International Journal of Pharmacology

ISSN 1811-7775

science alert

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Asian Network for Scientific Information

OPEN ACCESS International Journal of Pharmacology

ISSN 1811-7775
DOI: 10.3923/ijp.2022.983.993

Research Article
Prognostic Values of Tanshinone in Mediating the Activity of Human Umbilical Vein Endothelial Cells

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Abstract

Background and Objective: Little is known about the roles of tanshinone in the pathogenesis of disease onset of cardiovascular diseases. In this study, we aimed to identify disease-related genes and analyze prognostic values of tanshinone in the Human Umbilical Vein Endothelial Cells (HUVECs). **Materials and Methods:** The MTT assay was performed to investigate the cellular availability in the groups including the control group, vehicle group and treatment groups. The tanshinone group was treated with different concentrations of tanshinone. The MTT assay was performed to evaluate the cellular proliferation. Microarray expression profiles of GSE19804 were obtained from the gene expression omnibus (GEO) database. Clustering analysis was utilized to analyze the differentially expressed genes among these groups. The GO and KEGG pathway enrichment was analyzed based upon DEGs. **Results:** Tanshinone inhibited the proliferation of HUVECs. Tanshinone induced differential expression of genes *in vitro*. Enrichment analysis indicated that the ERK5 signalling pathway ranked first in the GO and KEGG analysis. **Conclusion:** Tanshinone played important role in mediating the signalling pathways in the HUVECs. It could induce differentially expressed genes.

Key words: Cardiovascular diseases, tanshinone, KEGG analysis, signalling pathways, differentially expressed genes, microarray, cellular proliferation

Citation: Tang, X., C. Chen, G. Cheng, Y. Chen, C. Li and X. Yang, 2022. Prognostic values of tanshinone in mediating the activity of human umbilical vein endothelial cells. Int. J. Pharmacol., 18: 983-993.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Tanshinone, a component obtained from the medicinal herb *Salvia miltiorrhiza*, has been reported to possess neuroprotective effects against the pathological features of several diseases[1,2]. Many active components have been confirmed to play important roles in the modulation of several conditions and diseases. For example, tanshinone IIA (Tan IIA), is an abietane-type diterpene quinone, extracted from the traditional Chinese herbal medicine *S. miltiorrhiza* (Danshen) which is a well-established medicine in the treatment of cardiovascular diseases[3,4]. To date, increasing *in vivo* evidence indicates that Tan IIA shows neuroprotective roles in the cholinergic system, which then results in improvements in the pathological changes caused by Alzheimer disease (AD)[5], however, the underlying mechanisms are largely unclear after a comprehensive literature search.

Recently, gene microarray and bioinformatics analysis were widely used to identify potential biomarkers of several diseases including cancer[6]. Interestingly, studies were performed to identify disease-related genes, which were associated with the prognosis of breast cancer, by using integrated bioinformatics methods[7]. Similarly, some studies were performed to find important key genes in lung cancer[8]. Nevertheless, rare studies have been conducted to identify the differentially expressed genes after treating with tanshinone.

In this study, we aim to identify disease-related genes and analyze prognostic values of tanshinone in the Human Umbilical Vein Endothelial Cells (HUVECs).

MATERIALS AND METHODS

Study area: The study was carried out at Department of Gerontology, The First Affiliated Hospital of Shandong First Medical University and Shandong Provincial Qianfoshan Hospital from January, 2012 to December, 2015.

Grouping: The HUVEC were purchased from Maide Biotech (Jinan, China) and were cultured according to the previous description. Tanshinone was purchased from Baili Biotech (Shanghai, China). The cells were divided into three parts: Control group, vehicle group and tanshinone group. The tanshinone group was treated with different concentrations of tanshinone.

MTT assay: Cells (5×10^3) in each group were incubated for 48 hrs, followed by MTT assay as previously described[9]. Briefly, 20 µL MTT (5 mg mL^{-1}) was added to the cell mixture, followed by removal of the culture about 4 hrs later. Then DMSO (100 µL) was added and then vortexed for 3-5 min. Finally, the cellular proliferation was evaluated by determining the OD value using a micro reader at a wavelength of 490/570 nm.

Microarray data: Data about the GSE19804 microarray expression profile were obtained from the gene expression omnibus (GEO) database. The GSE19804 was established based on the GPL570 platform (Affymetrix Human Genome U133 Plus 2.0 Array, CA, USA), which included 60 samples obtained from nonsmoking female NSCLC patients and 60 normal samples.

Clustering analysis: To remove redundancy among the amino acid sequences, clustering analysis was performed using the BLASTclust program in the BLAST package from NCBI (http://www.ncbi.nlm.nih.gov/BLAST/download.shtml). The BLASTclust program was run with the sequence identity threshold set to 25% and the longest sequence in each cluster was selected for the non-redundant dataset, PRINR25.

Encoding method: To investigate the association between GO terms or KEGG pathways and drug compounds, the enrichment theory of GO terms and KEGG pathways were utilized to represent each drug compound. For a certain drug compound d, G(d) served as a protein set with human proteins showing associations with d.

