate of children asthma also shows a rising trend (Gu et al., 2012). Asthma has caused serious impact on children, their families as well as social economy and has become a serious public health problem which has attracted great concern in the world (Da Silva et al., 2016).

Capsaicin receptor (Caterina et al., 1997) was cloned by receptor activated by capsaicin, which is also known as vanilloid receptor (VR1). Capsaicin receptor has ion channel features and belongs to transient receptor potential channel super family, and its English name is specified as transient receptorpotential vanilloid 1 (TRPV1). TRPV1 is a member of cation channel superfamily, subfamily V, and it expresses widely in mammalian respiratory system (Lieu et al., 2012). Extensive researches suggested that TRPV1 plays a crucial role in children bronchial asthma. With deeper understanding of respiratory system inflammation mechanism by people recently, signaling regulation of TRPV1 gene on asthma has been a research hotspot (Geppetti et al., 2006; Guibert et al., 2011). Bronchial asthma is a chronic airway inflammatory disease with participation of multiple cells and cellular components. Moreover, there are many studies (Liu, 2014; Li et al., 2014) showing that cytokine detection was significant for children bronchial asthma to some extent.

Signal transduction pathway of TRPV1 and related cytokines in the pathogenesis of children bronchial asthma, however, is still unclear currently. This study screened the correlated cytokines to children bronchial asthma through analysis of clinical data, and with KEGG database and retrospective analysis, this study also established a preliminary regulatory network of TRPV1 and related cytokines on children bronchial asthma, aiming to provide some theoretical basis for the pathogenesis and treatment of children bronchial asthma.

2. Material and methods

2.1. Subjects

Two hundred outpatients and inpatients with children bronchial asthma (new cases or patients with atopy) at the People's Hospital of Zhengzhou University from May 2012 to December 2015 were collected as case group, and 200 healthy children who came to the hospital for physical examination as control group. The general data of the two groups are listed in Table 1, and their age, gender and weight are similar, which means those materials are comparable. All included children in control group have neither family or personal history of allergic reaction, nor asthma history. And their guardians have agreed and signed the informed consent, besides, this study has been approved by the People's Hospital of Zhengzhou University ethic committee.

2.2. Sample collection

Before and after receiving treatment, 5 ml peripheral vein blood of children in a fasting state of the two groups was collected, from which 1 ml was mixed with EDTA for anticoagulation before being frozen in the refrigerator at $-20\,°C$ for total RNA extraction; and the rest of the blood was made into blood smear for serum preparation, then after one-hour quiescence at room temperature it was centrifuged for 10 min at 2000 rpm, and then the serum that remained was placed in refrigerator at $-70\,°C$ for the following detection.

2.3. TRPV1 mRNA level of subjects by real-time quantitative PCR detection

First, 1 ml peripheral vein blood was mixed with EDTA for anticoagulation, and then RNA was extracted from peripheral leukomonocytes by the total RNA quick extraction kit. With oligo DT as primer and total RNA as template, cDNA was composed by reverse transcriptase according to operation manual.

According to TRPV1 gene sequence listed in GenBank, the upstream primer sequence and the downstream primer sequence of TRPV1 mRNA were synthesized. Upstream primer sequence: 5′-GGCTGTCTTCATCATCCTGCTGCT-3′; downstream primer sequence: 5′-GTTCTTGCTCTCCTGTG CGATCTTGT-3′; and the size of PCR product was 117 bp. With GAPDH as reference gene, real-time quantitative PCR composed the upstream primer sequence and the downstream primer sequence of GAPDH; the upstream primer sequence: 5′-TGCACCACCAACTGCTTAGC-3′, the downstream primer sequence: 5′-GGCATGGACTGTGGTCATGAG-3; and its product size was 87 bp. Reaction cycle parameters were as follow: pre-denaturation at $94\,°C$ for 10 min, denaturation at $94\,°C$ for 30 s, annealing at $56\,°C$ for 30 s, extension at $72\,°C$ for 30 s. These procedures were repeated for totally 40 cycles among which 30 cycles later, extension was performed at $72\,°C$ for 7 min. Confidence interval and relative quantification were determined by Wilcoxon Rank Sum Test; $2-\Delta\Delta C$ T(Licak) was used to compare the TRPV1 relative expression of children in case group and control group.

Table 1 General data of the subjects.

