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ORIGINAL ARTICLE

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Network-based transcriptomic analysis reveals novel melatonin-sensitive genes in cardiovascular system

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Abstract

Purpose Heart disease is a major cause of mortality and disability worldwide. Melatonin is a neuroendocrine hormone and has been found to be protective in heart disease. However, the molecular basis underlying this cardioprotective effect is not fully understood. Here we aim to investigate melatonin-sensitive genes in cardiovascular system using public gene expression databases.

Methods An innovative genomic analysis method, the weighted gene co-expression network analysis (WGCNA) combined with differential gene expression analysis, was used in this study. The algorithm was implemented in R/Bioconductor.

Results Using this method, we provide a comprehensive characterization of transcriptional profiles associated with melatonin treatment. We found that 357 differentially expressed genes (DEGs) were highly sensitive to melatonin in mouse myocardium. Enrichment analysis showed that these 357 genes were mostly related to GO:0051984 (positive regulation of chromosome segregation), GO:0016605 (PML body) and GO:0006281 (DNA repair). We further obtained 5 hub genes from the 357 DEGs, including Set, Dhx40, Scaf11, Cfh, and Nup43.

Conclusions We identified numerous melatonin-sensitive genes and further identified five hub genes. The five novel genes are possibly associated with the myocardial benefits of melatonin.

Keywords Melatonin · Transcriptomic analysis · Network pharmacology · WGCNA

Introduction

Heart and cardiovascular diseases are the leading cause of morbidity and mortality in most countries worldwide and have caused great economic burdens [1]. For the past 20 years, the prevalence of this kind of disease is rapidly increasing around the world, especially in the elderly [1, 2]. Melatonin is a pleiotropic hormone synthesized at night by the pineal gland. The effect of melatonin on the cardiovascular system is well established. It has been found melatonin can be used as a direct free radical scavenger or an indirect antioxidant to protect against cardiac diseases [3]. Melatonin regulates the expression levels of a host of genes associated with cardiac function [4]. However, digging genes sensitive to melatonin treatment is still rudimentary and not fully investigated. Besides, the effect of melatonin is probably that they act via modulation of multiple proteins rather than single targets. Therefore, it is important to accurately identify melatonin-affected proteins and their interaction networks.

In the present study, we explored the transcriptome profiling of melatonin-treated mouse myocardium. By

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combining weighted gene co-expression network analysis (WGCNA) with differential gene expression analysis, we found numerous transcripts that were sensitive to melatonin treatment in mouse myocardial tissue and further identified five hub genes, including Set, Dhx40, Scaf11, Cfh, and Nup43. WGCNA is a systems biology method for describing the correlation patterns among genes and relating these gene sets (modules) to sample traits (here it is "melatonin treated or not") [5]. Genes screened via WGCNA were more likely to be of functional importance. The identified five novel genes are possibly associated with the myocardial benefits of melatonin.

Materials and methods

Data pre-processing and differentially expressed gene (DEG) screening

Raw data for reanalysis comprising 12 samples (6 control and 6 melatonin treated) were obtained from the Gene Expression Omnibus database under the accession number GSE115569 (Supplementary Table 1). Raw data were normalized via the Robust Multi-array Average method. The R/Bioconductor package "Limma" was used to screen DEGs [6]. Genes with a false discovery rate (FDR) of <0.05 were considered differentially expressed. A brief overview of data analysis procedure was illustrated in Supplementary Fig. 1.