Database for Annotation, Visualization and Integrated Discovery (DAVID, version 6.7, www.david.ncifcrf.gov) were used to analyze the GO and KEGG pathway enrichment based upon DEGs. As to the biological process, the DEGs were significantly enriched in the regulation of cell proliferation, cell adhesion, biological adhesion, vasculature development and blood vessel development. About the cellular component, the DEGs were significantly enriched in the extracellular region part, extracellular region, extracellular space, cell surface and proteinaceous extracellular matrix. The most enriched GO terms included carbohydrate-binding, growth factor binding, pattern binding, calcium ion binding and polysaccharide binding. The most enriched KEGG pathway terms were focal adhesion, ECM-receptor interaction, neuroactive ligand-receptor interaction, cell adhesion molecules, as well as complement and coagulation cascades.

Statistical analysis: The statistical analysis was performed using the SPSS 20.0 software. Numeration data were presented as Mean±Standard deviation. Measurement data were presented as percentages or proportions. The student's t-test was performed for the inter-group comparison. The p-value of less than 0.05 was considered to be statistically significant.

RESULTS

Tanshinone inhibited the growth of HUVECs *in vitro*: In this section, we determined the growth of HUVECs under various conditions. Compared with the vehicle group, the Tanshinone group could significantly inhibit the growth of HUVECs. There was no dose-depending pattern in the inhibitory effects of tanshinone based on the growth of HUVECs (Fig. 1).

Tanshinone modulated the expression of various genes: Real-time PCR was conducted to determine the expression of 30 genes. Among these genes, 18 genes were up-regulated after tanshinone treatment. In addition, 12 genes were down-regulated in the tanshinone group compared with the vehicle group.

The genes that were differentially expressed in the treatment group were marked in red colour. Then further clustering analysis was conducted on these genes. Figure 2 presented the distribution of differentially expressed genes in the treatment group and control group. The red colour presented the differentially expressed genes with an expression variation fold of >2 fold ($p<0.05$).

Clustering analysis of the gene spectrum: The differentially expressed genes in the treatment group and control group were analyzed as shown in Fig. 3. The red colour represented the genes that were up-regulated, while the green colour represented the down-regulated genes. The black colour represented moderate expression. The arbour in the upper part was drawn based on the clustering analysis of the samples. The arbour in the left part was drawn based on the signals of the differentially expressed genes.

Selection of genes involved in the signalling pathway: The differentially expressed genes regulated signalling pathways were summarized based on the IPA in the signalling pathway and the metabolic pathway. All the signalling pathways were arranged in this section based on the p-values. Signalling pathways marked in a yellow colour represented a Z score of more than 0, while those marked in a blue colour represented a Z score of less than 0. A Z score of >2 indicated that the signalling pathways were activated. A Z score of less than -2 demonstrated that the signalling pathways were inhibited. The ratio represented the proportion of the number of differentially expressed genes and the total number of genes involved in the signalling pathway. As shown in Fig. 4, the ERK5 signalling pathway was significantly inhibited with a Z score of -2.530.

Figure 5 indicated the signalling transmission of the predicted signalling pathways. Through molecular activation prediction, we predicted the inhibition and activation of some genes. Among the signalling pathways with a |Z-score|>2, the Extracellular-Signal-Regulated Kinase 5 (ERK5) signalling pathway ranked first in the enrichment analysis.

Disease and functional analysis: Figure 6 indicated the enrichment of the differentially expressed genes in certain diseases and functions. All the diseases and functions were

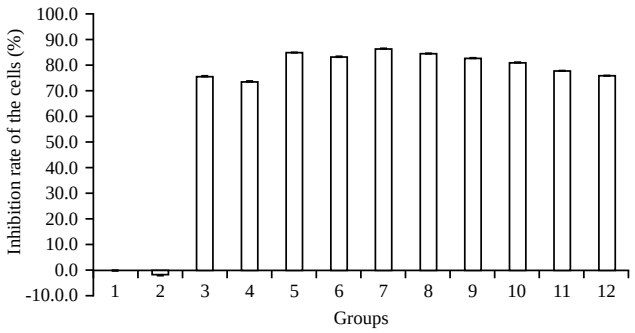

Fig. 1: Inhibition rate of cells in different treating groups

Group 1: HUVECs cultured under aerobic conditions, Group 2: HUVECs cultured under anaerobic conditions, Group 3 and 4: HUVECs treated with tanshinone (100 μg mL^{-1}) solution under aerobic and anaerobic conditions, Group 5 and 6: HUVECs treated with tanshinone (50 μg mL^{-1}) solution under aerobic and anaerobic conditions, Group 7 and 8: HUVECs treated with tanshinone (25 μg mL^{-1}) solution under aerobic and anaerobic conditions, Group 9 and 10: HUVECs treated with tanshinone (12.5 μg mL^{-1}) solution under aerobic and anaerobic conditions, Group 11 and 12: HUVECs treated with tanshinone (6.25 μg mL^{-1}) solution under aerobic and anaerobic conditions

