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Identifying significant crosstalk of pathways in tuberous sclerosis complex

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Abstract. – OBJECTIVE: Tuberous sclerosis complex (TSC) is the second most common phakomatosis and is characterized by the formation of benign hamartomas and low-grade neoplasms in multiple organ systems. In this study, our objective here was to explore the interaction and crosstalk between pathways in response to tuberous sclerosis complex.

MATERIALS AND METHODS: We enriched the significant pathways and made the crosstalk analysis of the significant pathways.

RESULTS: The results showed that ECM-receptor interaction was a significant pathway in TSC. In addition, insulin-signaling and mTOR signaling also have been identified involved in TSC here, which have been well characterized. Further analysis indicated that there was a crosstalk between ECM-receptor interaction and antigen processing and presentation, ECM-receptor interaction and apoptosis, and leishmaniasis-oxidative phosphorylation-pancreatic cancer. In this study, a network-based approach was used to analyze the crosstalk among TSC related pathways. The crosstalk of pathways is found and analyzed using the PPI datasets and expression profiles.

CONCLUSIONS: Our work showed that comprehensive and system-wide analysis could provide evidence for TSC pathway and complement the traditional component-based approaches. The crosstalk identified might provide new alternative insights into the TSC pathology, which may contribute to the development of novel therapeutic targets for TSC.

Key Words:
ECM-receptor interaction, Pathway crosstalk, Tuberous sclerosis complex, Leishmaniasis-oxidative phosphorylation-pancreatic cancer, Benign hamartomas.

Introduction

Tuberous sclerosis complex (TSC) is an autosomal dominant inherited disease occurring in roughly 1.5 million individuals worldwide, and is especially prevalent in newborns with an estimated occurrence of one in 6, 000 with an estimated prevalence of one in 6,000 newborns[1,2]. It is characterized by the development of benign tumors, named hamartomas, in many organs, including the kidneys, heart and skin[3]. The most common manifestations of TSC are seizures, epilepsy, mental retardation, cognitive impairment, challenging behavioral and skin abnormalities, and autism.

The molecular genetic basis of TSC is inactivating mutation of two suppressor genes, namely, *TSC1* and *TSC2*[4]. Of them, *TSC1* is located on chromosome 9q34 and encodes a 130-kDa hydrophilic protein hamartin, while. *TSC2* was identified on chromosome 16p13 and encodes a 200-kDa protein, tuberin, which can exhibit GTPase-activating protein function[5,6]. Many different types of mutation are common in the *TSC2* gene, including missense, inframe deletion and large deletion mutations, but very rare in *TSC1*[7,8]. Therefore, TSC2 *tsc2* mutations are much more common than TSC1 *tsc1* mutations (in a ratio of 4.2:1 ratio)[9]. Hamartin and tuberin bind to each other via their respective coiled-coil domains to form a functional hetero-dimer (TSC2-TSC1)[10]. Mutations in either *TSC1* or *TSC2* gene would result in the dissection of TSC2-TSC1complex and cause increased cell proliferation, cell size, cell adhesion and migration through insulin/mTOR pathway[11], cell cycle[12] and apoptosis[13] regulation pathway, cytoskeleton regulation[14], and focal adhesion pathways[15].

Given the complex nature of biological systems, pathways often need to function in a coordinated fashion in order to produce appropriate physiological responses to both internal and external stimuli[16]. Therefore, research on the interaction and crosstalk between pathways is impor-

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tant for understanding of potential pathogenesis of TSC and exploringe some new therapeutic targets. In this study, we used the similar strategy bioinformatics methods to explore crosstalk among pathways crosstalk in tuberous sclerosis complex disease.

Materials and Methods

Data Sources

We download all the pathways from KEGG[17] and protein-protein interaction (PPI) datasets from HPRD[18] and BIOGRID[19] database.

Then we constructed an ensemble protein-protein interaction (PPI) network by integrating two above existing PPI databases mentioned above in human. Total 326, 119 unique PPI pairs were collected, in which 39,240 pairs are were from HPRD and 379, 426 pairs are were from BIOGRID.