General data		Case group	Control group	χ^2/t	P
n		200	200		
	⩽3	37 (18.50%)	26 (13.00%)	4.21	0.122
Age	>3–⩽7	103 (51.50%)	97 (48.50%)		
	>7–⩽14	60 (30.00%)	77 (38.50%)		
Gender	Male	125 (62.50%)	109 (54.50%)	2.636	1.104
	Female	75 (37.50%)	91 (45.50%)		
Weight (kg)		19.2 ± 7.3	18.7 ± 8.1	0.648	0.517

2.4. Collecting and determining of targeting SNP sites

In reference to NCBI SNP data base, assuming MAF > 5% and r2 > 0.8, the specific SNP of UTR-3 gene was screened. SNP detection: PCR reaction product was directly sequenced by Shanghai Yingjun Biotechnical Company Limited.

2.5. Measurement of blood smear cell count and total serum IgE level

With help of high magnification (400x), eosinophils count of blood smear was performed; the total serum IgE level was measured by double-antibody sandwich ELISA.

2.6. Cytokine detection

ELISA was used to detect the IFN-γ, IL-2, IL-17, IL-4 and IL-5 levels in serum of children in case group before and after the treatment and in control group. All the detection steps were completely consistent with the instruction.

2.7. Data statistics and analysis

SPSS16.0 software was adopted for statistical analysis; comparison of means between the two groups was performed by t-test; measurement data was made by $\chi 2$ test. $P < 0.05$ means the difference has statistical significance. Then the correlative factors of bronchial asthma in children were analyzed by Logistic regression analysis, and the corresponding regulatory network was drawn referring to KEGG database and literature retrieval.

2.8. Protein analysis of TRPV1

Swiss-model website online tool was used to predict the tertiary structure of protein; online software of miRBase was employed for prediction of miRNA binding sites.

3. Results

3.1. TRPV1 gene expression level and SNP site analysis

TRPV1 was performed real-time quantitative PCR, whose results are listed in Table 2. TRPV1 expression level between the two groups was significantly different ($P < 0.01$), in particular, TRPV1 expression level in peripheral blood of children with asthma was higher than that in healthy children.

According to the gene sequencing, genotype distribution of two SNP sites (rs4790521 and rs4790522) was significantly different in the two groups. According to the gene sequencing and

distribution of rs4790521 site, case group was different from control group in distribution of three genotypes, CC, CT and TT; alleles C in case group were much more than those in control group ($P < 0.05$). While based on genotype distribution of rs4790522 site, three genotypes, CC, AC and AA, were notably different in the two groups, and alleles C in case group was much more than that in control group ($P < 0.05$) (see Table 3).

3.2. Correlative cytokines levels of bronchial asthma in children and eosinophile granulocyte count

As shown in Table 4, IgE content, EOS number, IFN-γ, IL-2, IL-17, IL-4 and IL-5 levels of children in case group before treatment were significantly different from that after treatment and that in control group ($P < 0.01$).

3.3. Logistic regression analysis of cytokines correlated to bronchial asthma in children and TRPV1 gene

Previous $\chi 2$ test and t-test showed that differences between case group and control group were statistically significant in aspects of TRPV1 mRNA expression, gene frequency of polymorphic sites rs4790521 and rs4790522, EOS count in peripheral blood and IgE, IFN-γ, IL-2, IL-17, IL-4 and IL-5 levels ($P < 0.05$). Through Logistic regression analysis on these indexes and step-by-step parameter screening, results showed that the risk factors for bronchial asthma in children were EOS count in peripheral blood, TRPV1 mRNA expression and IL-4 and IL-5 levels (see Table 5).

3.4. Regulatory network of bronchial asthma in children in which TRPV1 gene was included

Through KEGG signal pathway database, TRPV1 and cytokines linked to bronchial asthma in children including IL-2, IL-4, IL-5, IL-10 and IL-17 were analyzed, and then based on related literatures, a preliminary regulatory network of bronchial asthma in children was drawn (see Fig. 1). According to Fig. 1, the regulatory network of bronchial asthma in children was quite complicated, and TRPV1 and cytokines of TH1, Th2 and Th17 all can cause bronchial asthma in children. The relationship among TRPV1 and Th1, Th2 and Th17 cytokines was complex and mutually influenced. Specifically, Th1 might promote TNF-β, IFN-γ and IL-2 levels; Th2 might promote IL-4, IL-5 and IL-10 levels; Th17 might promote IL-17 level which would influence IL-1β affecting TRPV1. Besides, some macroelements or tracheal mucosal damage all can affect TRPV1, which could cause immune function disorder or peripheral sensitization, finally resulting in bronchial asthma in children.

