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Valvular Heart Disease-Related Feed-Forward Loop Network Reveals Some Biological Features for Disease Development

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Abstract. This study is to uncover VHD-related important biological features for understanding molecular of this disease. The system analysis strategy is applied to generate the first VHD-related FFL network. Then, the network topology is mapped into the biological space to refine the biological features of VHD. 4 miRNA, 4 TF and 34 gene hubs are identified and confirmed to play important roles in the occurrence and development of VHD. The signal pathways mediated by miRNA hubs are also predicted, where the processes that MAPK pathway mediated by has-let-7c induces VHD are explained. This study provides some new computational evidences for the understanding of VHD molecular mechanism, and also has important implications in terms of improving current therapeutic strategies of VHD.

Introduction

The miRNAs, TFs and genes interact to form a network system, in which miRNAs and TFs are major regulators and they are closely linked to regulate common target genes and to participate in many important biological processes, including cell proliferation, differentiation, apoptosis, etc.

Many complex diseases are associated with disorders of TFs and miRNAs [1], which will cause that the biological regulatory system of an organism appears to adjust abnormal, the internal homeostasis of system is broken and the symptoms of the disease in vitro are shown. So far, a lot of efforts have been made to construct the regulatory network associated with an objective disease based on interaction relationships among miRNA, TF and their target genes, such as the ovarian cancer [2], the glioblastoma multiforme [3], the osteosarcomas [4], etc. The existing studies showed that: (i) the analysis of complex diseases can benefit from systems biological approaches; (ii) FFLs are conservative and have basic biological functions in the complex regulatory relationships among miRNAs, TFs and genes, meanwhile it can be used as a basic unit to construct a regulatory network.

VHD is the function or structural abnormality of one or more valve structures due to

multiple causes, such as inflammation, mucus degeneration, degenerative changes, congenital malformations, ischemic necrosis, trauma, etc. It is a major cause of morbidity and premature death from cardiovascular diseases and still remains highly prevalent. Although this disease has been verified to have various genomic alterations [5-7], its molecular mechanism is not fully understood yet. Researches on VHD-related network consisting of miRNAs, TFs and their targets and the control mechanisms are still in the blank state at present. To end this, this paper applied a system analysis strategy to construct FFL network related to VHD. Through analyzing the network topology and mapping it into biological space, some important biological features associated with the development of VHD were found. This work can provide the theoretical evidences for understanding interaction mechanisms and regulation patterns among miRNAs, TFs and target genes in VHD development.

Materials and Methods

MiRNAs Related to VHD

VHD-related miRNAs were collected through performing a comprehensive literature searching and reviewing. First, a group of relevant articles were compiled from Google scholar (http://scholar.google.com.hk/) using the search phrase "valvular heart disease" and from PubMed (http://www.ncbi.nlm.nih.gov/pubmed/) using the search phrase "valvular heart disease&miRNA". After carefully analyzing the related literatures, 15 VHD-related miRNAs that have been experimentally verified and met the requirements of this study were ultimately selected. The information of 15 VHD-related miRNAs is shown in Table 1.

Table 1. The information of 15 VHD-related miRNAs

No.	Symbol	source	No.	Symbol	source	No.	Symbol	source
1	hsa-miR-30b	[8]	6	hsa-miR-125b	[12]	11	hsa-miR-99b	[12]
2	hsa-miR-141	[9]	7	hsa-miR-127	[12]	12	hsa-miR-328	[12]
3	hsa-miR-21	[10]	8	hsa-miR-199a-3p	[12]	13	hsa-miR-744	[12]
4	hsa-miR-23a	[11]	9	hsa-miR-204	[12]	14	hsa-miR-26a	[13]
5	hsa-let-7c	[12]	10	hsa-miR-320	[12]	15	hsa-miR-195	[13]

Predicting Relationships of miRNA-gene, TF-gene/miRNA and miRNA-TF

In order to minimize the false positives and to increase the reliability of prediction results, we predicted the target genes of each VHD-related miRNA using TargetScan [14], miRanda [15] and PicTar [16] databases. Only those relationships simultaneously appearing in the three databases were retained in this study.

To predict the regulatory relationships between TFs-genes and TFs-miRNAs, we

first downloaded the defined promoter region (-2000/+2000 around CDS) of VHD-related miRNAs and their target genes. Then, we performed a binding sites search using the Match^{TM} [17] software that is integrated in TRANSFAC Professional [18]. For the purpose of this study, we used the pre-calculated cut-offs to minimize the false positive matches and create a high-quality matrix. To restrict the search, we set a core score of 1.00 and a matrix score of 0.95, and selected TFs associated with the human genome only. Finally, we established the relationships of miRNA-TF through their common target genes.