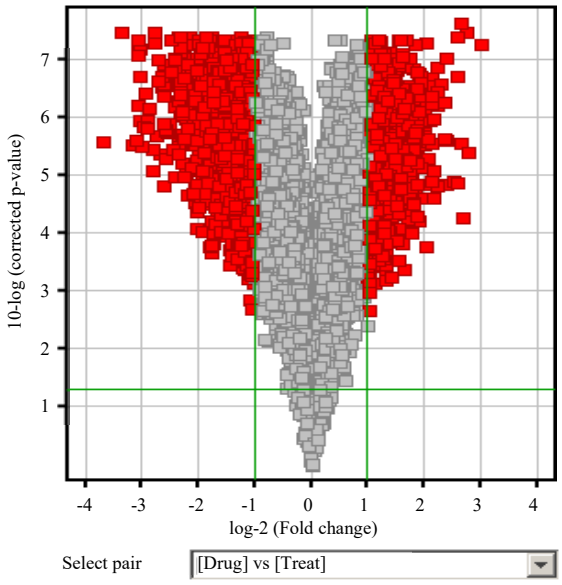

Fig. 2: Clustering analysis of the genes

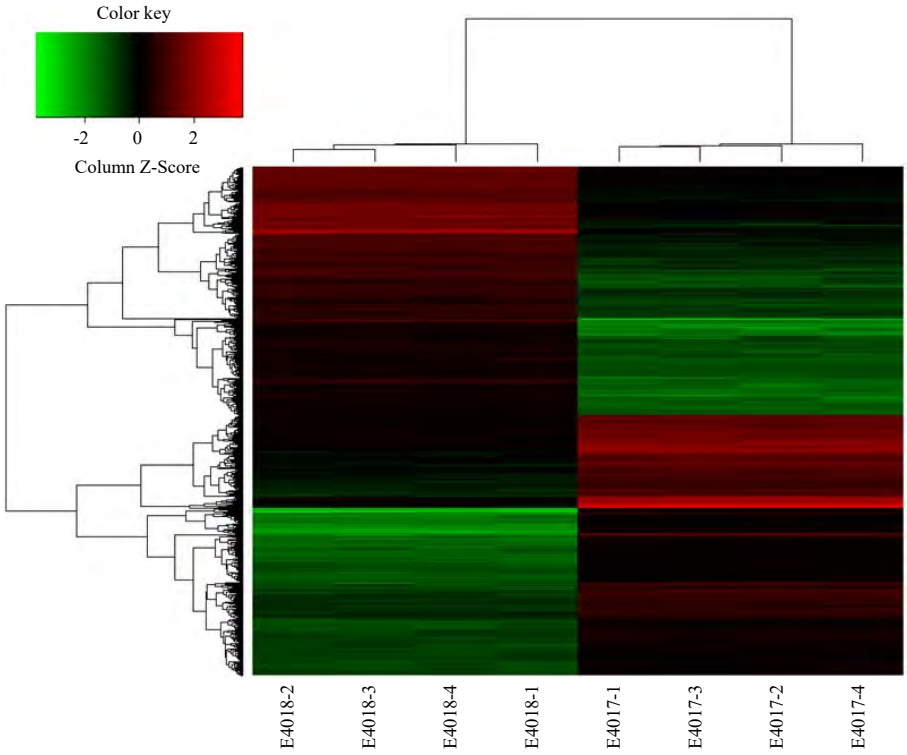

Fig. 3: Differentially expressed genes in each group

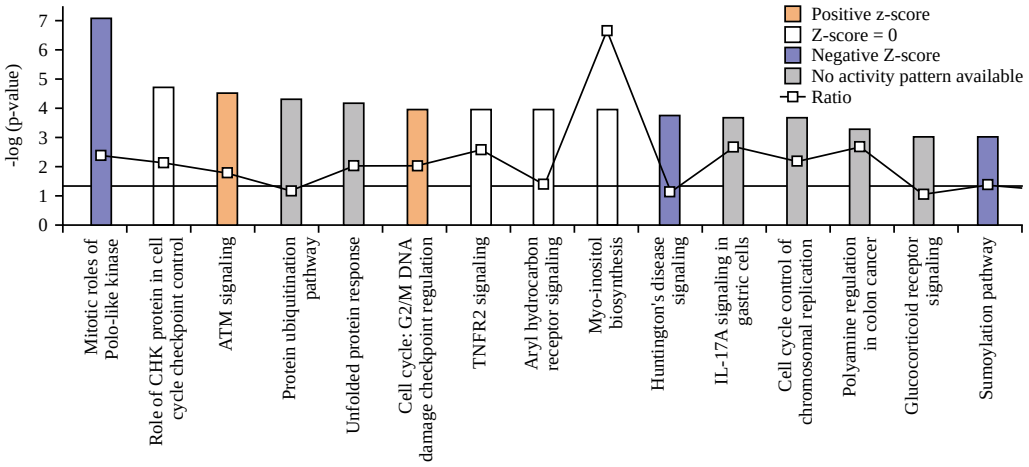

Fig. 4: Z-score of the signalling pathways mediated by tanshinones

ranked according to the -Log (p-value). The thermography of the disease and function represented the activation and/or inhibition of the differentially expressed genes. According to the Z score, the formation of gamma H2AX nuclear focus and cancer was activated with a Z score of 3.356 and 3.060, respectively. In contrast, the infection of cells and infection by RNA virus was inhibited with a Z score of -3.842 and -3.566, respectively.

Up-stream regulator analysis: The upstream regulators involved in this section included the cytokines, transcriptional factors, small RNA, kinase, receptors, drugs and chemical molecules. Based on the activation Z-score, we predicted the activation and/or inhibition of the upstream regulators using IPA. This data showed that *LY294002* was activated. For the *LY294002*, 73 genes were consistently activated and CSF2 was inhibited (Fig. 7).

