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Research on the molecular mechanism of Seretide treatment to asthma disease

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Abstract. – INTRODUCTION: Asthma is one of the most common chronic diseases in children. It is attributable to complicated coactions between various genetic factors and environmental allergens.

AIM: We attempt to unfold the mechanism of asthmatic disorder and research the molecular mechanism of Seretide on asthmatic disease.

MATERIALS AND METHODS: Using the GSE31773 microarray datasets downloaded from Gene Expression Omnibus database, we first screened the differentially expressed genes between healthy control and asthmatic samples cells based on classical *t*-test and false discovery rate < 0.05 as significant threshold. The underlying molecular mechanisms were investigated by Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis. In addition, the crosstalk network of pathways was also constructed.

RESULTS: A total of 2011 differentially expressed genes were obtained by comparing asthmatic sample treated with Seretide and healthy controls. A total of 403 differentially expressed genes were collected between asthma samples untreated by Seretide and healthy sample controls. The enriched pathway of differentially expressed genes included signal transduction disorder (such as TGF-beta signaling pathway) and metabolism disorder (such as Phenylalanine metabolism). There were 27 pathway crosstalk pairs among 13 pathways.

CONCLUSIONS: Our findings will help to clarify the molecular mechanism of Seretide and offer advices for asthma pathogenesis, Seretide therapy and follow-up treatment.

Key Words:
Seretide, Asthmas disorder, mRNA, Expression profile, Respiration.

Introduction

Asthma is one of the most common chronic diseases in children. It is attributable to complicated coactions between various genetic factors and environmental allergens[1]. Despite recent progresses in the development of anti-asthmatic medication, asthma continues to be a major health problem worldwide. Asthma symptoms include wheezing, chest tightness, cough and shortness of breath together with airway hyper-responsiveness, which, together with airway hyperresponsiveness, previously have been attributed to a dysfunction of airway nerves. However, research during the last two decades identifies Th2-sensitization and the subsequent allergic reaction to innocuous environmental antigens as a basic immunological mechanism leading to chronic airway inflammation. Recent evidence indicates that the development of allergic asthma is influenced by events and circumstances in early childhood and even in utero. Allergen, ozone or stress exposure, as well as RSV (Respiratory Syncytial Virus) infection in early life could be able to induce irreversible changes in the developing epithelial-mesenchymal trophic unit of the airways. The company of chronic inflammation with neural dysfunction has recently drawn attention to the involvement of interaction pathways between the nervous and the immune system in the airways. Concentrated basic research has accumulated morphological and functional evidence for the interaction between nerves and immune cells. Neuropeptides and neurotrophins have become focus of attention as the key mediators of neuroimmune interactions, which lead to the development of several pharmacological compounds specifically targeting these molecules[2].

Seretide (Advair [North America], GlaxoSmithKline, Durham, NC, USA) is an inhaler combination formulation intended for the maintenance therapy of obstructive airways disease.

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Seretide is developed and made available initially as three multi-dose, dry powder inhaler formulations delivering 50 microg/puff of the long acting beta(2) agonist salmeterol and either 100, 250 or 500 microg/puff of the inhaled corticosteroid fluticasone propionate[3]. The costs of asthma and chronic obstructive pulmonary disease (COPD), the two most common chronic respiratory illnesses, are substantial and rising. The fixed-dose combination of fluticasone and salmeterol has been a safe and effective therapy for these diseases. To review the pharmacoeconomic impact of the fixed-dose combination of inhaled fluticasone and salmeterol in asthma, a systematic screen of the expression profiles of asthma patients was carried out to identify molecular mechanism with salmeterol and fluticasone (Seretide, Advair, Viani)[4].

Materials and Methods

Asthmatic RNA Expression Data

For research on the effect mechanism of Seretide treatment on asthma, we downloaded mRNA expression data from GEO (Gene Expression Omnibus) database (access number: GSE31773)[5]. This dataset contains 40 samples, which includes 16 healthy donors, 8 non-severe asthma and 16 severe asthmatic samples. The samples either Seretide treated or not are 8. The following analysis used healthy donor and severe asthmatic samples only, and applied Affymetrix Human Genome U133 Plus 2.0 Array platform for test.

Pretreatment of Expression Data

We downloaded the original gene expression data from GEO database, used RMA (robust Multiarray Analysis) method for pretreatment, including background correction, standardization, and probe set summary process. Then we applied ComBat R software package for batch effects correction. After the ID transformation between probes and genes, we selected probe mean as the final expression value of this gene for mapping of different probes to the same gene (entrez gene ID). Finally we obtained the gene sample expression matrix of genes (19803*32).

