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Identification of microRNAs present in congenital heart disease associated copy number variants

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Abstract. – PURPOSE: Congenital heart disease (CHD) is the most common congenital anomaly in newborns and about 1.35 million infants are born with CHD each year worldwide. Recently a large category of copy number variants (CNVs), were established to be a major contributor of the pathophysiology of CHD. To date most studies focused on the analysis of CNV categories or the protein coding regions without investigation on the impact of non-coding regulatory microRNAs (miRNAs).

MATERIALS AND METHODS: Here with an array comparative genome hybridisation data set and a gene expression profile data set, we investigated the contribution of miRNAs in CNVs towards the development of CHD.

RESULTS: Approximately 18% of the identified high frequency CNV loci were shown to harbor miRNAs. According the expression profile analysis, 52 target genes of 16 miRNAs showed association with CHD. Targets of hsa-miR-650 was reported to be enriched with genes of cardiac dysfunctions and heart failure categories previously. In the constructed network, all 12 miRNAs directly or indirectly interacts with CHD related genes and hsa-miR-570 showed the highest degree.

CONCLUSIONS: Our study highlights the significance of CNV-microRNAs and their target genes in the pathogenesis of CHD. This knowledge will facilitate the identification of miRNA biomarkers and the development of new therapeutics for CHD.

Key Words:
MicroRNA, Congenital heart disease, Copy number variants.

Introduction

Congenital heart disease (CHD) refers to abnormalities in the heart's structure or function that arise during cardiac embryogenesis. CHD is the most common congenital anomaly in newborns and about 1.35 million infants are born with CHD each year worldwide[1]. Despite the improved treatment and prognosis of CHD patients, the aetiology of CHD remains unknown. About 20% of CHD can be attributed to chromosomal aberrations, Mendelian syndromes, single gene defects or environmental risk factors[2], while the majority of CHD arise through various multiple genetic contributors.

Array comparative genome hybridization (CGH) has been widely used to identify copy number variations (CNVs) in a test genome relative to a reference genome. CNVs have in the past been associated with CHD in both syndromic[3-6] and non-syndromic[7] CHD patients. Nevertheless, most previous CNVs studies on CHD focused on the analysis of CNV categories or the protein coding regions, with the aim to identify variant patterns or genes which may correlated with CHD. In fact, non-coding regions may also contribute to the pathogenesis of CHD. Recently, microRNAs (miRNAs), which are a class of 21-25 nucleotide non-coding RNAs, have been identified to act as "rheostats" and "switches" in modulating multiple facets of cardiac development, function and disease[8]. For example, as the most abundantly expressed miRNA in the heart, miR-1 was shown to play important roles in controlling heart development[9,10] and heart rhythm[11]. Besides, cardiac-specific depletion of functional mature miRNAs using the Cre-LoxP system leads to dilated cardiomyopathy or heart failure in mice[12,13], clearly established crucial roles for miRNAs during cardiovascular development. Thus, global survey of miRNAs in CNVs region may help us gain new insights into the pathogenetic mechanisms underlying CHD and provide a potential targets for future studies on CHD.

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Here we used an array Comparative Genomic Hybridization (CGH) data set downloaded from the Gene Expression Omnibus (GEO) data base to detect CNVs in CHD patients. Subsequent annotation of miRNAs in CNVs and prediction of miRNA target genes were carried out to identify candidate miRNAs which may contribute to the pathogenesis of CHD. For the miRNA target genes, using another data set of gene expression profile which was also from the GEO database, investigation of whether they were differentially expressed in CHD patients were carried out to confirm their roles in CHD. Our results here may clarify the mechanisms of CHD and propose new directions for future therapeutic investigations.

Materials and Methods

Data Sets

Two data sets from the GEO database were used in this study, including an array CGH data set (GSE7527) and a gene-expression microarray data set (GSE34459). A total of 104 CHD patients were included in GSE7527, which was used to identify CNVs in CHD patients. The array CGH data set for CHD patients was based on the GPL5000 platform: MPIMG Homo sapiens 44K ArrayCGH4. Series matrix files of GSE34459 were used to show whether the target genes of identified miRNAs were differentially expressed in CHD patients. Expression profile data of GSE34459 were based on two platforms, GPL6102 and GPL6947. On the GPL6102 platform, 21 down syndrome (DS) patients with CHD (DS/CHD+) and 22 DS patients without CHD (DS/CHD-) were included. On the GPL6947 platform, 11 DS patients and 12 healthy controls were included.

