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Subpathway Strategy used to Extract Significant Subpathways Competitively Regulated by lncRNAs in Atopic Dermatitis Treated by Cyclosporine A Based on lncRNA-mRNA Expression Data and Pathway Topologies

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ABSTRACT The aim of this study was to extract significant subpathways to further investigate the molecular mechanism of cyclosporine A (CsA) in patients with atopic dermatitis (AD) using subpathway strategy. Candidate lncRNA-mRNA interactions were reweighted using Pearson Correlation Coefficient (PCC). Condition-specific lncRNA competitively regulated signal pathways (LRSP) were established, and then lncRNA-regulated subpathways were dissected. Subsequently, the significance of candidate subpathways was assessed to further identify the significant subpathways. To further detect key AD-relevant lncRNAs, degree analysis was conducted for all nodes of the LRSP. Overall 61 significant lncRNAs competitively regulating subpathways involved in 41 complete pathways were identified in the LRSP. The top three subpathways included apoptosis, MAPK signaling pathway, and HIF-1 signaling pathway. There were 6 hub lncRNAs, including YLPM1, UBXN8, ERVK13-1, TTTY15, C14orf169, and EPB41L4A-AS1. Subpathways of apoptosis, MAPK signaling pathway, and HIF-1 signaling pathway might play crucial roles in AD after treatment with CsA.

INTRODUCTION

Atopic dermatitis (AD), also known as atopic eczema, is a complex inflammatory skin disorder, which is characterized by erythema, pruritus, excoriations, as well as serous exudate (Wollenberg et al. 2016). Worriedly, AD is constantly a comorbid condition with other atopic disorders, for example, asthma, and food allergies (Bingefors et al. 2013; Mauro et al. 2016). Although there is the increasing incidence of AD globally, suitable therapeutic options for patients suffering from moderate-to-severe AD are limited (Guttman-Yassky et al. 2011; Guttmanyassky et al. 2013). Clinically, pruritus in AD patients is frequently tolerant to conventional treatments (histamine H 1 receptor antagonists). Such intractable itch has crucial impairment in the quality of life in AD patients (Dalgard et al. 2015). Hence, there is a substantial unmet need for management of AD.

Cyclosporine A (CsA) is a strong immunosuppressant which acts on T-cells by suppressing calcineurin, and thereby signal transduction regulated by TCR activation, and it has been indicated to affect B-cells and dendritic cells (DCs), and inhibit several growth-associated pathways in keratinocytes (Tsuda et al. 2012). Significantly, the clinical efficacy of CsA in AD has been reported in several trials and it is used as a choice to treat severe AD (Gnarra et al. 2017; Kitayama et al. 2017). For example, a previous study has suggested that clinical resolution of AD with CsA is related to strong inhibition of immune disease phenotypes (Khattri et al. 2014). Moreover, CsA has been indicated to have treatment efficacy in AD via decreasing the epider-

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mal nerve density (Ko et al. 2016). However, the antiprutitic mechanisms of CsA are poorly understood.

In recent years, long non-coding RNAs (lncRNAs) have been identified to play important roles in many significant biological processes, such as tumorigenesis, post-transcriptional regulation, and human disease (Liu et al. 2015; Thomas et al. 2017). A growing body of evidence implicated that lncRNAs could competitively regulate mRNAs expression by sharing mutual miRNA binding sites with mRNAs (Tay et al. 2014). As reported, keratinocytes without vitamin D response have changed the expression of lncRNAs in a pattern associated with malignant transformation in other tissues (Jiang and Bikle 2014). LncRNAs have been implicated to competitively regulate biological pathways and play key roles in the progression of diseases (Liang et al. 2015), thus it is necessary to integratively analyze the joint effect of mRNAs and lncRNAs that competitively mediate them by taking into consideration of pathway topologies. Furthermore, RNA-RNA competing interaction network has been demonstrated to be able to regulate oncogenic pathways in disease (Sumazin et al. 2011). Of note, focusing on subpathways instead of entire pathways might gain more biologically meaningful pathways and extract the functional roles of lncRNAs. Moreover, Lin et al. (2013) proposed a strategy of subpathway, instead of the entire pathways, which is more explainable for exploring the etiology of diseases.