Weighted gene co-expression network analysis

By calculating the median absolute deviation (MAD), we selected the top 5000 most variant genes to generate a weighted co-expression network. WGCNA was performed using R package "WCGNA." To weight highly correlated genes, the soft thresholding power (β) was set as 10 (Supplementary Fig. 2C), and the minimal module size was set as 30. To define clusters of genes in the data set, the adjacency matrix was used to calculate the topological overlap measure (TOM), which shows the degree of overlap in shared neighbours between pairs of genes in the network. $1 - \mathrm{TOM}$ was used as the dissimilarity measure for hierarchical clustering and module detection. Modules of clustered genes were then selected using the Dynamic Tree Cut algorithm within WGCNA. To identify modules that are significantly associated with the measured clinical traits, expression profiles of each module were summarized by the module eigengene (ME) and the correlation between the module and the trait was calculated. The associations of individual genes with the melatonin-treated samples were quantified by Gene Significance (GS) value. Genes with both GS >0.9 and differentially expressed after melatonin treatment were considered as hub genes. For each module, module membership (MM) was defined as the correlation of the ME and the gene expression profile.

Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis

GO and KEGG pathway enrichment analysis were done by Metascape (http://metascape.org/). Metascape is a set of reliable, productive and intuitive tools to analyse gene/protein lists and make better data-driven decisions. GO covers three domains: molecular function (MF), cellular component (CC), and biological process (BP). p Value <0.05 was considered to be significant enrichment. DEGs in the darkturquoise module were imported into Metascape to enrichment analysis.

Results

WGCNA and DEG analysis

To screen genes closely related and sensitive to melatonin treatment, we applied WGCNA and DEG analysis. First, we used WGCNA. WGCNA is a systems biology method for describing the correlation patterns among genes or probes and relating gene/probe sets (modules) to sample traits (here it is "melatonin treated or not"). Gene expression data should be filtered prior to WGCNA as low-expressed or non-varying genes usually represent noise. Filtering genes/probes by MAD have been used in most WGCNA studies, so we filtered 5000 probes by the MAD method.

WGCNA was performed in R software. Twelve samples were clustered in a dendrogram (Supplementary Fig. 2A). Then 5000 probes across the 12 samples were clustered into distinct modules depending on topological overlap (Supplementary Fig. 2B). We finally got 45 modules ranging in size from 31 to 592 probes with a median of 91 (Supplementary Table 2). Overall, 4969 probes were assigned to modules and only 31 probes were not classified into any modules (designated as grey) (Supplementary Fig. 2B and Supplementary Table 2). To visualize the results, we plotted a heatmap that depicted the topological overlap matrix among these probes (Supplementary Fig. 2D). ME represented the expression of all probes classified into that module. Next, we assessed ME relationship to melatonin treatment and identified six down-regulated modules significantly correlated with melatonin treatment (Fig. 1). In addition, we further found that MM and GS were strongly correlated in darkturquoise module ($r = 0.77$, $p = 3.1\mathrm{e}{-117}$), suggesting that genes in darkturquoise module were highly related to melatonin treatment (Supplementary Fig. 3). Likewise, genes in red module (Fig. 1, $r =$