Identification of Significantly High Frequency CNVs

For the analysis and visualization of CGH array data, R software (V. 2.15.1) was used. Raw data were normalized by LOWESS algorithm with no background subtraction. For the assessment of copy number gains and losses, the conservative log2-ratio thresholds were set as 0.3 and –0.3, respectively. The number of CNVs in a region in the population is influenced by region length and the background CNV rate. Thus, to identify significantly high frequency CNVs, we calculated the probability of the number of CNVs under the given CNV rate and region length using the following set of calculations. First, we estimated background CNV rate per unit length. Second, the number of CNVs in the population was counted for each detected region. Finally, the expected number of CNVs in each region was calculated by the region length and the background CNV rate. Statistical test of significance for each region were performed by assuming a Poisson distribution, and multiple testing were adjusted by using Benjamini-Hochberg method[14]. All positions of detected CNVs based on human reference genome hg17 coordinates were transformed to hg19 (GRCh37) coordinates.

MiRNA Annotation and Target Gene Prediction

MiRNAs and genes in detected CNVs based on hg19 coordinates were annotated according to the miRbase release 19 (http://www.mirbase.org/ftp.shtml) and the UCSC database (ftp://hgdownload.cse.ucsc.edu/goldenPath/hg19/database/refGene.txt.gz), respectively. Target genes of the miRNAs were predicted using Targetscan 6.2[15], TAREF 2.0[16] and miRDB (http://mirdb.org/miRDB/). To reduce false positive rate, predicted genes supported by all the three methods were kept as miRNA target genes for further analysis.

mRNA Expression Profile Analysis

To verify whether the identified miRNAs are related with CHD, gene expression profile analysis was carried out for the target genes of these miRNAs. Target genes which showed differential expression in the DS/CHD+ vs DS/CHD- pair were considered as candidate genes which may contribute to the pathogenesis of CHD. Expression analysis for these differentially expressed genes in the DS vs normal controls pair was also carried out to check whether their association with CHD were related with the DS status. All the differential expression analysis were performed using the limma package (V. 3.12.1) in the R (V. 2.15.1) software.

Interaction Network

To illustrate the relationships among the identified miRNAs, the miRNA target genes and genes in the CNVs, a network was constructed by using Cytoscape (V. 2.8.3)[17] and the NCBI database (http://ftp.ncbi.nlm.nih.gov/gene/GeneRIF/, 2013-2-25).

Results

For the array CGH analysis, a total of 8440 CNVs were identified and 208 of them were found to be significantly high frequency with 37 CNV loci harboring miRNAs. As shown in Table I, the frequency of these 37 CNVs are all over 22/104. According to the annotation results, 20 miRNAs were found in these regions. The target gene prediction results using Targetscan 6.2[15], TAREF 2.0[16] and miRDB showed that 124 genes are related with these miRNAs.

Expression profile analysis was carried out in the DS/CHD+ vs DS/CHD- pair for all the target genes to confirm their relationship with CHD. As shown in Table II, 52 target genes of 16 miRNAs were found to be differentially expressed in the DS/CHD+ vs DS/CHD- samples (p-value < 0.01). To confirm whether their correlation with CHD involve the DS status, expression profile analysis in the DS vs normal controls pair was carried out. Of the 52 genes, signals of 20 genes were not detected due to low susceptibility or low probe specificity of the GPL6947 platform (Table II). For the 32 genes which could be detected in both platforms, 30 of them were only differentially expressed in the DS/CHD+ vs DS/CHD- pair, suggesting that their relationships with CHD may not related with the DS status. Eleven of the target genes have been reported to be related with CHD or normal heart development and function according to previous studies (Table II).

Table I. Information of the identified significantly high frequency CNVs.