We extracted the gene expression profile data on tuberous sclerosis complex (TSC) patients with normal controls from BIOGRID Interaction Database[20], which were deposited in NCBI GEO (http://www.ncbi.nlm.nih.gov/geo/) database (ID: GSE16969). The Gene expression profiles of cortical tubers were compared with autopsy control specimens and perituberal tissue from the same patients to study the significantly expressed pathways and connection among them in the TSC.

The bioconductor packages such as limma method[21] was used to identify differentially expressed genes (DEGs). The original expression datasets from all conditions were processed into expression estimates values using the RMA method with the default settings implemented in Bioconductor, and then construct the linear model was constructed. The DEGs only with the fold change value larger than 2 and p-value less than 0.05 were selected.

Pathway Crosstalk Analysis

Here the crosstalk pathways are defined as those pathways which have the overlapping genes and edges with each other. The overlapping genes represent genes that are included in both two pathways while mean both of the two pathways included and the overlapping edges mean represent PPI interaction edges which two pathways both involve in both of the two pathways included the PPI interaction edges.

To determine the co-expressed significance of a gene pair in disease cases, we used the Pearson's correlation coefficient (PCC) test to calculate the *p*-value. Then we Mmapped those p-values to the nodes and edges in the PPI network collected from the HPRD[18] and BIOGRID[19] database. The following formula is used to define a function as the combination of statistical significance of an interaction by a scoring scheme. The detail desprcition could be seen in Liu et al[22].

$$S(e) = \mathrm{f}\,[\mathit{diff}\,(x), \mathit{corr}\,(\mathrm{x}, \mathrm{y}), \mathit{diff}\,(y)]$$

$$= -2 \sum_{\mathrm{i}=1}^{\mathrm{k}} \log_e(pi)$$

The diff(x) and diff(y) are differential expression assessments of gene x and gene y, respectively. cCorr(x,y) represents their the correlation between gene x and gene y and . f is a general data integration method that can handle multiple data sources differing in statistical power. In the formula, Wwhere k = 3, p1 and p2 are the p-values of differential expression of two nodes, p3 is the p-value of their co-expression.

Significant Pathways Analysis

$$Sp = \sum_{e \in P} S(e)$$

The frequency of scores that are larger than Sp is used as the significance *p*-value of pathway P to describe its importance.

We also used the Database for Annotation, Visualization and Integrated Discovery (DAVID)[23] for the pathway enrichment analysis with the *p*-vlaue value < 0.1 through inputting the DEGs dataset.

Then, the overlap pathways of the two enrichment analysis methods were selected as the significant.

Pathway Crosstalk Analysis

The detailed analysis of crosstalk of relationships among pathways is then investigated, especially of that with overlap of two significant pathways analysis results. For detail analysis of the crosstalk between the significant pathways, the hypergeometric test was used to find the significant GO terms in each pathway with the *p*-value < 0.05.

To define the interaction significance between pathways, we summarized all the scores of edges S(e) of all non-empty overlaps. Specifically, the

interaction score between two pathways is estimated by their overlapping status of weighted pathways in the following formula:

$$C(pi, pj) = \sum_{e \in Oij} S(e)$$

where Pi pi and Pj pj are two pathways, and O is their overlapping.

To estimate the significance of the overlapping between different pathways, we assigned a random sample which is 10^6 times of the same size in two pathways in the edges of pathway network and calculated their overlapping scores. The frequency that larger than C is regarded as the interaction significance *p*-value.

Significant GO Enrichment Analysis in Each Pathway

The functional enrichment among proteins in one pathway is defined as:

$$P = 1 - \sum_{i=0}^{k-1} \frac{\binom{f}{i}\binom{n-f}{m-i}}{\binom{n}{m}}$$

where n is the number of nodes in the network, *f* is the number of proteins annotated with a particular GO function, m is the number of proteins involved in the pathway and k is the frequency of the GO term. We identified the significant pathways through this GO function enrichment of the pathways respectivelyanalysis.

Results and Discussion

Significant Pathway Analysis

The bioconductor packages were used to detectedA total of 23 DEGs (detail see the methods)were detected. Use the DAVID with the DEGs to find the significant pathways. At last, we and only find one significant pathway, ECM-receptor interaction (hsa04512) with the *p*-value = of 0.0799 was identified using the DAVID method.