Objective

Thus, in the current study, to better understand the antiprutitic mechanisms of CsA in AD, the researchers used the subpathway strategy to dissect lncRNAs competitively regulated signal subpathways in AD treated by CsA based on the combination of lncRNA-mRNA expression profile and pathway topologies. In detail, taking KEGG reference pathways as backbone, the researchers listed the undirected graphs in which genes represented nodes and regulated interactions denoted edges. Subsequently, the researchers established the condition-specific lncRNA competitively regulated signal pathways (LRSP) on the basis of the matched lncRNA-mRNA expression data and the common miRNAs. After that, the researchers mapped the interesting lncRNAs and genes to the LRSP, and then they located the subpathways into pathways relying on "lenient distance" similarity method (Lin et al. 2013), following by the evaluation of the significance of candidate subpathways using Wallenius approximation (Epstein et al. 2012). Ultimately, the identification of hub lncRNAs in the LRSP was conducted. The specific analysis steps were shown in Figure 1.

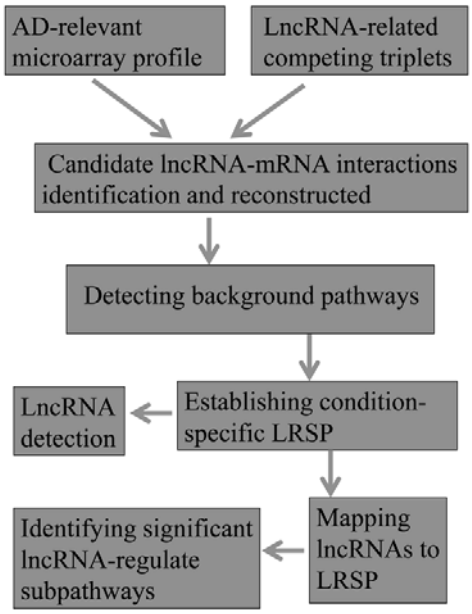

Fig. 1. Pipeline overview of subpathway strategy
Source: Author

METHODOLOGY

AD Dataset

In order to explore the antiprutitic mechanisms of CsA on AD progression, the researchers collected gene expression profile about AD (Access ID: GSE58558) (Khattri et al. 2014) from Gene Expression Omnibus (GEO) database, which was deposited in the GPL570 platform of [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array. In this microarray profile, there were 35 AD patients and 74 patients with AD treated with CsA. Original probe annotation data were downloaded for further analysis.

The researchers utilized the Affy package to pretreat the gene expression data of GSE58558 (Gautier et al. 2004). Data in CEL format were transformed to expression measures, and then

background correction was conducted, following by quartile data normalization. Then, probe was aligned to the gene symbols. Overall, the expression matrix including 20,514 genes was obtained.

Establishing Candidate lncRNA-mRNA Interactions

To begin with, the researchers obtained lncRNA-related competing triplets (lncRNA-miRNA-mRNA interactions). Concretely, lncRNA-miRNA interactions were collected from StarBase database (version 2.0) (Li et al. 2014), and experimentally proved mRNA-miRNA interactions were retrieved from mirTarBase, miRecords (V4.0), TarBase, as well as mir2Disease. Relying on the common miRNAs, candidate lncRNA-mRNA competitively regulated relationships were established. For ensuring the reliability of data, the candidate competing mRNAs for each lncRNA were identified and reserved based on the two conditions. One criteria was that hypergeometric test of common miRNAs was under the threshold of false discovery rate (FDR) < 0.05. On the other hand, Jaccard Coefficient of lncRNA-mRNA interactions ordered at top 20 percent. Based on these two conditions, candidate lncRNA-mRNA competitively regulated relations included 7693 lncRNAs-mRNA interactions, 835 lncRNAs and 1749 mRNAs. Then, taking the intersection between 20,514 genes of the microarray profile and 7693 lncRNAs-mRNA interactions, a total of 1650 mRNAs and 165 lncRNAs were extracted.