Constructing VHD-related FFL Network

The FFLs have mainly the three types: TF-FFL, miRNA-FFL and composite-FFL [3]. In a TF-FFL motif, a TF regulates both miRNA and gene and a miRNA represses its target gene. In a miRNA-FFL motif, a miRNA represses both TF and its target gene, as well as a TF regulates a gene. In a composite-FFL motif, a TF regulates both miRNA and gene, as well as a miRNA represses both TF and gene.

We first tallied all the relationship pairs among miRNAs, TFs and genes above. Then, according to the tallied results, we constructed the TF-FFL motifs, the miRNA-FFL motifs and the composite-FFL motifs. Next, we calculated the P-values of miRNA-TF pairs in each type of the FFLs using the cumulative hypergeometric test shown in the equation (1):

$$P = \sum_{i=(|N_{(miR)}| \mathrm{I} |N_{(TF)}|)}^{\min(|N_{(miR)}|,|N_{(TF)}|)} \left\{ \binom{|N_{(miR)}|}{i} \binom{|\mathrm{Total} - N_{(miR)}|}{|N_{(TF)}| - i} \right\} \Big/ \binom{Total}{|N_{(TF)}|} \quad (1)$$

where $N_{(\mathrm{miRNA})}$ is the number of genes targeted by a given miRNA, $N_{(\mathrm{TF})}$ is the number of genes regulated by a given TF, and Total is the number of common genes between all human genes targeted by human miRNAs and all human genes regulated by all human TFs. Furthermore, the multiple test with false discovery rate (FDR) of less than 0.05 [19] is performed. Only those FFLs with the miRNA-TF pairs of $P < 0.05$ in each type of FFLs were screened. Finally, through blending the three kinds of FFLs with significant miRNA-TF pairs, the VHD-related FFL network is constructed.

Mapping Network Topology into Biological Space for Critical Biological Features

A biological network is with scale-free, that is, the degree distribution of nodes obeys power-law distribution, indicating that most nodes had a low degree, while only a small portion of nodes had a high degree. Therefore, we can observe only a few of miRNAs, genes and TFs to exhibit a high degree in a biological network. These critical molecules act as hubs that might play important roles in maintaining the overall connectivity of a disease-related network [20].

According to the above views, we firstly count the degree of each node on the VHD-related FFL network. Then, we rank the nodes according to their degree numbers and types. Next, we extracted miRNAs and TFs arranged in the top 25% of the degrees and genes arranged in the top 5% of the degrees as the hubs, and run the functional annotation clustering Tools GO and KEGG to obtain the critical biological features of the hubs. Finally, we analyze the roles of key nodes in the cell for understand important molecular mechanism of the VHD disease from the activation of biological molecules, to opening of the corresponding biological signaling pathways, and to influence of specific biological process behavior on diseases.

Results

The Topology Characteristics of Constructed VHD-related FFL Network

The topology characteristics of VHD-related FFL network constructed are shown in Table 2.

Table 2. The topology characteristics of VHD-related FFL network

	FFLs	Gene	miRNA	TF	miRNA-gene	miRNA-TF	TF-gene	TF-miRNA
Number	1016	691	12	23	845	21	893	24

The degree distribution curve of genes is shown in Fig. 1, which obeys the power law distribution. Because the numbers of miRNAs and TFs in this network are too low, their degree distribution curves lack statistical significances.

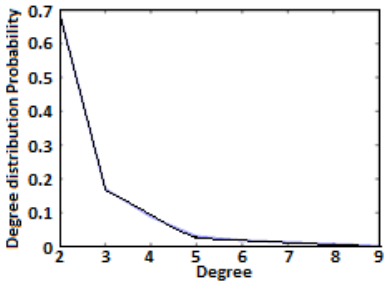

Fig. 1. The degree distribution curve of genes in VHD-related FFL network.

The Critical Hubs and Their Biological Features

Those miRNAs, TFs and genes that their degrees are ranked in the top 25% and 5% respectively are identified as the hubs. The results are shown in Table 3, including 4 miRNAs (hsa-let-7c, hsa-miR-23a, hsa-miR-26a and hsa-miR-195), 4 TFs (Nkx2-5, Oct-1, FOXD3 and HNF-3beta) and 34 genes.