We also use the Sp to evaluate the importance of pathways (detail see the methods)The outcomes from evaluation of pathways by Sp indicated that . Ttotal 55 pathways were detected to be with the *p*-value < 0.1. Notablely, ECM-receptor interaction (hsa04512) was detected with a Sp = of 0.0176 was detected.

There is evidence that the extracellular matrix (ECM)-receptor interaction pathway is related to the progression of TSC. For instance, increased expression levels were observed for laminins, integrins, collagens, and extracellular matrix protein 2 in cortical tubers. In addition, robust expression of integrin β1 was observed in giant cells in tubers. This supports the previously suggested hypothesis that giant cells in TSC retain an immature and possibly stem cell-related phenotype. Low expression levels of integrin β1 were observed in the dysplastic neurons. Loss of integrin β1 results in excessive neuronal migration and a disorganized marginal zone .

In addition, it is well-characterized that insulin signaling and mTOR signaling pathway were involved in TSC. The role of the TSC2-TSC1 hetero-dimer in the insulin-signaling pathway was first revealed by genetic epistasis experiments in Drosophila. In brief, binding of insulin or insulin-like growth factors (IGFs) to their receptors results in recruitment and phosphorylation of the insulin receptor substrate and subsequent activation of phosphoinositide 3-kinase (PI3K). Activated PI3K converts phosphatidylinositol (4-5) bi-phosphate (PIP2) into phosphatidylinositol (3-5) tri-phosphate (PIP3), and leads to recruitment of Akt to the plasma membrane where it is phosphorylated and activated by PDK1. Activated Akt phosphorylates other proteins TSC2, and this phosphorylation results in disruption of the TSC2-TSC1 complex. This process in turn releases mTOR from its state of inhibition (by TSC2-TSC1 complex), and continue to mTOR pathways. Activated mTOR then phosphorylate p70S6K and 4E-BP1. P70S6K is responsible for increased ribosome synthesis through phosphorylation of the ribosomal S6 protein. Binding of 4E-BP1 with eIF4E inhibits 5′ cap-dependent mRNA translation and thus is an important regulator of protein translation. Loss of TSC1 or TSC2 results in mTOR-dependent increased phosphorylation of ribosomal protein S6, p70S6K, and 4E-BP1 and led to enhanced protein synthesis

Crosstalk Among the Pathways

We considered the pathway crosstalk between ECM-receptor interaction (hsa04512) and other significant pathways detected by the overlaping score. The detailed description of every pathway was is listed in the Table I. We find that these 18 significant pathways crosstalk were identified among these pathways (see Figure 1).

Table I. Significant pathway analysis.