Reconstructing the lncRNAs-mRNA Interactions

It is well known that Pearson Correlation Coefficient (PCC) is an index to measure the correlation between two variables, which ranges from -1 to 1 (Nahler 2009). Thus, the researchers used PCC to analyze the co-expression possibility for any pair of interactions in the candidate lncRNA-mRNA network using the matched lncRNA and mRNA expression profiles. Using Fisher's r-to-Z transform (Best and Roberts 1975), the interaction with r value reaching a significant positive threshold were remaining ($P<0.05$).

Detecting Background Pathways

Kyoto Encyclopedia of Genes and Genomes (KEGG) database can provide a reference knowledge to further understand cellular processes using pathway aligning (Kanehisa et al. 2008). Thus, in this study, the researchers downloaded all the KEGG reference pathways based on KEGG database. After that, the mRNAs of the reconstructed lncRNAs-mRNA interactions were mapped to the KEGG reference pathways, and then background pathways were obtained. The researchers used FDR to adjust the raw P values by means of Benjamini-Hochberg procedure (Benjamini and Hochberg 1995). Background pathways were selected using the criteria of a FDR<0.01.

Establishment of Condition-specific LRSP

The background pathways identified above were converted into undirected graphs which maintained original pathway structure by means of R packages (Li et al. 2013). The lncRNAs in the reconstructed lncRNAs-mRNA interactions were inserted into the pathway graphs where lncRNAs were as nodes and they connected to their mediated-mRNAs. Then, the condition-specific LRSP including lncRNA nodes and lncRNA-mRNA competitively regulated edges were established.

Locate Subpathways Competing Regulated by LncRNAs

LncRNAs have been demonstrated to act as signature nodes, because these lncRNAs were involved in the interested genes and competing regulation. Integration of the lncRNAs and the topology characteristics of LRSP can be helpful for us to effectively locate lncRNA-mediated subregions. The signatures nodes were first aligned to the LRSP, and then the subpathways competing regulated by lncRNAs were identified using "lenient distance" similarity as well as the topology feature of the LRSP. Briefly, the researchers analyzed the shortest path between any two signature nodes, on condition that the quantity of molecules between each pair of signatures was less than n, and then these nodes would be merged into one. The quantity of nodes in the molecules involved in a given pathway more than s were considered as candidate subpahtways mediated by lncRNAs. The parameters of n and s respectively controlled the strength of regulated signals and the size of candidate subpathways. The researchers employed $n = 1$ and $s = 8$ in their study to detect the candidate subpathways.

Extraction of Significant Subpathways

To evaluate whether the candidate subpathways were competitively regulated by lncRNAs, the researchers utilized the Wallenius approximation to estimate the significance of candidate subpathways based on BiasedUrn (Epstein et al. 2012). Several parameters offering different insights were employed: (1). The count of mRNAs of interest (e); (2). The count of background mRNAs (f); (3). The count of background mRNAs annotated in a given subpathways (x1); (4). The number of interesting mRNAs enriched in this given subpathway (x2); and (5). The weight score of this subpathway (w). The weight index meant the strength of competing regulation by lncRNAs participating in this subpathway. The raw P values were computed using the formula of P was equal to F (e, x1, x2, f, $weight_i$). Then, FDR was used to correct the original P values, and the significant subpathways were identified based on the threshold of a FDR<0.01.

The weight value was calculated using the following equation:

$$w = 1 + \beta \left(-\log_2 \left(\frac{G_L}{P_G} \right) \right)$$

Where, P_G stood for the gene number of this subpathway, as well as G_L represented the number of genes competitively mediated by lncRNAs involved in this subpathway. β denoted the parameter of control (β = 1 herein).