Chr	Start	End	miRNAs in region	Num	*p*-value
2	87343490	87559220	hsa-miR-4771	44	1.49E-39
2	87825504	87995925	hsa-miR-4435	59	8.18E-66
2	87832573	88003938	hsa-miR-4435	33	3.95E-29
2	87833539	88047229	hsa-miR-4435	52	2.19E-50
2	110692019	110852124	hsa-miR-4267; hsa-miR-4436b-5p; hsa-miR-4436b-3p	22	1.27E-16
2	110696493	110841550	hsa-miR-4267	23	1.05E-18
2	110817636	110972451	hsa-miR-4267; hsa-miR-4436b-5p; hsa-miR-4436b-3p	58	1.15E-66
2	110817636	110989964	hsa-miR-4267; hsa-miR-4436b-5p; hsa-miR-4436b-3p	40	3.19E-38
2	110820120	110989964	hsa-miR-4267; hsa-miR-4436b-5p; hsa-miR-4436b-3p	45	2.18E-45
3	195419363	195589698	hsa-miR-570-3p; hsa-miR-570-5p	40	1.70E-38
8	117784467	117977826	hsa-miR-3610	22	6.53E-15
14	106193680	106362176	hsa-miR-4539; hsa-miR-4507; hsa-miR-4537	71	7.98E-85
14	106196304	106364513	hsa-miR-4539; hsa-miR-4507; hsa-miR-4537	75	2.57E-91
14	106196304	106364513	hsa-miR-4539; hsa-miR-4507; hsa-miR-4537	75	2.57E-91
14	106254813	106430900	hsa-miR-4539; hsa-miR-4507; hsa-miR-4537	88	3.05E-111
14	106265158	106446398	hsa-miR-4539; hsa-miR-4507; hsa-miR-4537	81	1.32E-98
15	20808193	21984411	hsa-miR-3118; hsa-miR-5701	45	2.05E-45
15	20856954	21984411	hsa-miR-3118; hsa-miR-5701	55	7.44E-68
15	20873568	22053666	hsa-miR-3118; hsa-miR-5701;	49	1.35E-50
15	21035721	21191264	hsa-miR-3118; hsa-miR-5701	56	1.80E-63
15	21040508	21203279	hsa-miR-5701	64	1.04E-74
15	21087094	21191892	hsa-miR-5701	67	5.00E-92
15	21110951	21269468	hsa-miR-5701	64	1.97E-75
15	21894915	22063115	hsa-miR-3118	48	8.05E-50
15	21897602	22088226	hsa-miR-3118	43	1.78E-40
15	21950131	22114361	hsa-miR-3118	41	2.13E-40
15	21954363	22137847	hsa-miR-3118	49	1.82E-49
15	22348218	22544993	hsa-miR-1268a	31	8.06E-25
15	22385699	22558428	hsa-miR-1268a	42	6.79E-41
15	22432010	22592818	hsa-miR-1268a	40	2.16E-39
15	22465132	22592818	hsa-miR-1268a	30	4.31E-29
15	22469156	22592818	hsa-miR-1268a	33	1.15E-33
15	22469156	22592818	hsa-miR-1268a	23	3.00E-20
16	70040606	70241577	hsa-miR-1972	32	3.00E-20
19	774182	962290	hsa-miR-4745-5p; hsa-miR-4745-3p; hsa-miR-3187-3p;	95	2.01E-120
22	23070750	23222730	hsa-miR-650	47	2.14E-50
22	23070750	23222730	hsa-miR-650	44	4.80E-46

Note: Chr: Chromosome; Num: number of the patients with the identified CNVs.

Table II. Information of differentially expressed miRNA target genes.