Identifing Differentially Expressed Genes

We applied the canonical *t*-test method to severe asthmatic (Seretide severe asthmatic and non-Seretide severe asthmatic groups) and healthy donor samples for differential analysis. All the genes got their corresponding *p* values after *t*-test. For decreasing false positive, we applied multiple test correction (Benjamini& Hochberg method) on p value. The differentially expressed gene is limited when BH $p < 0.05$, so we chose genes with $p < 0.01$ as the objects for the follow-up analysis.

KEGG Pathway Enrichment Analysis

For identifying the differentially expressed genes of asthma and the linked biological function, we applied hyper-geometric distribution[6] for KEGG pathway enrichment analysis. The KEGG pathway is downloaded on April 26th, 2011, including 232 pathways. Assuming that KEGG pathway contains N genes wholly, n differentially expressed genes, and m genes involved in certain pathway, so the possibility for overlapping at least k genes is.

$$p = 1 - \sum_{o}^{k-1} \frac{c_k^m c_{n-k}^{N-m}}{c_n^N}$$

Synergic Analysis of Pathways

For identifying the synergic relationship between pathways, we constructed the similarity network among pathways. Jaccard index is used for measuring crosstalk between pathways[7]. The intersection of pathway A and B is #intersection (A, B), and the union of pathway A and B is #union (A, B). The formulation is as follows,

$$\text{Jaccard (path A, path B)} = \frac{\#\text{Intersection (path A, path B)}}{\#\text{Union (path A, path B)}}$$

For these pathway pairs, not only their expression happens to be disordered significantly, but also the interaction between them changed. All of these are important for the pathogenesis of asthma research.

Results

Differentially Expressed Genes of Asthmatic Patients and their Function Analysis

After *t*-test method application, the 8 asthmatic samples after Seretide treatment were compared to 16 healthy controls and 2011 differentially expressed genes were obtained. Of which 1497 genes were down regulated and 514 genes were up

regulated, and all of their FDR (False Discovery Rate) were less than 0.1. When compared the 8 asthma samples untreated by Seretide to the 16 healthy sample controls, we obtained 403 differentially expressed genes, within which 260 genes were down-regulated and 143 genes were up-regulated. For further research on the related biological functions of differentially expressed genes in asthma, we applied hyper-geometry enrichment analysis method to both Seretide treated and untreated differentially expressed genes in asthma. Because of sample size limitation, the Statistical Power is not enough and we can't get significant functions under rigid FDR control. Therefore, we applied $p < 0.05$ for control. The differentially expressed genes associated with Seretide treatment were enriched in disorder of 16 pathways, including mainly disordered signal transduction pathways such as "TGF (Transforming Growth Factor)-beta signaling pathway", "ErbB signaling pathway", and "PPAR signaling pathway", metabolism disorder such as "Phenylalanine metabolism" and "Nitrogen metabolism". The most significant disordered metabolism pathway was "Neuroactive ligand-receptor interaction" (see Table I). The differentially expressed genes untreated by Seretide were also enriched in significant disorder of 12 signal pathways, including commonly disordered signal pathways "TGF-beta signaling pathway", "Axon guidance", "ECM (Extracellular Matrix)-receptor interaction", "ABC (ATP-Binding Cassette) transporters", and cell adhesion related signal pathway "Tight junction". The most significant disordered pathway was "Axon guidance" (see Table II).

Compare of Enriched Pathways of Asthma With and Without Seretide Treatment

For further research on the differences between asthmatic patients under Seretide treatment and non-treated by Seretide and the normal individuals, we observed both the similar function influences and different function influences after enrichment analysis. The similarities of asthma treated and not with Seretide involved three signal or signal transduction pathways disorder including "TGF-beta signaling pathway", "ECM-receptor interaction", "Axon guidance" and a human disease pathway "Amoebiasis". We could clearly see that Seretide treated asthma involved more biological pathways. For example, some classical signal pathways were present in the asthmatic patients treated by Seretide, such as "ErbB signaling pathway", "PPAR (Peroxisome Proliferator-Activated Receptor) signaling pathway", and the significantly disordered signal transduction pathway "Neuroactive ligand-receptor interaction" and those metabolism pathways of "Nitrogen metabolism" and "Phenylalanine metabolism".

The Network Analysis of Asthmatic Disorder Treated by Seretide and the Disorder Mechanism Analysis

To identify the synergic relationship among pathways and construct the similar function network between pathways, we applied Jaccard index for measuring inter-pathway crosstalk. The nodes in the network are the pathways and the sides are Jaccard value. The sides represent the existence of crosstalk between pathways. There

Table I. The pathways enriched of asthma after Seretide treatment.