Identifying Hub lncRNAs in AD-associated LRSP Network

As we all know, hub nodes in the network frequently reflect the key roles of the network. In biological network, the degree connectivity of a node was determined as the total number of edges linking all the nodes. Therefore, in the current study, the researchers measured the degree distribution of the LRSP network and the top ten percent of the lncRNAs having the highest degree connectivity in the LRSP network were regarded as hub nodes.

RESULTS

Detection of Background Pathways

Before identifying the background pathways, the researchers firstly analyzed the co-expression probability for any pair of relationships of the candidate lncRNA-mRNA interactions according to the matched lncRNA and mRNA expression profiles using the PCC. Using the criteria of P<0.05, a total of 88 lncRNAs, 302 mRNAs and 378 pair co-expressed interactions were reconstructed. After obtaining the co-expressed interactions, the researchers putted those 302 mRNAs to all the KEGG reference pathways to further extract the background pathways. Finally,

Table 1: Background pathways using the threshold of false discovery rate (FDR) < 0.01

Pathways	*FDR*
Hepatitis B	7.74E-14
Bladder cancer	6.23E-12
PI3K-Akt signaling pathway	9.57E-11
Chronic myeloid leukemia	4.42E-11
Prostate cancer	3.12E-11
p53 signaling pathway	9.55E-10
Glioma	4.43E-10
HTLV-I infection	2.25E-10
Pathways in cancer	1.89E-10
Cell cycle	1.03E-10
Small cell lung cancer	6.63E-09
Pancreatic cancer	4.91E-09
Focal adhesion	5.90E-08
Melanoma	1.53E-08
Transcriptional misregulation in cancers	7.77E-07
Non-small cell lung cancer	2.44E-07
Colorectal cancer	5.58E-06
Endometrial cancer	5.58E-06
Viral carcinogenesis	2.66E-06
Thyroid cancer	1.30E-06
Measles	2.64E-05
Acute myeloid leukemia	4.28E-04
ErbB signaling pathway	1.85E-04
Apoptosis	1.65E-04
Adherens junction	1.54E-04
VEGF signaling pathway	1.50E-04
Chagas disease (American trypanosomiasis)	9.35E-03
Toxoplasmosis	8.75E-03
Legionellosis	6.46E-03
Epithelial cell signaling in Helicobacter pylori infection	5.97E-03
Neuroactive ligand-receptor interaction	5.38E-03
Axon guidance	5.29E-03
MAPK signaling pathway	5.27E-03
TGF-beta signaling pathway	4.89E-03
Wnt signaling pathway	4.38E-03
Tight junction	3.87E-03
Natural killer cell mediated cytotoxicity	3.63E-03
Rheumatoid arthritis	3.37E-03
Neurotrophin signaling pathway	3.34E-03
Gap junction	3.10E-03
HIF-1 signaling pathway	2.93E-03
Long-term depression	2.73E-03
Oocyte meiosis	2.01E-03
Ribosome biogenesis in eukaryotes	2.01E-03
Epstein-Barr virus infection	1.57E-03
NF-kappa B signaling pathway	1.07E-03