Entrez ID	Gene symbol	DS/CHD+ vs DS/CHD–		DS vs normal		miRNAs
		Fold change	*p*-value	Fold change	*p*-value	
9400	RECQL5	0.1734	8.85E-03	–	–	hsa-miR-1268
25852	ARMC8	-0.1018	4.74E-03	0.0304	6.61E-01	hsa-miR-1972
2026	ENO2	0.4946	7.00E-03	–	–	hsa-miR-1972
4957	ODF2	0.1958	1.24E-03	-0.0755	4.01E-01	hsa-miR-1972
5467	**PPARD**	0.2203	3.89E-04	-0.0119	8.71E-01	hsa-miR-1972
6717	**SRI**	-0.3174	3.53E-03	0.0597	5.30E-01	hsa-miR-1972
943	TNFRSF8	0.3079	7.47E-03	-0.0299	6.77E-01	hsa-miR-1972
5058	**PAK1**	0.2109	7.02E-03	–	–	hsa-miR-3187-3p; hsa-miR-3187-3p
84196	USP48	0.3514	6.65E-05	0.0709	4.31E-01	hsa-miR-3610
10294	**DNAJA2**	0.2035	4.06E-03	–	–	hsa-miR-4267
8605	PLA2G4C	0.5949	1.93E-04	-0.0421	4.28E-01	hsa-miR-4267
401944	LDLRAD2	0.6651	2.55E-03	–	–	hsa-miR-4267; hsa-miR-1972
57610	RANBP10	0.1388	3.90E-03	0.1142	2.45E-01	hsa-miR-4267; hsa-miR-3187-3p; hsa-miR-4435; hsa-miR-4267; hsa-miR-3187-3p
6941	TCF19	-0.3035	1.00E-03	–	–	hsa-miR-4267; hsa-miR-4436b-5p; hsa-miR-4435; hsa-miR-4267; hsa-miR-4436b-5p; hsa-miR-3187-3p
9567	GTPBP1	0.1265	3.46E-03	–	–	hsa-miR-4435
7086	TKT	0.1776	7.00E-03	-0.5242	6.16E-03	hsa-miR-4435
55294	FBXW7	0.2662	8.22E-03	-0.0752	4.41E-01	hsa-miR-4435; hsa-miR-3187-3p
253980	KCTD13	0.2320	3.54E-03	–	–	hsa-miR-4435; hsa-miR-3187-3p
83892	KCTD10	-0.3429	3.60E-03	–	–	hsa-miR-4436b-3p
113419	TEX261	-0.2140	1.15E-03	-0.2074	2.56E-02	hsa-miR-4436b-3p
7127	TNFAIP2	0.5103	2.48E-03	-0.3072	4.03E-01	hsa-miR-4436b-3p
80727	TTYH3	0.2669	3.58E-03	0.0516	3.11E-01	hsa-miR-4436b-3p
79065	ATG9A	0.1819	8.81E-03	-0.1026	2.27E-01	hsa-miR-4436b-3p; hsa-miR-4435; hsa-miR-4436b-3p
7461	CLIP2	0.2773	1.10E-04	0.0032	9.79E-01	hsa-miR-4436b-5p
57171	DOLPP1	-0.1601	7.70E-03	–	–	hsa-miR-4436b-5p
30001	**ERO1L**	0.2895	7.79E-03	0.0209	8.07E-01	hsa-miR-4436b-5p
51278	IER5	0.2677	7.06E-04	0.0151	8.44E-01	hsa-miR-4436b-5p
23609	MKRN2	0.2125	5.42E-03	–	–	hsa-miR-4507
777	**CACNA1E**	0.5626	9.30E-03	–	–	hsa-miR-4539
598	**BCL2L1**	0.2940	9.88E-03	0.0119	8.93E-01	hsa-miR-4539; hsa-miR-4435; hsa-miR-4267; hsa-miR-4436b-3p; hsa-miR-4539; hsa-miR-1972
25855	BRMS1	-0.2494	1.87E-03	0.0049	9.63E-01	hsa-miR-4745-5p
933	CD22	0.4038	1.20E-03	0.2562	8.01E-03	hsa-miR-4745-5p
54470	ARMCX6	-0.2947	1.20E-03	-0.1580	5.96E-02	hsa-miR-4771

Table comtinued

The effect of miRNAs on the pathogenesis of CHD may also involve genes in the CNVs. To illustrate interactions of the indentified miRNAs, differentially expressed target genes and genes in the CNVs, a network was constructed as shown in Figure 1. The degree of each node was mea-

Table II *(Continued)*. Information of differentially expressed miRNA target genes.