Pathid	Size	Node	Edge	Sp	Description
hsa00020	31	45	70	0	Citrate cycle (TCA cycle)
hsa00480	50	1	1	0	Glutathione metabolism
hsa00604	15	1	1	0	Glycosphingolipid biosynthesis - ganglio series
hsa00785	3	1	1	0	Lipoic acid metabolism
hsa03022	36	91	464	0	Basal transcription factors
hsa04062	189	299	814	0	Chemokine signaling pathway
hsa04514	136	206	501	0	Cell adhesion molecules (CAMs)
hsa04612	79	235	539	0	Antigen processing and presentation
hsa04660	108	77	156	0	T cell receptor signaling pathway
hsa04670	118	39	74	0	Leukocyte transendothelial migration
hsa04730	70	75	152	0	Long-term depression
hsa05020	35	40	65	0	Prion diseases
hsa05131	64	103	216	0	Shigellosis
hsa05140	74	491	1693	0	Leishmaniasis
hsa05211	70	1	1	0	Renal cell carcinoma
hsa05212	70	653	2276	0	Pancreatic cancer
hsa05215	89	82	162	0	Prostate cancer
hsa05219	42	1	1	0	Bladder cancer
hsa05416	75	369	975	0	Viral myocarditis
hsa04020	178	205	489	1.00E-05	Calcium signaling pathway
hsa00030	27	9	11	2.00E-05	Pentose phosphate pathway
hsa04320	25	16	30	2.00E-05	Dorso-ventral axis formation
hsa04150	52	51	86	8.00E-05	mTOR signaling pathway
hsa04140	35	31	93	0.00013	Regulation of autophagy
hsa04662	75	51	147	0.00016	B cell receptor signaling pathway
hsa00190	134	9	14	0.00039	Oxidative phosphorylation
hsa04120	138	263	766	0.00039	Ubiquitin mediated proteolysis
hsa00600	40	8	10	0.00045	Sphingolipid metabolism
hsa00232	7	5	28	0.00058	Caffeine metabolism
hsa04114	114	407	1469	0.00065	Oocyte meiosis
hsa04621	62	116	314	0.0023	NOD-like receptor signaling pathway
hsa04146	78	70	176	0.00291	Peroxisome
hsa04810	216	71	140	0.00373	Regulation of actin cytoskeleton
hsa04210	88	94	247	0.0053	Apoptosis
hsa04130	36	65	213	0.00555	SNARE interactions in vesicular transport
hsa05120	68	5	7	0.00602	Epithelial cell signaling in Helicobacter pylori infection
hsa04620	102	37	85	0.00697	Toll-like receptor signaling pathway
hsa03020	29	59	220	0.0104	RNA polymerase
hsa00603	14	2	2	0.01146	Glycosphingolipid biosynthesis - globo series
hsa04512	84	99	246	0.0176	ECM-receptor interaction
hsa04610	69	112	289	0.02157	Complement and coagulation cascades
hsa02010	44	23	39	0.02293	ABC transporters
hsa05016	184	259	629	0.02799	Huntington's disease
hsa04060	265	161	453	0.02852	Cytokine-cytokine receptor interaction
hsa00460	7	4	4	0.03993	Cyanoamino acid metabolism
hsa05014	53	206	465	0.04399	Amyotrophic lateral sclerosis (ALS)
hsa04910	137	37	76	0.05021	Insulin signaling pathway
hsa04630	155	69	120	0.05334	Jak-STAT signaling pathway
hsa00532	22	4	4	0.05568	Glycosaminoglycan biosynthesis - chondroitin sulfate
hsa00730	8	3	3	0.05895	Thiamine metabolism
hsa00250	32	33	51	0.06265	Alanine, aspartate and glutamate metabolism
hsa00051	34	32	43	0.09198	Fructose and mannose metabolism
hsa04623	56	5	8	0.09304	Cytosolic DNA-sensing pathway
hsa00592	19	3	3	0.09807	alpha-Linolenic acid metabolism
hsa00410	22	2	2	0.0989	beta-Alanine metabolism