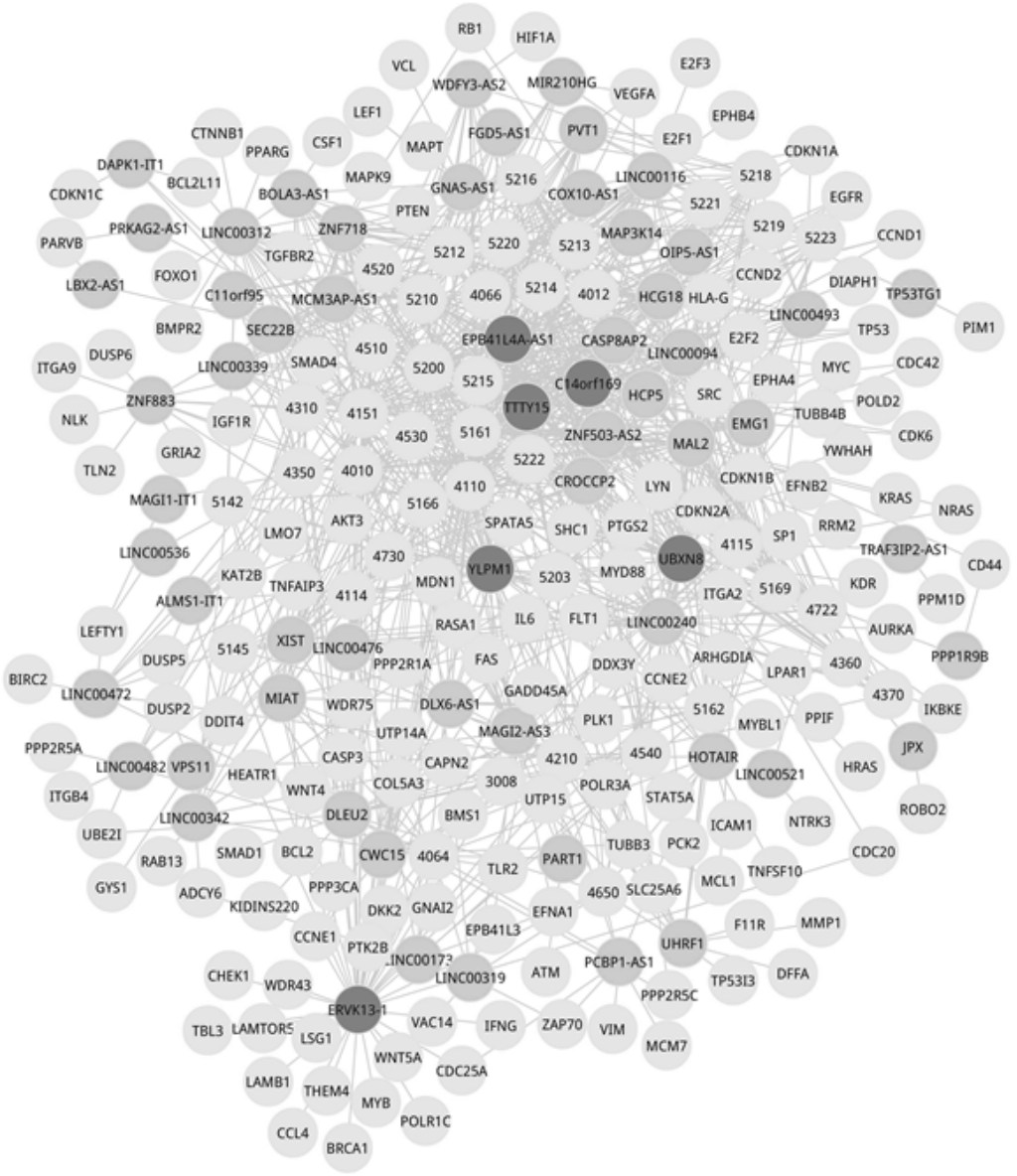

Fig. 2. AD-relevant lncRNA competitively regulated signal subpathway network. Pink nodes were mRNAs, and purple nodes denoted pathway IDs, green and yellow nodes stood for lncRNAs. Significantly, yellow ones denoted hub lncRNAs
Source: Author

a total of 46 background pathways were dissected using the threshold of FDR less than 0.01, as shown in Table 1. Significantly, most of these background pathways were found to be associated with cancer and inflammation.

Detecting Significant Subpathways Competitively Regulated by lncRNAs

After getting the background pathways, these background pathways were transformed

into undirected graphs, and the researchers putted those 88 lncRNAs in the reconstructed lncRNAs-mRNA interactions into the pathway graphs as nodes by linking to their regulated-genes. Thus, the condition-specific LRSP was established, which covered lncRNAs, pathways, as well as lncRNA-mRNA competitively regulated edges. The LRSP structure data is exhibited in Figure 2. From this LRSP network, the researchers observed that overall 61 significant lncRNAs competitively regulating subpathways involved in 41 complete pathways with a FDR<0.01. These 41 complete pathways including apoptosis, MAPK signaling pathway, HIF-1 signaling pathway and other pathways were listed in Table 2.