Class III	Pathway-name	#Gene	#DEGs	*p*-value
hsa04080	Neuroactive ligand-receptor interaction	318	54	1.62E-06
hsa04360	Axon guidance	130	19	0.020304
hsa04270	Vascular smooth muscle contraction	126	19	0.014925
hsa05146	Amoebiasis	106	17	0.011595
hsa04350	TGF-beta signaling pathway	85	16	0.002914
hsa05410	Hypertrophic cardiomyopathy (HCM)	87	15	0.009007
hsa04974	Protein digestion and absorption	81	15	0.004574
hsa04512	ECM-receptor interaction	85	13	0.036025
hsa04012	ErbB signaling pathway	87	13	0.042459
hsa05215	Prostate cancer	89	13	0.04966
hsa03320	PPAR signaling pathway	70	11	0.043291
hsa04978	Mineral absorption	51	9	0.0337
hsa05110	Vibrio cholerae infection	54	9	0.046572
hsa00601	Glycosphingolipid biosynthesis – lacto and neolacto series	26	6	0.023682
hsa00910	Nitrogen metabolism	23	5	0.047812
hsa00360	Phenylalanine metabolism	17	5	0.013668

Table II. The pathways enriched of asthma without Seretide treatment.

Class III	Pathway-name	#Gene	#DEGs	*p*-value
hsa04360	Axon guidance	130	7	0.007492
hsa04530	Tight junction	133	6	0.028622
hsa05146	Amoebiasis	106	6	0.010368
hsa04350	TGF-beta signaling pathway	85	5	0.016223
hsa04512	ECM-receptor interaction	85	5	0.016223
hsa05218	Melanoma	71	4	0.035248
hsa02010	ABC transporters	44	3	0.041145
hsa04320	Dorso-ventral axis formation	25	3	0.009067
hsa04744	Phototransduction	29	3	0.013695
hsa05216	Thyroid cancer	29	3	0.013695
hsa05219	Bladder cancer	42	3	0.036563
hsa00120	Primary bile acid biosynthesis	16	2	0.031058

were 16 enriched pathways mentioned above. After the filtration treatment, we obtained 27 pathway pairs with 13 pathways involved. There were three classes of pathways closely connected. Ranking with the interaction rate between pathways, we can exclude the human disease pathways. It is considerable that "Vascular smooth muscle contraction" pathway connects most closely with other pathways because of the smooth muscle transformation involves in the pathogenesis of asthmatic disease (Figure 1).

As shown in Figure 1, there were different rates of interactions between pathways which facilitate the pathogenesis of disease. For further unfolding the disorder mechanism of asthmatic disease, we focused on the function of differen-