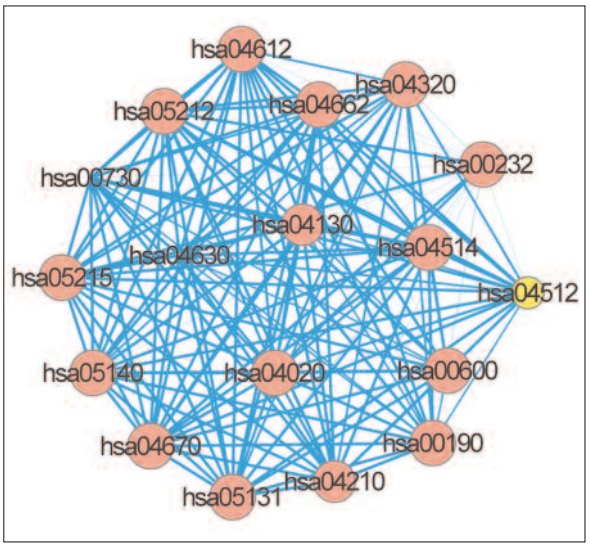
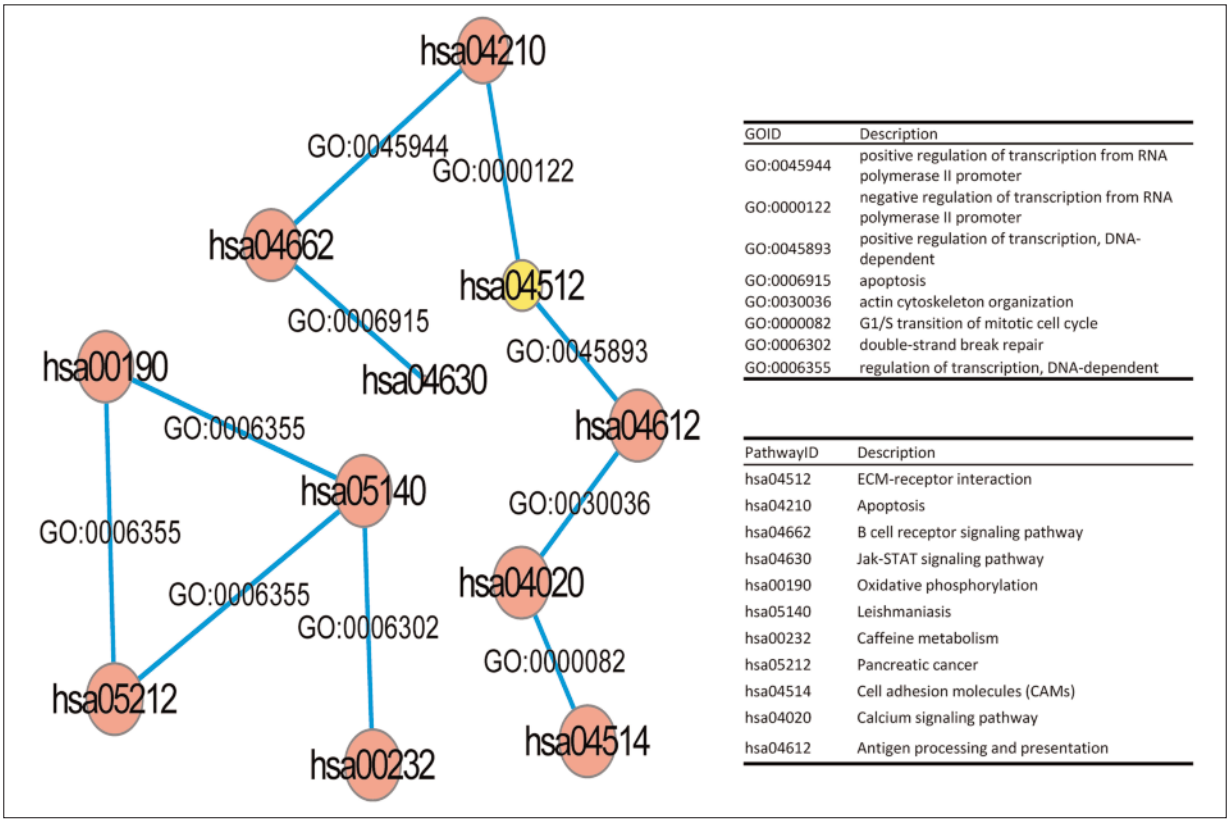


Figure 1. Crosstalk of TSC related pathways. The yellow node is the overlap of two significant pathway analysis results. The size of the nodes represents the significance of the pathways. The width of the edges represents the significance relationship between the two pathways.

Crosstalk of in GO Relationships Analysis Among Pathways

For detail analysis the crosstalk between the significant pathways, using the hypergeometric test to find the significant GO terms in each pathway with the p-value < 0.05, respectively. The results of the top five GO terms in part of the pathways are used to construct the connection among pathways. In As shown in the Figure 2, ECM-receptor interaction (hsa04512) crosstalk with the Apoptosis (hsa04210) pathway was negatively regulated through the transcription from RNA polymerase II promoter (GO: 0000122), whereas the hsa04512 crosstalk with the pathway of and Antigen processing and presentation (has04612) was positively regulated via the DNA-dependent transcription. through the negative regulation of transcription from RNA polymerase II promoter (GO: 0000122) and positive regulation of transcription, DNA-dependent (GO: 0045893), respectively. The pathway oxidative phosphorylation (hsa00190), leishmaniasis (hsa05140) and pancreatic cancer (hsa05212) crosstalk were pre-



GOID	Description
GO:0045944	positive regulation of transcription from RNA polymerase II promoter
GO:0000122	negative regulation of transcription from RNA polymerase II promoter
GO:0045893	positive regulation of transcription, DNA-dependent
GO:0006915	apoptosis
GO:0030036	actin cytoskeleton organization
GO:0000082	G1/S transition of mitotic cell cycle
GO:0006302	double-strand break repair
GO:0006355	regulation of transcription, DNA-dependent