In further analysis, the researchers only focused on the top three subpathways with the smallest FDR values (Fig. 3). The most significant subpathway was path: 04210_1, which was a subregion of pathway of apoptosis (Fig. 3A). From this subpathway, the researchers found that this subpathway was competitively mediated by 2 lncRNAs (ERVK13-1, and YLPM1). Among the two lncRNAs, ERVK13-1, directly regulated 10 genes.

The second was the significant subpathway path: 04010-1, an important sub region in MAPK signaling pathway (Fig. 3B). Within this subregion, there were 21 lncRNAs which competitively mediated the subpathway of MAPK signaling subpathway. Significantly, MYC gene was simultaneously regulated by three lncRNAs (MAL2, UBXNB, and CASP8AP2). Similarly, TP53 were also synergistically controlled by three lncRNAs (LINC00094, C14orf169, and EPB41L4A-AS1). Interestingly, the lncRNA MAGI2-AS3 mediated the expression of three genes (CASP3, GADD45A and RASA1).

The third significant subpathway, path: 04066_1, was a part of HIF-1 signaling pathway (Fig. 3C). Based on this subpathway, mRNA CDKN1A was competitively regulated by five lncRNAs, LINC00116, OIP5-AS1, LINC00493, UBXN8 and MAP3K14. Moreover, four lncRNAs simultaneously regulated one gene VEGFA.

Based on these results, the researchers indicated that this strategy could extract the biological meaningful subpathways, and dissect several key lncRNAs in AD.

Table 2: Subpathways list based on FDR < 0.01

Pathway ID	*Pathways*	*FDR*
04210_1	Apoptosis	1.01E-18
04010_1	MAPK signaling pathway	1.06E-18
04066_1	HIF-1 signaling pathway	1.59E-18
04110_1	Cell cycle	1.73E-18
04114_1	Oocyte meiosis	3.26E-18
04115_1	p53 signaling pathway	8.35E-18
04151_1	PI3K-Akt signaling pathway	9.90E-18
03008_1	Ribosome biogenesis in eukaryotes	5.14E-17
04310_1	Wnt signaling pathway	9.03E-17
04360_1	Axon guidance	9.25E-17
04510_1	Focal adhesion	1.01E-16
04540_1	Gap junction	2.69E-16
05161_1	Hepatitis B	3.26E-16
05166_3	HTLV-I infection	3.43E-16
05200_1	Pathways in cancer	3.62E-16
05210_1	Colorectal cancer	3.95E-16
05212_1	Pancreatic cancer	8.42E-16
05213_1	Endometrial cancer	8.49E-16
05214_1	Glioma	2.16E-15
05215_1	Prostate cancer	2.44E-15
05218_1	Melanoma	2.71E-15
05219_1	Bladder cancer	2.81E-15
05220_1	Chronic myeloid leukemia	1.36E-14
05222_1	Small cell lung cancer	4.82E-14
05223_1	Non-small cell lung cancer	3.09E-12
05162_2	Measles	1.62E-11
05203_1	Viral carcinogenesis	1.16E-09
04520_1	Adherens junction	1.16E-09
04722_1	Neurotrophin signaling pathway	1.16E-09
04350_1	TGF-beta signaling pathway	3.91E-09
04370_1	VEGF signaling pathway	1.66E-08
05216_1	Thyroid cancer	8.06E-08
04012_1	ErbB signaling pathway	1.42E-07
04530_1	Tight junction	7.25E-07
04730_1	Long-term depression	1.88E-06
05221_1	Acute myeloid leukemia	7.03E-06
04064_1	NF-kappa B signaling pathway	7.45E-06
04650_3	Natural killer cell mediated cytotoxicity	7.03E-06
05142_1	Chagas disease (American trypanosomiasis)	7.45E-06
05169_3	Epstein-Barr virus infection	2.38E-05
04650_2	Natural killer cell mediated cytotoxicity	6.27E-04