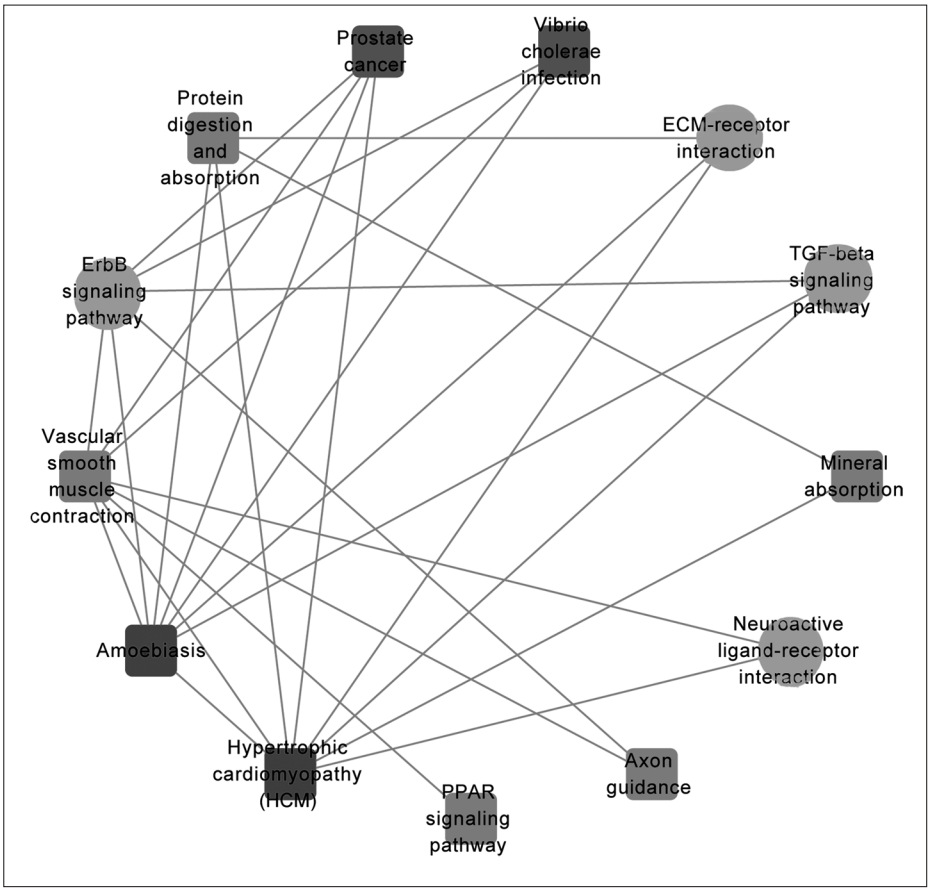

Figure 1. Disorder network of asthmatic disease treated with Seretide. The oval represents the pathways of "Environmental Information Processing" class; the gray represents "Organismal Systems" class; The black represents the pathways of "Human Diseases" class.

tially expressed genes in "Vascular smooth muscle contraction", "ErbB signaling pathway" and the mostly disordered "Neuroactive ligand-receptor interaction". Vascular smooth muscle contraction involved 19 differentially expressed genes, ErbB signaling pathway involved 13 differentially expressed genes and Neuroactive ligand-receptor interaction involved 54 differentially expressed genes.

Discussion

From our research above, the differentially expressed genes both present in asthma patients after Seretide treatment and those not were enriched in the TGF-beta signaling pathway, Axon guidance, Amoebiasis, and ECM-receptor interaction. There are two ways to explain these general characters. One possible explanation is that these pathways are not the core change events in asthma disease, or in other words, they are not responsible for asthma pathologic changes. Considering the accumulation of evidence of the association between TGF-beta signaling pathway and asthma disease before[8], the other explanation seems more reasonable. It seems that Seretide cannot wholly rejuvenate the asthma pathologic changes or it is possible that some historical events on the pathogenesis progress are irreversible.

However, there is a trend of increasing number of differentially expressed genes among Axon guidance, TGF-beta signaling pathway and ECM-receptor interaction.Take example of the down-regulation of *TGFB2* in TGF-beta signaling pathway (hsa04350), Hypertrophic cardiomyopathy (hsa05410) and Amoebiasis (hsa05146) in asthma patients. It is accountable to believe that these are the rehabilitation to the part of TGF-beta signaling pathway the Seretide treatment brings about.

As is well-known, TGF is a kind of growth factor related to construction and holds the biological functions of wide regulating cellular proliferation[9], differentiation[10] and apoptosis[11]. Many researches before have proved that it is a critical regulator in asthma remodeling[8] since it regulates the untranslated region (UTR) of MMPS (Metalloproteinases) and some binding proteins which are related to the stability of mRNA and hence up regulates the level of MMP-9 (Matrix Metallopeptidase-9) and MMP-2 (Matrix Metalloproteinase-2). At the meantime, it can stimulate the cell division and proliferation of vascular smooth muscle, induce the transformation from fibroblast to myofibroblast, facilitate the collagen deposition in airway as well as the thickening of basal membrane and hence cause the airway remodeling via increasing the proteins protein synthesis of connective tissue[12].

Beside of these, the down-regulation of TGFA (Transforming Growth Factor-alpha) and PDGFD (Platelet Derived Growth Factor D) gene in Prostate cancer (hsa05215) and ErbB signaling pathway (hsa04012) in Seretide treated patients was also a meaningful discovery. It indicates the certain association between asthma disease and prostate cancer with the gene TGFA and PDGFD.

Peroxisome proliferator-activated receptors (PPAR) is the target for obstructive lung disease[3,4] and a well-known target for lots of other complex diseases[13,14]. Nitrogen metabolism may indicate the pharmacokinetics process of absorption, distribution, metabolism and excretion (ADME) of Seretide mainly through these pathways.

It is reported that the long-term repeated attacks of asthma causes the pathological changes of respiratory tract, or terms airway remodeling. The airway remodeling process has a very close relationship with airway inflammation, involving the participation of many cytokines such as transforming growth factor (TGF)[15], vascular endothelial growth factor (VEGF)[16,17], platelet-derived growth factor (PDGF), Epidermal Growth Factor (EGF)[18], Connective Tissue Growth Factor (CTGF)[19], Nerve Growth Factor (NGF)[20,21] and matrix Metalloproteinases (MMPS)[22], which play important roles in the degradation and reconstruction of Extracellular Matrix (ECM).

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