Table 2   mRNA expression level of TRPV1 in children of case group and control group.

Grouping	Before treatment	After treatment	Control group	t_1	P_1	t_2	P_2
TRPV1 mRNA level	6.63 ± 4.44	4.88 ± 3.21	3.04 ± 2.70	4.517	0	9.742	0

Note: P_1 indicates comparison in case group before and after the treatment; P_2 suggests comparison between case group before the treatment and control group.

Table 3 Genotype distribution comparison of rs4790521 and rs4790522 sites in the two groups.

	Grouping	n	Genotype distribution n (%)			Alleles n (%)	
			CC	CT	TT	C	T
rs4790521	Case group	200	64 (32.00)	60 (30.00)	76 (38.00)	188 (47.00)	212 (53.00)
	Control group	200	17 (8.50)	69 (34.50)	114 (57.00)	103 (25.75)	297 (74.25)
	χ^2		77.288			39.023	
	P		0			0	

	Grouping	n	Genotype distribution n (%)			Alleles n (%)	
			CC	AC	AA	C	A
rs4790522	Case group	200	112 (56.00)	81 (40.50)	7 (3.50)	305 (76.25)	95 (23.75)
	Control group	200	49 (24.50)	115 (57.50)	36 (18.00)	213 (53.25)	187 (46.75)
	χ^2		50.108			46.354	
	P		0			0	

Table 4 Level of cytokines correlated to asthma and eosinophils count.

Category	Case group		Control group	t_1	P_1	t_2	P_2
	Before treatment	After treatment					
IgE (IU/ml)	104.39 ± 28.13	79.56 ± 20.43	75.30 ± 26.38	10.100	0	10.667	0
EOS (×10⁶number/L)	414.61 ± 93.44	231.24 ± 98.51	192.48 ± 99.62	19.099	0	22.999	0
IFN-γ (ng/L)	31.71 ± 20.10	50.14 ± 19.89	55.64 ± 29.76	−9.271	0	−9.424	0
IL-2 (ng/L)	26.72 ± 13.58	29.74 ± 14.21	31.04 ± 16.07	−2.173	0.03	−2.901	0.004
IL-17 (ng/L)	6.16 ± 2.32	3.27 ± 1.98	2.77 ± 1.41	13.400	0	17.685	0
IL-4 (ng/L)	148.79 ± 22.48	69.14 ± 17.35	64.76 ± 16.12	−39.667	0	−42.96	0
IL-5 (ng/L)	50.93 ± 13.47	26.81 ± 10.84	22.81 ± 9.06	19.729	0	24.498	0

Note: P_1 indicates comparison in case group before and after the treatment; P_2 suggests comparison between case group before the treatment and control group.

Table 5 Logistic regression analysis of the factors correlated to bronchial asthma in children.

Inclusive parameters	Regression coefficient	Wald	P	OR	95%CI
EOS	0.005	4.071	0.044	1.005	1.000–1.009
IL-4	0.06	34.105	0	1.062	1.041–1.084
IL-5	0.039	3.242	0.072	1.039	0.997–1.084
TRPV1 mRNA	0.147	3.846	0.05	1.158	1.000–1.341

3.5. Specific localization of mutant sites rs4790522 and rs4790521

Mutant sites rs4790522 and rs4790521 of TRPV1 gene were both located in 3′UTR of gene (see Fig. 2).

3.6. Prediction of miRNA binding sites before and after mutation

The above mutant sites were located in 3′UTR region of TRPV1 gene, which thus had no influence on TRPV1 gene's animo acid sequence. By changing miRNA bidding sites, however, mRNA level can be regulated. After prediction of miRNA's binding sites before and after TRPV1 gene, results showed that mutation of rs4790522 caused disappearance of binding site miR-141-3p and that mutation of rs4790521 caused addition of binding sites miR-6802-3p and miR-551b-

5p as well as the disappearance of binding site miR-6807-3p. And the specific information on miRNA is shown in Fig. 3.