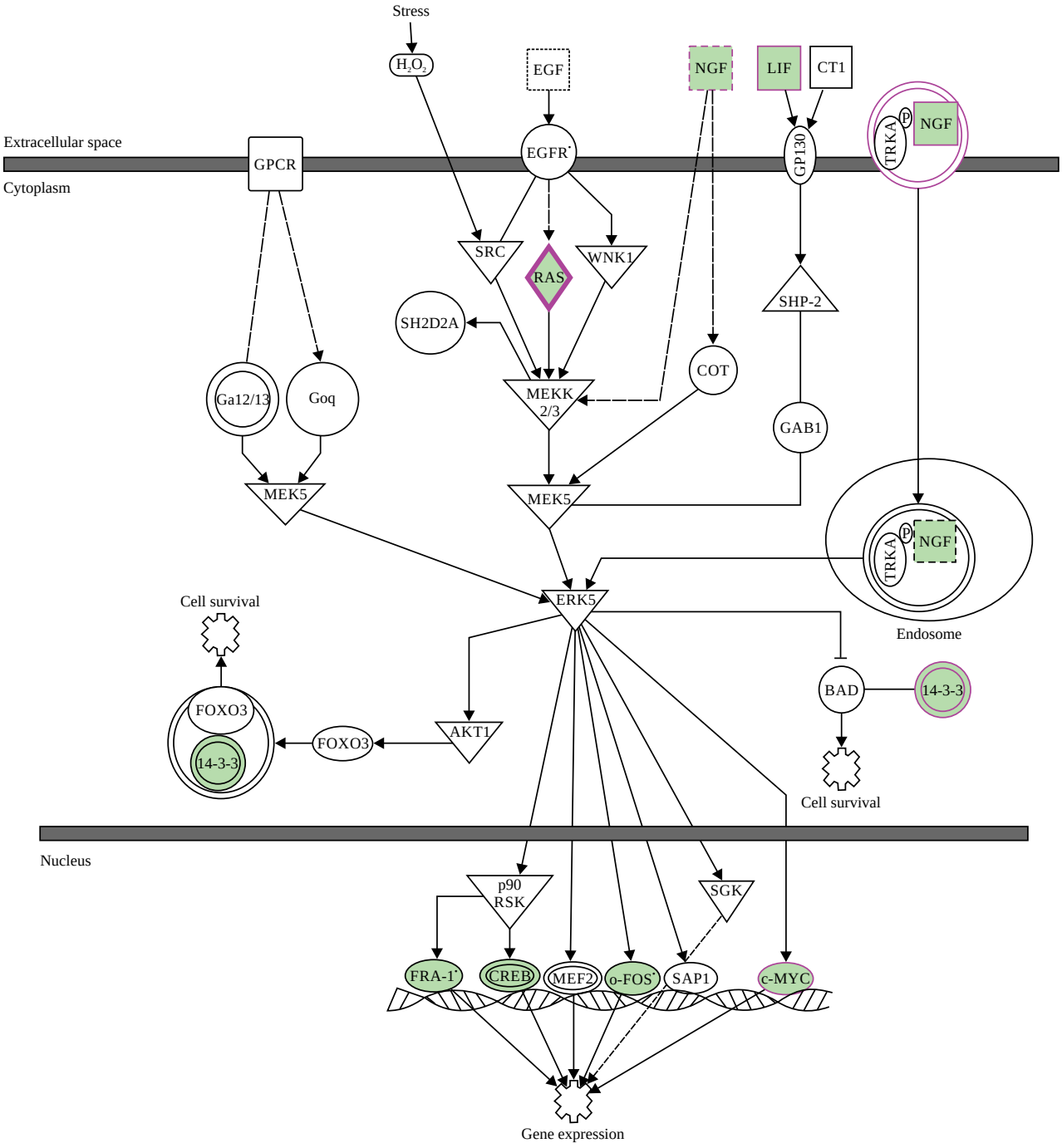

Fig. 5: Signalling transmission of the predicted signalling pathways

Figure 8 indicated the mutual interaction between upstream regulators and the downstream molecules. The orange line represented the consistent activation of the upstream regulators and the genes, while the blue line represented the consistent inhibition of the upstream regulators and the genes. The yellow line indicated the non-consistent expression of the upstream regulators and the genes. The grey line represented the non-availability of prediction information of the expression state. Colony-Stimulating Factor 2 (CSF2) could promote the expression of RELA mRNA, while in the treatment group, the expression of RELA showed obvious up-regulation. Therefore, there might be a consistent expression state for the CSF2 and IL1B.

Figure 9 showed the activation and inhibition correlation of the gene and function. The gene function or disease of the