Dissecting Hub lncRNAs in AD-relevant LRSP Network

To further identify key AD-relevant lncRNAs, the researchers conducted degree analysis for all nodes of the condition-specific LRSP. On the basis of the degree distribution, a total of 6 hub lncRNAs were identified, including YLPM1 (degree = 46), UBXN8 (degree = 44), ERVK13-1 (degree = 42), TTTY15 (degree = 39), C14orf169 (degree = 35), and EPB41L4A-AS1 (degree = 33).

DISCUSSION

LncRNAs have been observed to competitively regulate gene expression and to maintain normal cellular functions, and this regulatory

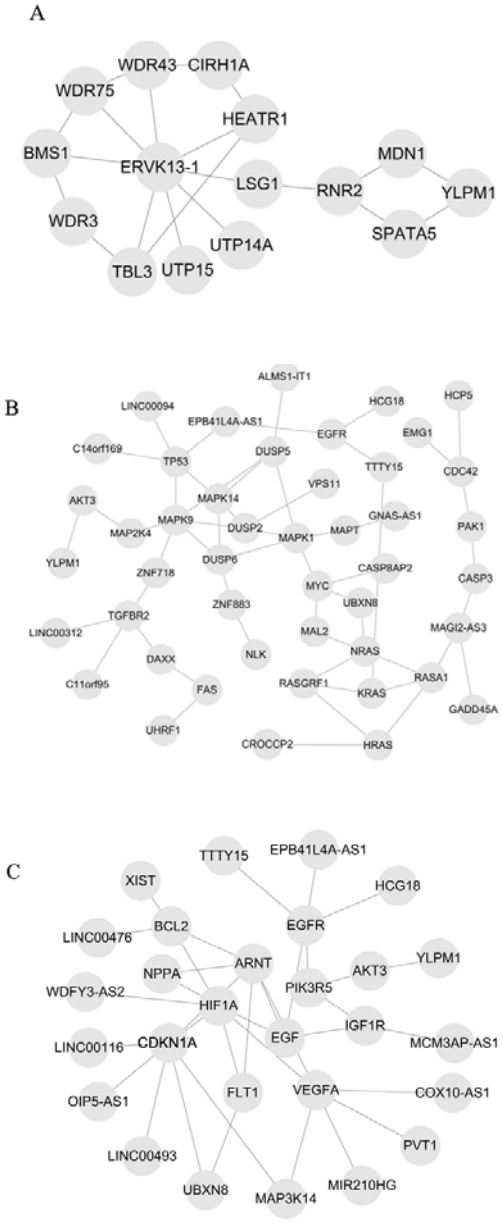

Fig. 3. Top 3 subpathways identified using the subregion method. Pink and green nodes were mRNAs and lncRNAs, respectively
(A) Apoptosis subpathway (path: 04210_1, FDR = 1.01E-18)
(B) MAPK signaling pathway (path: 04010_1, FDR = 1.06E-18)
(C) HIF-1 signaling pathway (path: 04066_1, FDR = 1.59E-18)
Source: Author

phenomenon may be beneficial for us to understand the biological complexity (Liang et al. 2011; Tay et al. 2014). Thus, a better understanding the disruption of the lncRNAs competitively mediated biological pathways might offer novel chance for developing new treatments. So far, few approaches have been proposed to detect the lncRNAs competitively regulated functions, and the biological roles of these lncRNAs have not be well revealed in disease condition. Hence, except analyzing the joint effect of genes and lncRNAs, pathway topologies must be taken into consideration. More specifically, concentrating more attention on subpathways (called subpathway strategy) rather than entire pathways, might extract more meaningful pathways and identify the biological roles of lncRNAs (Shi et al. 2016). Thus, in this study, this subregion strategy was used to detect lncRNAs competitively regulated subpathways for AD before and after treatment with CsA, thereby to further explore the antiprutitic mechanisms of CsA.