4. Discussion

Over these years, relationship between TRPV1 and children bronchial asthma has been a research hotspot in pathological mechanism of bronchial asthma, and much related achievements has been made. Study by Geppetti et al. (2006) indicated that TRPV1 might be the key signaling regulation factor inducing children asthma. Besides, TRPV1 plays an important role in bronchoconstriction, protein secretion, edema of tunica mucosa tracheae, inflammatory cell chemotaxis (Liu, 2014) and cough reflex (Smit et al., 2012; Patberg, 2011). Recent study further suggested that TRPV1 exists in immunohisto-chemical T-cells and can regulate the activity of CD4+ and the specificity of inflammatory cells (Baker et al., 2016). Fur-
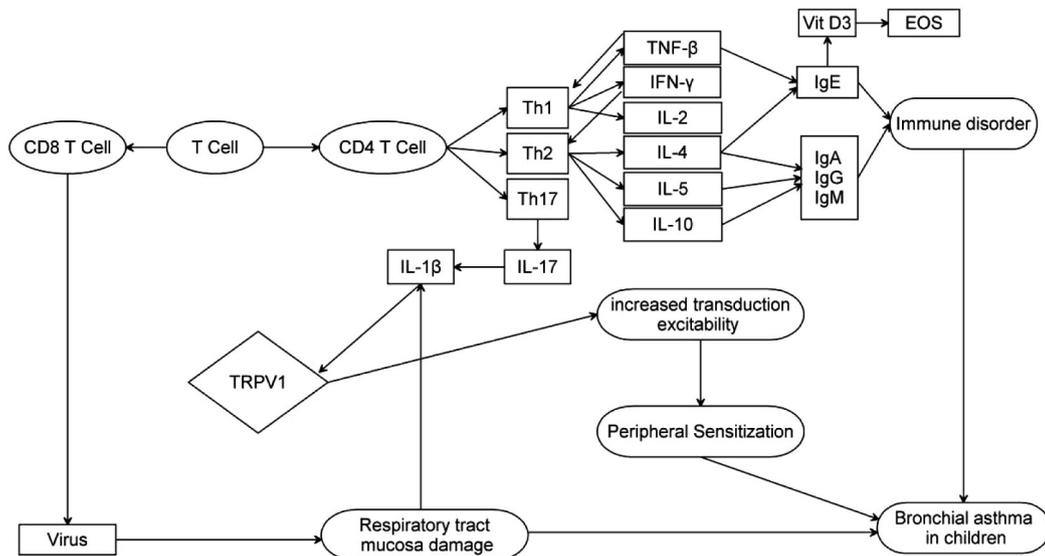
Figure 1 Partial regulatory network of bronchial asthma in children.

Figure 2 Specific localization of mutant sites rs4790522 and rs4790521. Note: gene sequence between two vertical red lines was CDS region of TRPV1 gene; base in blue box was mutant base; gene sequence in dotted line was the disappeared binding site of miRNA after mutation; and gene sequence in straight line was new binding site of miRNA after mutation.

Figure 3 Specific information on binding sites of miRNA before and after TRPV1 gene mutation.

thermore, McGarvey et al. (2014) found that functional TRPV1 presents in epithelial cell of human airway and has a high expression in airway of patient with refractory bronchial asthma. What's more, correlation of TRPV1 to bronchial asthma has been proved in some studies. Cantero-Recasens et al. (2010) found that children with functional TRPV1 channel caused by genetic deletion have low risk of exercise-induced

asthma. In this study, mRNA level of TRPV1 in case group before treatment was statistically different from that after treatment and that in control group ($P < 0.05$); and according to TRPV1 gene sequencing, it was found that genotype distribution of the two sites (rs4790521 and rs4790522) was obviously different between case group and control group. Thus it can be concluded that mRNA level of TRPV1 and the two

SNP sites all may be a predictor of bronchial asthma in children.

Recent study showed that one of key mechanisms in the pathogenesis of asthma is correlated to imbalance of Th1/Th2/Th17/Treg cytokines (Holgate, 2012; Umetsu et al., 2002; Ji et al., 2014; Shi et al., 2011). And Th1 type cytokines mainly participate in cellular immune; Th2 type cytokines always cause some pathological responses like increasing IgE level in plasma, elevating EOS in airway, hyperplasia and hypertrophy of airway smooth muscle, and airway mucus hypersecretion, etc. (Ngoc et al., 2005) Th17 type cytokines play a significant role in pro-inflammatory reaction and autoimmunity; Treg type cytokines can regulate and inhibit immune responses through secretion of IL-10 or cell-contact mechanism(CTLA-4 and GITR). This study shows that both interior-group and inter-group differences of IgE level in peripheral blood and EOS count were significant, which indicates that IgE level and EOS count play certain role in bronchial asthma in children. Besides, interior-group and inter-group differences of IFN-γ, IL-2, IL-4, IL-5 and IL-17 levels were both statistically significant.