Table 3. The identified hubs according to the degree ranking

miRNA Hubs	**TF Hubs**	**Gene Hubs**				
hsa-let-7c	Nkx2-5	QKI	KPNA4	CAB39	WHSC1	FUT4
hsa-miR-23a	Oct-1	CDC25A	CXCL12	DCBLD2	ATP2A2	ELF2
hsa-miR-26a	FOXD3	PLAGL1	GPRC5B	LYPLA2	PPARGC1A	RBM24
hsa-miR-195	HNF-3beta	POM121	ANKHD1	DMTF1	PRDM1	SOX5
		CPEB3	CAMTA1	PPP1R15B	STK35	LRIG1
		HAS2	ATXN1	SLC1A1	RAP2C	CCND1
		MTPN	PURB	TNKS2	SLC7A1	

We predicted 140 target genes of hsa-let-7c and respectively obtained 116, 100 and 122 terms of biological processes, cellular components and molecular functions through GO analysis. In summary, the enriched biological processes mainly included protein kinase cascade (29/116, 25%), molecular signaling pathway (24/116, 20.7%), protein modification (12/116, 10.3%), and metabolism regulation (28/116, 24.1%); The enriched cellular components mainly included proteinaceous extracellular matrix (7/100, 7%) and plasma membrane part (30/100, 30%); the enriched molecular function terms have mainly protein kinase activity (12/122,9.8%). For hsa-miR-23a, we predicted its 119 target genes and respectively obtained 106, 78 and 95 terms of biological processes, cellular components and molecular functions through GO analysis. In summary, the enriched biological processes mainly included protein kinase cascade (20/106, 18.9%), regulation of transcription (17/106, 16.0%), and regulation of metabolic process (39/106, 36.8%); the enriched cellular components are mainly cell junction (9/78, 11.5%), etc. the enriched molecular function terms have mainly protein

kinase activity (13/95, 13.7%) and transcription regulator activity (18/95, 18.9%). For hsa-miR-26a, we predicted its 124 target genes and respectively obtained 98, 84 and 96 terms of biological processes, cellular components and molecular functions through GO analysis. In summary, the enriched biological processes are mainly involved in intracellular signaling cascade (22/98, 22.4%), protein amino acid phosphorylation (12/98, 12.2%) and metabolic process (21/98, 21.4%); the enriched cellular components are mainly cytoskeleton (17/84, 20.2%); The molecular function terms have mainly protein kinase activity (11/96, 11.5%) and molecular binding (46/96, 47.9%). For hsa-miR-195, we predicted its 120 target genes (Table S4) and respectively obtained 100, 81 and 94 terms of biological processes, cellular components and molecular functions through GO analysis. In summary, the enriched biological processes mainly are involved in regulation of cell cycle (17/100, 17%), protein catabolic process (11/100, 11%) and metabolic process (25/100, 25%); The enriched cellular components include plasma membrane (34/81, 42.0%) and cytoplasmic vesicle (10/81, 12.3%); The molecular function terms have mainly protein kinase activity (11/94, 11.7%) and phosphatase activity (6/94, 6.4%) .

The predicted signal pathways mediated by hsa-let-7c, hsa-miR-23a, hsa-miR-26a and hsa-miR-195 using KEEG analysis respectively, are shown in Table 4.

Table 4. The predicted signal pathways mediated by miRNA hubs

miRNA Symbol	The mediated signal pathway	*P* Value	Involved gene count (proportion)
Hsa-let-7c	MAPK signaling pathway	1.10E-02	8 (16.7%)
	Pancreatic cancer	2.80E-02	4 (8.3%)
	Axon guidance	3.00E-02	5(10.4%)
	Chronic myeloid leukemia	3.10E-02	4(8.3%)
Hsa-miR-23a	Heparan sulfate biosynthesis	1.70E-02	3(7.5%)
Hsa-miR-26a	mTOR signaling pathway	6.10E-03	4 (10.5%)
Hsa-miR-195	Cell cycle	1.30E-03	7(13.7%)
	Wnt signaling pathway	3.40E-03	7(13.7%)
	Pathways in cancer	4.10E-03	10(19.6%)
	Pancreatic cancer	5.20E-03	5(9.8%)
	Chronic myeloid leukemia	6.00E-03	5(9.8%)
	Axon guidance	3.70E-02	5(9.8%)
	Focal adhesion	4.60E-02	6(11.8%)
	Colorectal cancer	4.90E-02	4(7.8%)

By performing GO analysis of 34 critical genes, we obtained 30, 22 and 27 terms of biological processes, cellular components and molecular functions. respectively. In summary, the enriched biological processes are mainly involved in cell chemical property balance (6/30, 20%) and metabolic regulation (15/30, 50%); the important cellular component is nuclear membrane (3/22, 13.6%); the terms of molecular functions have mainly transcriptional regulator activity (8/27, 29.6%). No signal pathway is enriched on KEGG for 34 gene hubs.