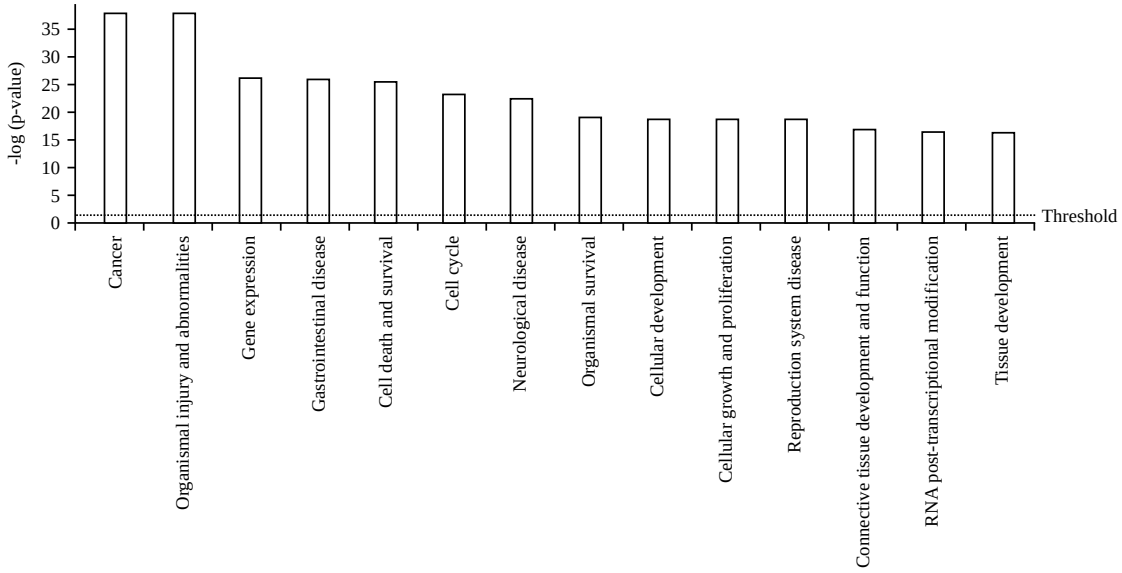

Fig. 6: Enrichment of the differentially expressed genes in certain diseases and functions

Upstream ...	Expr Fold ...	Molecule ...	Predicted Act...	Activation z-s...	p-value o...	Target mo...	Mechanisti...
TP53		transcription reg...		0.114	3.56E-38	↑AIFM2, ...all 252	549 (21)
CDKN1A		kinase	Activated	2.534	9.75E-33	↓ANLN, ...all 76	448 (15)
NUPR1		transcription reg...	Activated	2.228	1.28E-30	↑ABCC5, ...all 117	474 (9)
ERBB2		kinase	Inhibited	-5.733	4.46E-30	↓AHN... ...all 147	541 (19)
E2F4		transcription reg...		0.781	1.31E-23	↓ANLN, ...all 66	
CSF2	↓-7.057	cytokine	Inhibited	-7.337	6.72E-23	↑ABCA1, ...all 97	610 (21)
ESR1		ligand-dependen...	Inhibited	-3.315	8.95E-23	↓AASS, ...all 192	569 (20)
dextran sulfate		chemical drug		-1.195	2.02E-21	↓ANLN, ...all 65	449 (17)
EGF		growth factor	Inhibited	-6.296	5.45E-21	↓ALCA... ...all 100	465 (16)
PDGF BB		complex	Inhibited	-6.069	7.65E-21	↓ACAT2, ...all 76	480 (18)
TBX2		transcription reg...	Inhibited	-4.282	2.02E-20	↓ANLN, ...all 35	478 (16)
doxorubicin		chemical drug		-0.771	3.11E-20	↓AREG, ...all 84	537 (21)
E2F1	↓-2.025	transcription reg...	Inhibited	-5.139	3.44E-20	↑ATG14, ...all 97	365 (15)

Fig. 7: Up-stream regulators of the differentially expressed genes

datasets was summarized, to illustrate the up-regulation and down-regulation situation, as well as the prediction interaction based on the literature in the Ingenuity database. The orange line presented the activation of the differentially expressed gene on the functional module. The blue line presented inhibition of differentially expressed genes on the functional module. The yellow line presented the non-consensus of the effects of gene expression on the functional module. The grey line presented the predictive information that was not available in the database. The organismal death was not activated, which showed the highest |Z-score| in the function and disease.

Analysis of regulatory effects: Figure 10 showed the upstream regulatory network and the downstream function involved by the differentially expressed genes. Consistency score was the upstream regulator in the network. The IL1A regulator could inhibit the cell movement of tumour cell lines through the *ASIC1* gene.

Figure 11 summarized the interaction between the gene and regulator as well as the function in the datasets. The IL1A could inhibit the cell movement of tumour cells through modulating the expression of ASIC1, CCL20, CSF2, CXCL8, CYR61, FOS, IL1B, ITGAV, JUN, LIF, MYC, NGF, PLAU, RELA, SERPINA1, SERPINE1.