The most significant subpathway was subregion of pathway of apoptosis in the present study. Apoptosis is part of the normal process of epithelial cell renewal, and increased keratinocyte (KC) apoptosis has been implicated to be an important cause of AD (Gu et al. 2017). Remarkably, CsA has been noted to increase the apoptosis of KCs of rats (Tu et al. 2009). Targeting KCs apoptosis might indicate the severity of the lesion and the effectiveness of any treatment in AD (Kao et al. 2017). Therefore, the current study suggested that disruption of CsA-regulated the KCs apoptosis might offer a novel target for the therapy of AD.

The second significant subpathway was the subregion in MAPK signaling pathway. As demonstrated, dendritic cells (DCs) exert important functions in the initiation and progression of AD (Noordegraaf et al. 2010). Significantly, Shi et al. **(**2015) have reported that inhibiting DCs migration can effectively prevent AD development through desceasing the capacity of DCs to stimulate the cytokine generation. During the process of DCs differentiation and migration, MAPK signaling pathway play key roles (Li et al. 2017). MAPK signaling is one of the mechanisms affected during immune suppression (Li et al. 2017), which is involved in the regulation of CKR and COX-2 expression (Luo et al. 2003; Nagano et al. 2002). Moreover, another study has indicated that CsA-mediated suppression

of MAPK activation might be partly responsible for the inhibition of COX-2 and CKR expression observed in CsA-treated DCs (Chen et al. 2005). Thus, the researchers infer that CsA might be capable of treating AD effectively via suppressing the MAPK signaling pathway, and thus decreasing the DCs migration.

The third significant subpathway was a part of HIF-1 signaling pathway. As reported, HIF-1 enhances the transcription level of some genes which promote inflammation, including COX-2 (Marks et al. 2017). COX-2 is the key enzyme involved in prostaglandins (PG) generation in mediator-implicated inflammation. Moreover, PGE2 produced by COX-2 has been suggested to be increased in the skin of AD patients, and to enhance the development of TH2 cells (Lim et al. 2018). Interestingly, Laouini et al. (2005) have implicated that COX inhibitors may regulate the allergic skin inflammation in AD patients via mediating the systemic TH2 response. Of note, it has long been demonstrated that CsA could suppress the expression of COX-2 which in turn down-regulates the production of PGE2 (Elgowelli et al. 2014). Thus, these indicated that CsA might be effective for the management of AD via regulating the HIF-1 signaling pathway to further prevent the skin inflammation response.

In the AD-relevant LRSP network, lncRNA YLPM1 owned the highest degree connectivity, and this lncRNA stimultaneously participated in the top three subpathways. YLPM1 plays a key role in the reduction of telomerase activity during differentiation of cells (Blalock et al. 2014). More importantly, decrease of telomerase activity is related to cell cycle arrest (Rana et al. 2015). CsA, which block T cell activation and proliferation, produce an equivalent cell cycle arrest in cells of the immune system (Kawahara et al. 2015). Thus, the researchers further confirm that this subpathway method can not only dissect the lncRNA competitively regulated signal subpathways underlying disease status, but also help to reveal the biological roles of lncRNAs.

CONCLUSION

In short, the researchers successfully identified 61 significant lncRNAs competitively regulating subpathways involved in 41 complete pathways. Based on these results, the researchers indicated that the top 3 subpathways (apoptosis, MAPK signaling pathway, and HIF-1 signaling pathway) might play important roles in the treatment process of CsA in AD.

RECOMMENDATIONS

Nevertheless, several limitations must be taken into consideration. To begin with, there were limited samples. Moreover, the findings in the current study have not been verified using experiments. Thus, the results described above should be validated using experiments in future work.

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