At present, some scholars have used the experimental methods to explore the pathogenesis of VHD disease at molecular level, including: extracellular matrix remodeling [21], cell gap junction [22], cell metabolism regulation [23] and abnormal cell signals, such as TGF-β[24], Wnt [25], mTOR [26], MAPK [27], cell cycle signal

[28], etc. Thus, the biological features of the identified 4 miRNA and 34 gene hubs at biological processes, cellular components and molecular functions have high matching degree with the verified pathological factors above. This illustrates that these hubs might play a major role in the occurrence and development of VHD disease.

The Roles of MAPK Signal Pathways Mediated by Hsa-let-7c in VHD Development

Using the Go analysis results above and the existing evidences for the biological functions of MAPK pathway, we summarized the process that hsa-let-7c mediates MAPK signal pathway to induce VHD, as shown in Fig. 2. Once the receptor proteins on cell membrane like CACNA1E, ACVR1B and TNFR bind the relevant cell external active proteins, MAPK signal is activated. On the one hand, the active CACNA1E receptor can jointly activate a series of kinases and then acts on NRAS, which is an important Ras protein in MAPK signaling pathway and can connect the upstream pathway Ras-GAPs and the downstream pathway Rho/Rac. The active Ras protein can indirectly activate ERK, an extracellular signal-regulated kinase. MAPK/ERK pathway is a classical signal transduction pathway and it can mediate the cell proliferation and differentiation. On the other hand, the membrane receptor ACVR1B can trigger the kinases in connection with P38 signaling pathway, meanwhile TNFR acts on CASP, an enzyme related to apoptosis. CASP further activates PAK1, an important transfer enzyme, which can initiate JNK signaling enzymes as well as NLK and MAP3K3. P38 and JNK alternately motivate p53 signal, which is related to inflammatory reaction and adjusts apoptosis of cells. NLK and MAP3K3 further activate Wnt signal pathway, which mediates actin reorganization and remodels cytoskeleton via the complex cross-talking between signals. The existing study found that P38, JNK, ERK and P53 pathways change physiological structure of cardiac valve and take part in VHD development through affecting skeleton, fibrosis, mitosis and apoptosis of cardiac muscle cells [21]. This supports our explanation.

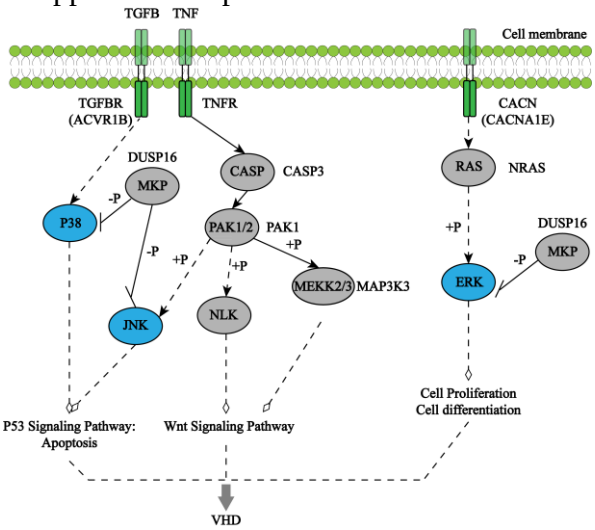

Fig. 2. The process that hsa-let-7cmediates MAPK signal pathway to induce VHD.

Conclusions

This study represents the first attempt to investigate FFL networks in the pathogenesis of VHD. 4 miRNA (hsa-miR-23a, hsa-miR-195, hsa-miR-26a and hsa-let-7c) , 4 TF (Nkx2-5, Oct-1, FOXD3 and HNF-3beta) and 34 gene hubs are identified through the analysis of the network topology. Since there are high matching degree between the

biological features of VHD-related hubs and the verified VHD pathological factors, these hubs might play an important role in VHD development. We predicted VHD-related signal pathways mediated by miRNA hubs, in which the roles of MAPK signal pathway mediated by hsa-let-7c in VHD development are analyzed. This study provides some new computational evidences for the understanding of VHD molecular mechanism.

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