Through a multi-factor Logistic regression analysis in terms of factors with significant differences between case group and control group, including TRPV1 mRNA, the two SNP gene sites' genotypes, IgE level in peripheral blood, EOS count and cytokines, four factors (EOS, IL-1, IL-5 and TRPV1 mRNA leve)l were included in Logistic regression equation. The analysis results show that these four factors were the risk factors of bronchial asthma in children, which is different from previous studies by our research team (Chen et al., 2015). And the reason might lie in the sample size and quality; on the other hand it could be that too many factors were included in Logistic regression equation.

Based on our previous study, the preliminary regulatory network of genes and cytokines involved in this study was made through searching KEGG database and combing other literatures. The regulatory network for bronchial asthma in children is quite complex, and relations among TRPV1, Th1, Th2 and Th17 are complicated with mutual influence, which all eventually induce immune function disorder or peripheral sensitization and cause bronchial asthma in children. This network is just part of bronchial asthma in children, and this study could contribute to the whole regulatory network of bronchial asthma in children.

There are a lot of studies on miRNA and children bronchial asthma home and abroad, and most are about miRNA in signaling pathway of bronchial asthma or miRNA regarded as diagnostic marker for asthma. Huo et al. (2016) found that miR-181b-5p in epithelium and plasma is a potential biomarker for eosinophil increase in airway, and it can participate in eosinophilic airway inflammation through regulating pro-inflammatory cytokines' expression with targeting SPP1. Kärner et al. (2016) indicated that miR-323-3p participates in negative feedback loop so as to control production of IL-22 in IL-22/IL-17-producing T cells, which may affect T cell response in asthma. According to a study by Maes et al. (2016), miR-223-3p, miR-142-3p and miR-629-3p express high in the sputum of patients with severe asthma and they are correlated to neutrophil airway inflammation, hence this study indicated that these miRNAs can be regarded as markers of this kind of airway inflammatory phenotype. Moreover, it's found that mutant sites rs4790522 and rs4790521 are located

in 3′UTR of TRPV1, thus not affecting the protein structure of gene. 3′ regulatory region plays an important regulating role in gene expression; therefore, mutation in this region might affect gene's expression. Through prediction of miRNA biding sites before and after the mutation of TRPV1 gene, it's found that two mutant sites induce changes of four miRNA biding sites including miR-141-3p, miR-6802-3p, miR-551b-5p and miR-6807-3p, which is crucial for studies on posttranscriptional regulation of TRPV1 gene. miRNA is able to act on 3′-UTR region of target gene mRNA to inhibit expression of mRNA and even cause its degradation, and mRNA mainly regulates cell proliferation, differentiation, apoptosis and other cellular processes (Adlakha and Saini, 2016). But for the time being, most studies on the four miRNAs involved in our studies, miR-141-3p, miR-6802-3p, miR-551b-5p and miR-6807-3p, focus on tumor, for instance, miR-145-5p have been reported that it works in kidney cancer, ovarian cancer, liver cancer and prostate cancer (Iorio et al., 2007; Liu et al., 2014; Porkka et al., 2007). miR-551b-3p is closely linked to the growth and development of gastric cancer and pancreatic cancer (Wen et al., 2016; Kuśnierz-Cabala et al., 2015). miR-6802-3p and miR-6807-3p are two new subtypes of miRNA, and there is no relevant study on them at present(Ladewiq et al., 2012). miR-141-3p, miR-6802-3p, miR-551b-5p and miR-6807-3p have not been reported in children bronchial asthma, either, which need deep researches in future.

5. Conclusions

Combining with KEGG database and literature searching, this study establishes a preliminary regulatory network on children bronchial asthma in which cytokines correlated to children bronchial asthma screened out by analysis of clinical data were included; meanwhile, through analysis on molecular mechanism of TRPV1 in children bronchial asthma, this study also reveals that TRPV1 gene rs4790521 and rs4790522 mutation sites induce changes in corresponding miRNA, which may be the potential molecular mechanism of significant difference between case group and control group. The outcomes in this study provide certain theoretical basis for the pathogenesis of children bronchial asthma.

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