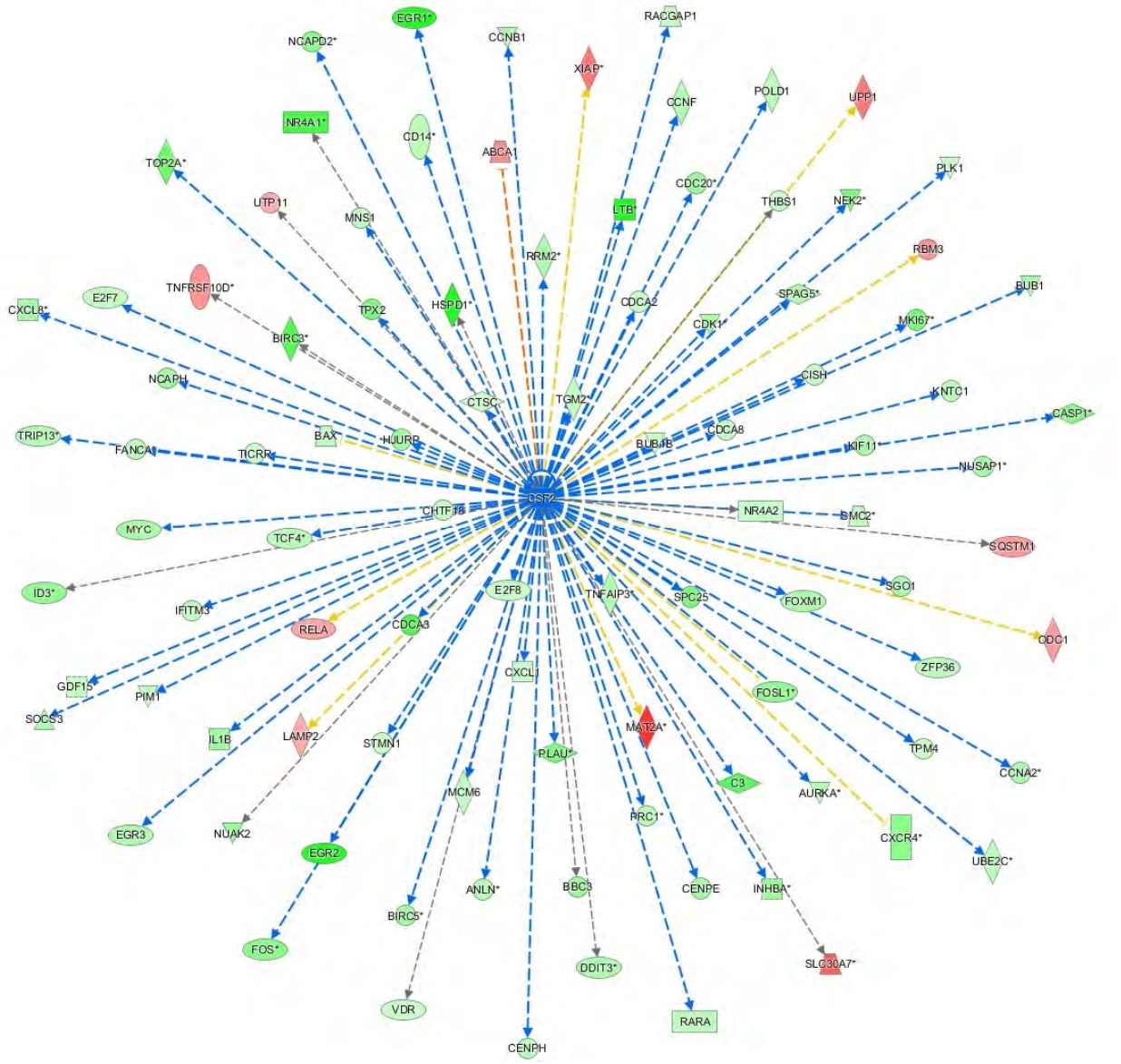

Fig. 8: Interaction between upstream and downstream regulators

DISCUSSION

In this study, a total of 1700 genes were screened, including 838 up-regulated genes and 862 down-regulated genes. Moreover, we selected two significant modules with several key DEGs in the regulatory network of nonsmoking female patients with cardiovascular diseases. Then clustering analysis was performed to analyze the situation of differential expression among these genes. The differentially expressed genes in the treatment group and control group may contribute to the investigation of the active components of Tanshinones in treating cerebral diseases.

In a previous study, the Tanshinone IIA (Tan IIA), one of the major components isolated from *Radix salvia miltiorrhiza*, exhibits potent antioxidant activity[10]. In addition, many experimental studies and clinical trials demonstrated that Tan IIA prevents oxidative stress-triggered atherogenesis as well as cardiac injury and hypertrophy[11]. In a previous study, the authors tried to illustrate the protective effect of Tan IIA on DOX induced cardiotoxicity[12], but the mechanisms are still not well defined. Besides, tanshinones showed protective roles against ischemic injury in cultured primary cortex neurons[13]. This study focused on the screening of genes that may involve in the proliferation and biological activity