Entrez ID	Gene symbol	DS/CHD+ vs DS/CHD-		DS vs normal		miRNAs
		Fold change	*p*-value	Fold change	*p*-value	
2932	**GSK3B**	0.1911	4.16E-03	–	–	hsa-miR-4771; hsa-miR-4771; hsa-miR-4267; hsa-miR-570
54841	BIVM	0.2162	9.24E-04	-0.0024	9.67E-01	hsa-miR-570
284611	FAM102B	0.3056	4.19E-04	–	–	hsa-miR-570
56262	LRRC8A	-0.1453	2.92E-03	-	-	hsa-miR-570
10133	OPTN	0.3111	3.57E-03	-	-	hsa-miR-570
54956	PARP16	0.1888	4.10E-03	-0.0266	7.75E-01	hsa-miR-570
22976	**PAXIP1**	-0.1818	8.89E-03	0.0624	3.05E-01	hsa-miR-570
55279	ZNF654	0.3200	3.66E-03	-0.0592	3.43E-01	hsa-miR-570
27101	CACYBP	-0.3753	3.65E-03	-	-	hsa-miR-5701
5682	PSMA1	-0.2678	4.89E-03	0.1045	1.89E-01	hsa-miR-5701
115294	PCMTD1	0.3581	7.26E-03	-	-	hsa-miR-570-5p
10477	UBE2E3	-0.1780	7.32E-03	-0.0326	7.26E-01	hsa-miR-570-5p
51699	VPS29	-0.1378	4.06E-03	-0.0546	3.93E-01	hsa-miR-570-5p
57706	DENND1A	0.1649	9.32E-03	–	–	hsa-miR-570-5p; hsa-miR-4507
7170	**TPM3**	-0.2166	9.31E-03	–	–	hsa-miR-570-5p; hsa-miR-570; hsa-miR-4507
58155	**PTBP2**	0.1611	2.74E-03	-0.0002	9.98E-01	hsa-miR-570-5p; hsa-miR-570; hsa-miR-4539
192111	PGAM5	-0.2778	9.96E-03	-0.0988	3.04E-01	hsa-miR-650
55147	RBM23	-0.1641	5.87E-03	0.0751	5.44E-01	hsa-miR-650
11078	TRIOBP	-0.2170	7.67E-04	-0.0191	7.19E-01	hsa-miR-650

Note: "-" indicates that the expression signal of the gene was not detected in the samples. Genes related with CHD or heart development and function according to previous studies are shown in bold.

sured by the number of links of the node in the network. Nodes with higher degrees are shown in bigger circles. 12 of the 16 miRNAs were included in the network.

Discussion

Chromosomal aberrations are well- known causes of syndromic[3-6] and non-syndromic[7] CHD. Previous CNVs studies on CHD mainly focused on the analysis of CNV categories or protein coding regions in the CNVs, without exploring miRNAs which have been shown to be important in controlling heart development. Here using an array CGH data set and a gene expression data set from the GEO database, we identified miRNAs that may play important roles in the pathogenesis of CHD through regulating its target genes.

Approximately 18% of the CNV loci (37 out of 208) were shown to harbor miRNAs. To confirm the relationship of these miRNAs and CHD, expression profile analysis was carried out to check whether their target genes were deregulated in the comparison of DS/CHD+ vs DS/CHD- pairs. 52 of the 124 target genes were found to be differentially expressed, which would be regulated by 16 miRNAs. Among the 52 genes, most of them did not show differential expression in the DS vs control pair, suggesting their association with CHD may not related with the DS status. Besides, none of the 16 miRNAs were previously reported to be associated with CHD. However, previous bioinformatics analysis of the modulated targets of hsa-miR-650 showed the enrichment of cardiac dysfunctions and heart failure categories[18]. Other miRNAs may also related with CHD through their modulation of their target genes since the target genes showed differential expression in the DS/CHD+ vs DS/CHD- samples. Of note, as shown in Figure 1, most of the selected miRNAs (12/16) were included in the network, suggesting that miRNAs may function together as a group of interconnected components. Of all detected miR-