PathwayID	Description
hsa04512	ECM-receptor interaction
hsa04210	Apoptosis
hsa04662	B cell receptor signaling pathway
hsa04630	Jak-STAT signaling pathway
hsa00190	Oxidative phosphorylation
hsa05140	Leishmaniasis
hsa00232	Caffeine metabolism
hsa05212	Pancreatic cancer
hsa04514	Cell adhesion molecules (CAMs)
hsa04020	Calcium signaling pathway
hsa04612	Antigen processing and presentation

Figure 2. Crosstalk with the overlapped top5 GO terms of pathways. Significantly enriched GO biological processes are identified in every pathway respectively. The size of the nodes represents the significance of the pathways. The edge of each pair of pathways represents the connection with the same GO terms.

sented to be regulated by the regulation of DNA-dependent transcription, DNA-dependent (GO: 0006355) among them. From the significant GO enrichments, we know the crosstalk of GO biological processes during the disease development among the pathwaysBased on the results indicated in the significant GO enrichment analysis, the crosstalk which may play a crucial role in the process of TSC development was identified.

Previous reports have demonstrated that there is crosstalk between the ECM-receptor interaction pathway and the antigen processing and presentation, in which hyaluronan (HA), as an ECM component, can be produced by dendritic cells (DC) and antigen-presenting cells (APCs) to promote antigen presentation and to augment T-cell activation and proliferation . HA promotes induction of Foxp3- IL-10-producing T cells with regulatory properties to abrogate inflammatory disease. This induction could be suppressed by another inflamed ECM component, osteopontin. Further, TR1 induction signals can be recapitulated using synthetic matrices, such as extracel, a commercially available HA and collagen (COL)-based hydrogel. Therefore, ECM is suggested as a biosensor for inflammatory micro-environments that plays a critical role in peripheral immune tolerance .

Identically, there is crosstalk between the ECM-receptor interaction pathway and apoptosis. The ECM of bone consists mainly of collagen type I, which induces osteoblastic differentiation and prevents apoptosis. Fas induces apoptosis in cells improperly adhering to ECM . In addition, etoposide and radiation induce G2/M cell cycle arrest in small-cell lung cancer (SCLC) cells prior to apoptosis and that ECM prevents this by overriding the upregulation of p21Cip1/WAF1 and p27Kip1 and the down-regulation of cyclins E, A and B. ECM activates phosphatidylinositol 3-kinase (PI3-kinase) signaling in SCLC cells and prevents etoposide-induced caspase-3 activation and subsequent apoptosis in a β1 integrin/PI3-kinase-dependent manner .

Mitochondria are dynamic intracellular organelles playing a central role in cell metabolism by generating ATP, through the oxidative phosphorylation system . Dysregulated mitochondrial fusion and fission events can now be regarded as playing a role in cancer onset and progression. Accordingly, mitochondria-shaping proteins seem to be an appealing target to modulate the mitochondrial phase of apoptosis in cancer cells. In fact, several cancer tissues, including pancreatic showed a pattern suggestive of enlarged mitochondria resulting from atypical fusion . Leishmaniasis is a complex disease, and is caused by over 15 different species of the protozoan parasite genus Leishmania . Mitochondrial oxidative phosphorylation is also the main source of ATP in Leishmania parasites. Antimicrobial peptide, histatin 5, produces a bioenergetic collapse of the parasite, caused essentially by the decrease of mitochondrial ATP synthesis through inhibition of F1F0-ATPase, with subsequent fast ATP exhaustion.

Discussion

There is evidence that the extracellular matrix (ECM)-receptor interaction pathway is related to the progression of TSC. For instance, increased expression levels were observed for laminins, integrins, collagens, and extracellular matrix protein 2 in cortical tubers. In addition, robust expression of integrin β1 was observed in giant cells in tubers. This supports the previous hypothesis that giant cells in TSC retain an immature and possibly stem-cell-related phenotype. Low expression levels of integrinβ1 were observed in the dysplastic neurons. Loss of integrin β1 results in excessive neuronal migration and a disorganized marginal zone[24].