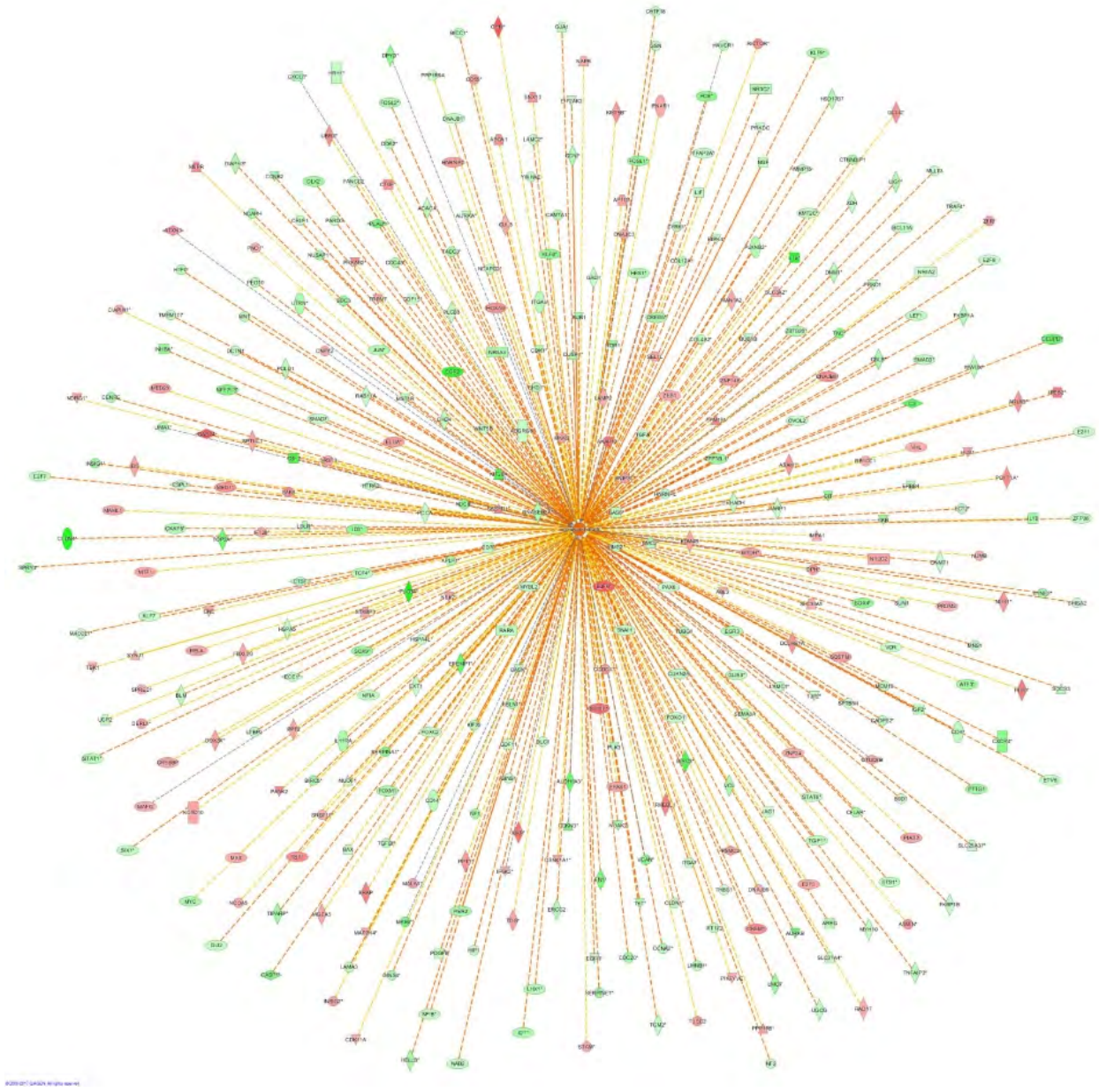

Fig. 9: Activation and inhibition correlation of the gene and function

ID	Consisten...	Node Total	Regulator...	Regula...	Target To...	Target ...	Disease ...	Diseas...	Known Re...
1	3.750	18	1	IL1A ...all 1	16	ASI....all 16	1	cell m....all 1	100% (1/1)
2	3.474	16	1	IL1A ...all 1	14	ASI....all 14	1	migra....all 1	100% (1/1)
3	3.464	14	1	carra... ...all 1	12	CX... ...all 12	1	cell m....all 1	100% (1/1)
4	3.317	13	1	FN1 ...all 1	11	CS... ...all 11	1	growt....all 1	100% (1/1)
5	3.317	13	1	pepti... ...all 1	11	AT... ...all 11	1	angio....all 1	0% (0/1)
6	3.182	10	1	AREG ...all 1	8	CCL... ...all 8	1	cell m....all 1	100% (1/1)
7	3.182	10	1	AREG ...all 1	8	CCL... ...all 8	1	cell p....all 1	100% (1/1)
8	3.182	10	1	FN1 ...all 1	8	CSF2, ...all 8	1	cell m....all 1	100% (1/1)
9	3.175	14	1	E. coli... ...all 1	12	CX... ...all 12	1	bindi... ...all 1	100% (1/1)
10	3.175	14	1	E. coli... ...all 1	12	CX... ...all 12	1	intera... ...all 1	100% (1/1)
11	3.175	14	1	KITLG ...all 1	12	BI... ...all 12	1	leuko... ...all 1	100% (1/1)
12	3.175	14	1	pepti... ...all 1	12	AT... ...all 12	1	devel... ...all 1	0% (0/1)
13	3.162	12	1	PRKCE ...all 1	10	BI... ...all 10	1	mitosis ...all 1	0% (0/1)
14	3.162	12	1	TAC1 ...all 1	10	CS... ...all 10	1	cell m....all 1	100% (1/1)

Fig. 10: Up-stream regulatory network and the downstream function involved by the differentially expressed gene