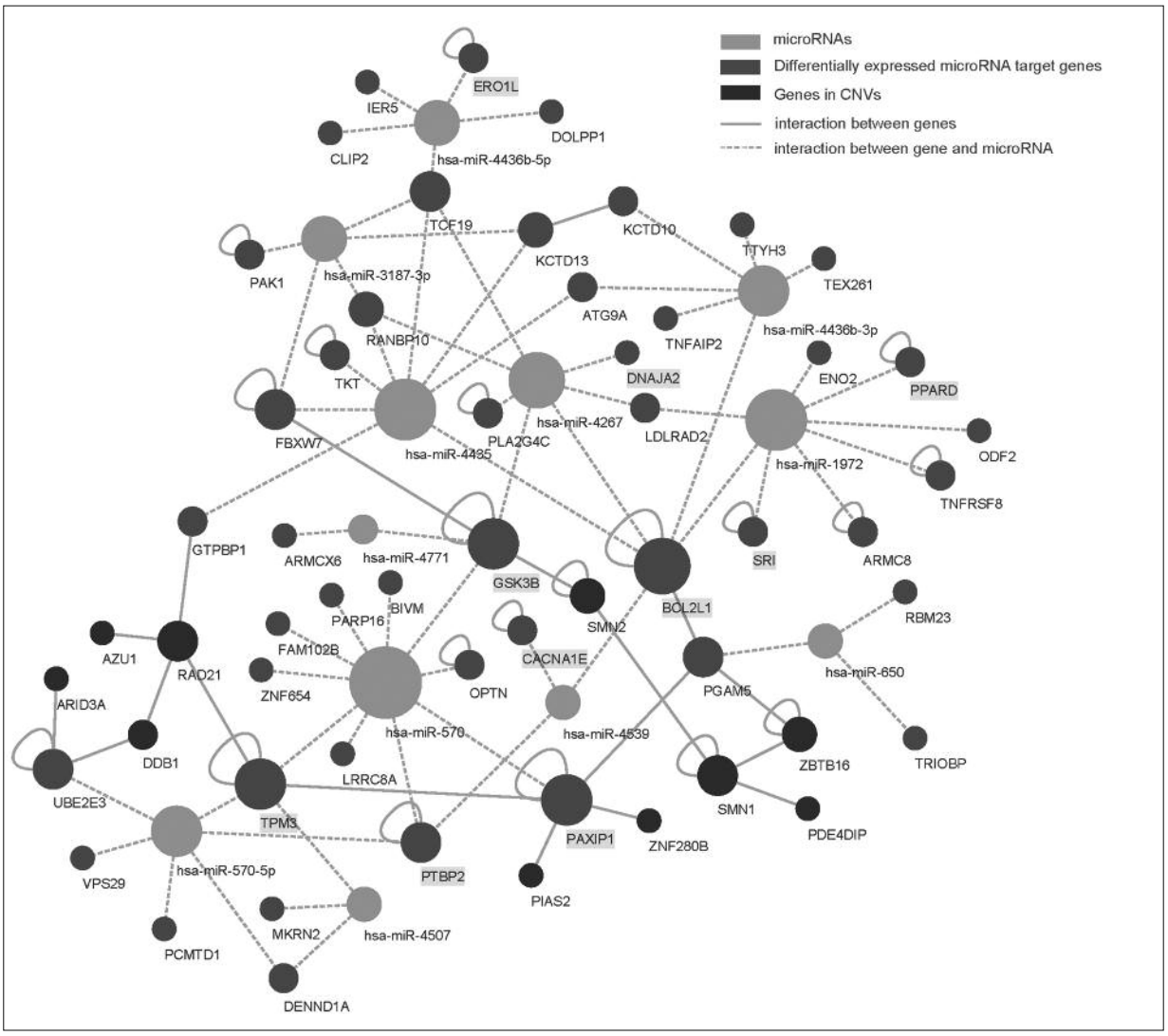

Figure 1. Interaction network of the miRNAs, target genes and genes in the CNVs. Only miRNAs whose targets showed differential expression in the expression profile analysis were included. Only genes or miRNAs with more than two direct or indirect links were shown. Target genes which have been reported to be related with CHD or heart function are marked in grey. Genes or miRNAs with higher degree (more links) are shown in bigger size.

NAs, hsa-miR-570 showed the highest degree in the network. Among its targets, protein encoded by *GSK3B* is a central regulator of embryonic cardiomyocyte proliferation and differentiation[19]. Besides, TPM3 protein participates the cardiac muscle contraction pathway (from KEGG, hsa04260) and PTBP2 protein mediates CaV1.2 exon mutations which caused severe arrhythmia disorder[20,21], implicating the potential roles of hsa-miR-570 in the development of CHD. Other miRNAs in the network were all directly or indirectly interacts with genes which have been reported to be related with CHD or normal heart development and function (Figure 1), suggesting their involvement in the pathogenesis of CHD.

Conclusions

Using an array CGH data set and a expression array data set from the GEO database, our study investigated the contribution of miRNAs in CNVs towards the development of CHD. Approximately 18% of the high frequency CNV loci were shown to harbor miRNAs. According the expression profile analysis, 52 target genes of 16 miRNAs showed association with CHD. Among the 16 miRNAs, targets of hsa-miR-650 was reported to be enriched with genes of cardiac dysfunctions and heart failure categories. All 12 miRNAs in the constructed network directly or indirectly interacts with CHD related genes while

hsa-miR-570 showed the highest degree. Our study suggests a possible mechanism that will account for the genetic heterogeneity and phenotypic variability of CHD. This knowledge will facilitate the identification of miRNA biomarkers and the development of new therapeutics.

Conflict of Interest

The authors have no financial conflicts of interest.

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