In addition, it is well-characterized that insulin signaling and mTOR signaling pathways were related to TSC. The role of the TSC2-TSC1 hetero-dimer in the insulin-signaling pathway was first revealed by genetic epistasis experiments in Drosophila[25]. In brief, binding of insulin or insulin-like growth factors (IGFs) to their receptors results in recruitment and phosphorylation of the insulin receptor substrate and subsequent activation of phosphoinositide 3-kinase (PI3K). Activated PI3K converts phosphatidylinositol (4, 5) bisphosphate (PIP2) into phosphatidylinositol (3, 4, 5) trisphosphate (PIP3), and leads to recruitment of Akt to the plasma membrane where it is phosphorylated and activated by PDK1. Activated Akt phosphorylates other proteins like TSC2, and this phosphorylation results in disruption of the TSC2-TSC1 complex. This process in turn releases mTOR from its state of inhibition (by TSC2-TSC1 complex), and continues to mTOR pathways[10,26]. Activated mTOR then phosphorylates p70S6K and 4E-BP1. P70S6K is responsible for increased ribosome synthesis through phosphorylation of the ribosomal S6 protein.

Binding of 4E-BP1 with eIF4E inhibits 5′ cap-dependent mRNA translation, which make 4E-BP1 an important regulator of protein translation[27]. Other researches demonstrated that loss of TSC1 or TSC2 results in mTOR-dependent increased phosphorylation of ribosomal protein S6, p70S6K, and 4E-BP1 and leds to enhanced protein synthesis[28-30]. Our results indicated that the pathways of DEGs from data on TSC patients was significantly enriched in ECM-receptor interaction (hsa04512), as well as insulin-signaling and mTOR signaling pathways, which suggesting that genes related to TSC may participate in these pathways through phosphorylation involved in TSC2-TSC1 complex.

Previous reports have demonstrated that there is a crosstalk between the ECM-receptor interaction pathway and the antigen processing and presentation, in which hyaluronan (HA), as an ECM component, can be produced by dendritic cells (DC) and antigen-presenting cells (APCs) to promote antigen presentation and to augment T-cell activation and proliferation[31]. HA is verified to be entitled to promote induction of Foxp3-IL-10-producing T cells with regulatory properties to abrogate inflammatory disease[32]. This induction could be suppressed by another inflamed ECM component, osteopontin. Further, TR1 induction signals can be recapitulated using synthetic matrices, such as extracel, a commercially available HA and collagen (COL)-based hydrogel. Therefore, ECM is suggested as a biosensor for inflammatory micro-environments that plays a critical role in peripheral immune tolerance[33].

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Mitochondria are dynamic intracellular organelles playing a central role in cell metabolism by generating ATP, through the oxidative phosphorylation system[37]. Dysregulated mitochondrial fusion and fission events can now be regarded as crucial roles in cancer onset and progression[38]. Accordingly, mitochondria-shaping proteins seem to be an appealing target to modulate the mitochondrial phase of apoptosis in cancer cells. In fact, several cancer tissues, including pancreatic showed a pattern which was suggestive of enlarged mitochondria resulting from atypical fusion[39]. Leishmaniasis is a complex disease, and is caused by over 15 different species of the protozoan parasite genus Leishmania[40]. Mitochondrial oxidative phosphorylation is also the main source of ATP in Leishmania parasites. Antimicrobial peptide, histatin 5, produces a bioenergetic collapse of the parasite, caused essentially by the decrease of mitochondrial ATP synthesis through inhibition of F1F0-ATPase, with subsequent fast ATP exhaustion[41]. In the present study, our results revealed the regulation relationships of crosstalks between TSC-related pathways, which may contribute to the illustration of regulatory mechanism involved in TSC.

Conclusions

In this study, a network-based approach was used to analyze the crosstalk among TSC related pathways. The crosstalk of pathways is found and analyzed using the PPI datasets and expression profiles. T and the results are were all in consistent with our prior knowledge. Taken together, the crosstalks identified and their relationships indicated in the present study might provide new alternative insights into the TSC pathology, which may contribute to the development of novel therapeutic targets for TSC, and may offer a foundation for the deeper investigation of pathway regulatory networks in TSC development.

The crosstalk of pathways presents new alternative insights for TSC pathology. Our work shows that comprehensive and system-wide analysis provides evidence for TSC and complements the traditional component-based approaches.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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