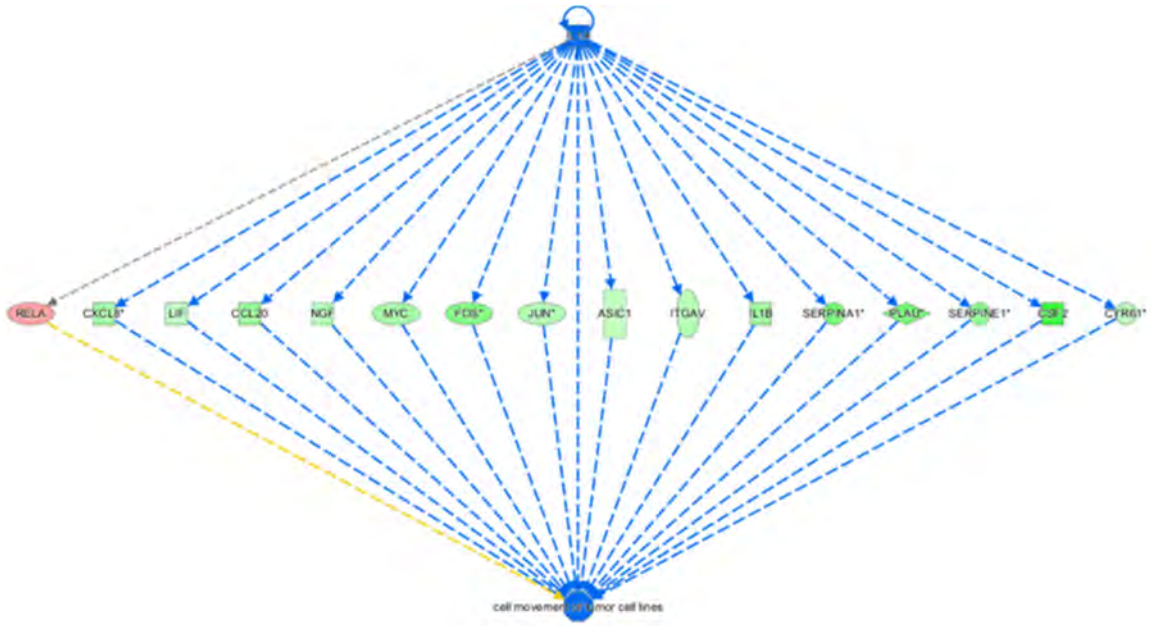

Fig. 11: Interaction between the gene and regulator and the function

of HUVECs of the Tanshinone. This data showed that E4018 and E4017 were differentially expressed.

Signalling pathway analysis was then conducted to analyze the genes involved in different signalling pathways. Our data showed that the ERK5 signalling pathway was significantly inhibited with a Z score of -2.530 in the treatment group compared with the control group and vehicle group, respectively. Big Mitogen-activated protein Kinase 1 (BMK1), also known as Extracellular Signal-Regulated Kinase 5 (ERK5), is the most recently identified member of the mammalian Mitogen-activated Protein Kinase (MAPK) family[14]. In the previous study, cellular activation of BMK1 is induced by a variety of stimuli including growth factors, oxidative stress and hyperosmolar conditions. These physiological mediators could activate cellular BMK1 through sequential activation of a signalling cascade that was consisted of MEK5, kinases MEKK3 and BMK1. Many studies indicated that some agents could attenuate cerebral injuries through modulating the ERK5 signalling pathway[15]. In a rat model of cerebral ischemia-reperfusion injury, Gu *et al.*[16] indicated that neuregulin1β could affect the brain tissues through regulating the ERK5-dependent MAPK pathway[16]. Meanwhile, the overexpression of MEK5 was reported to be associated with metastatic prostate cancer[17]. Furthermore, CX3CL1/CX3CR1 was reported to regulate the nerve injury-induced pain hypersensitivity by modulating the ERK5 signalling pathway[18]. These indicated that ERK5 played crucial roles in cerebral injuries and our data indicated that Tanshinone could inhibit the expression of the ERK5 signalling pathway.

The IL-1α is closely related to the development and metastasis of cancer and its polymorphisms have been reported to affect the susceptibility of malignancy tumours. In a previous meta-analysis, the authors investigated the association between IL-1α polymorphism and cancer risk was appraised by counting ORs and 95% CIs of overall and stratification analysis[19]. In all genetic models, there was a significantly upregulated risk caused by rs3783553, while the same tendency was still available in all cancer types. Meanwhile, there was a high risk of cancer susceptibility in patients with rs1800587 polymorphism of IL-1A, in which allelic and homozygote models in overall studies were extracted[20]. Moreover, emerging evidence demonstrated that polymorphisms of Interleukin-1 (IL-1) may involve in human tumorigenesis by regulating the production of a certain number of cytokines. The rs3783553 in IL1A 3'-UTR is inversely associated with CC risk, suggesting an important role IL-1α may play in cervical carcinogenesis. Our data showed that IL1A could inhibit the cell movement of tumour cells through modulating the expression of ASIC1, CCL20, CSF2, CXCL8, CYR61, FOS, IL1B, ITGAV, JUN, LIF, MYC, NGF, PLAU, RELA, SERPINA1, SERPINE1. Previously, rare studies have been conducted to investigate the roles of tanshinone in the IL-1A. In a study designed to investigate the effect of tanshinone IIA on IL-1β, IL-6 and TNF-α cytokines in immune vasculitis and platelets, Tanshinone IIA may diminish the inflammation damage of vessels in patients with immune vasculitis through the inhibition of cytokines and platelets[21].

CONCLUSION

In summary, tanshinone inhibited the proliferation of HUVECs and induced differential expression of genes *in vitro*. Enrichment analysis indicated that the ERK5 signalling pathway ranked first in the GO and KEGG analysis.

SIGNIFICANCE STATEMENT

This study discovers the roles of tanshinone in mediating the activity of human umbilical vein endothelial cells that can be beneficial for the investigate on the potential roles of tanshinone in treating cerebral conditions. This study will help the researchers to uncover the critical areas of differentially expressed genes after treating with tanshinone that many researchers were not able to explore. Thus, a new theory on the tanshinon in treating cerebral diseases may be arrived at.

ACKNOWLEDGMENT

This research was supported by the Shandong Natural Science Foundation Joint Fund (No. ZR2021LZY028); The Youth Program of National Natural Science Foundation of China (No. 81302940); General Program of Shandong Natural Science Foundation (No. ZR2021MH386); The National Natural Science Foundation of China (No. 30873323); Technology and Development Program of Traditional Chinese Medicine, Shandong Province (No. 2011-192); and Shandong Natural Science Foundation (No. ZR2015PH046).

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