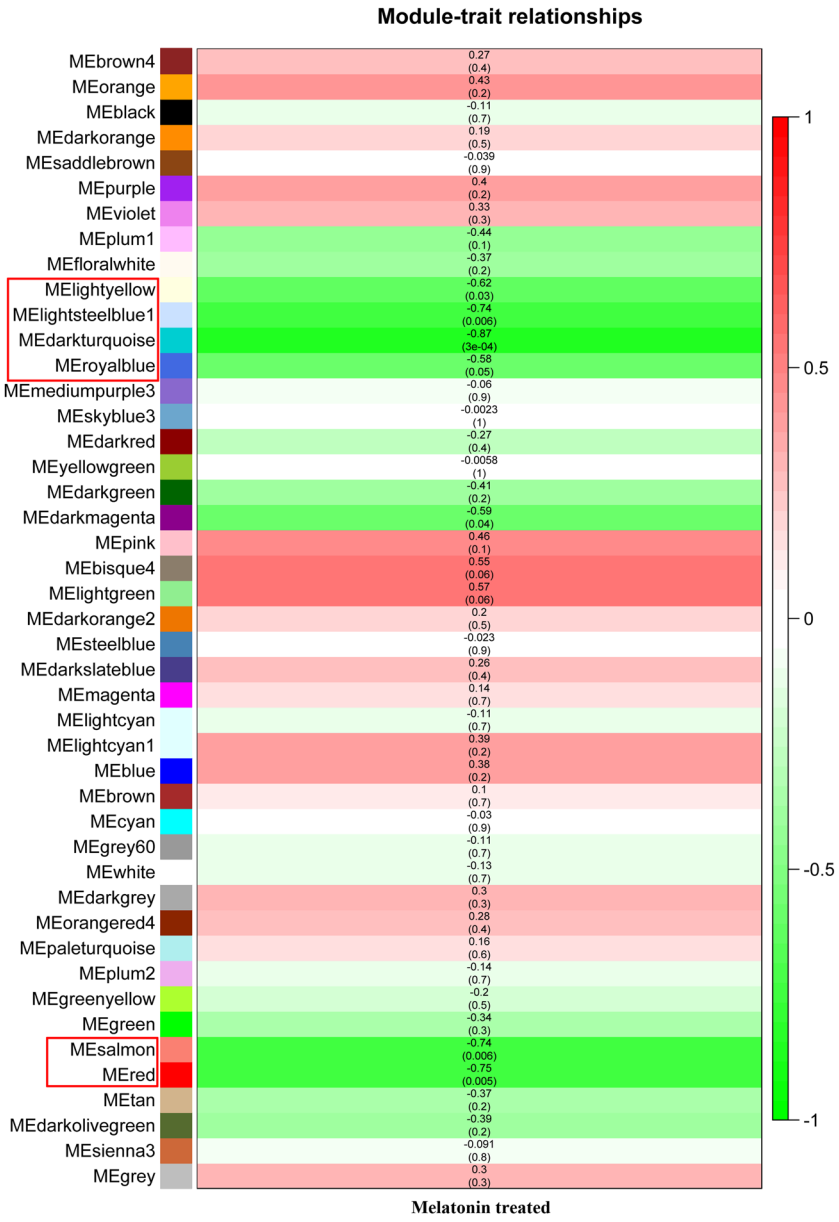

Fig. 1 Pearson correlation between module eigengenes and sample traits. Numbers in the table report the correlations with the p values printed below the correlations in parentheses. Red is positively correlated and green is negatively correlated

-0.75, $p = 0.005$) were also closely associated with the melatonin treatment (Supplementary Fig. 3, MM vs. GS, $r = 0.75$, $p = 3.5e{-}26$). In contrast, genes in the grey module (Fig. 1, $r = 0.3$, $p = 0.3$) and black module (Fig. 1, $r = -0.11$, $p = 0.7$) showed only weak correlations (Supplementary Fig. 3, MM vs. GS, Red: $r = 0.54$, $p = 0.0017$; Black: $r = 0.019$, $p = 0.83$).

Among the 45 WGCNA modules, darkturquoise had the strongest correlation ($r = -0.87$, $p = 0.0003$) with melatonin treatment (Fig. 1). Meanwhile, the darkturquoise module was the largest module that possessed 592 probes (Supplementary Table 2). Therefore, we selected the darkturquoise module for the next analysis. However, not all of those WGCNA-filtered genes were differentially expressed in the present data set. To identify DEGs, we used "Limma" package in R/Bioconductor software with the cut-off criteria of FDR<0.05. Two thousand five hundred and forty-five differentially expressed probes, including 1091 upregulated and 1454 downregulated probes, were obtained (Fig. 2). When we integrated the WGCNA and Limma results, we got 358 differentially expressed probes in the darkturquoise module (Fig. 2a). After converting Affymetrix probes to gene name and removal of duplicates, 357 DEGs remained in darkturquoise module (Supplementary Table 3).

Enrichment analysis of DEGs in darkturquoise module

To better understand the biological functions of genes in darkturquoise module, we conducted GO and KEGG

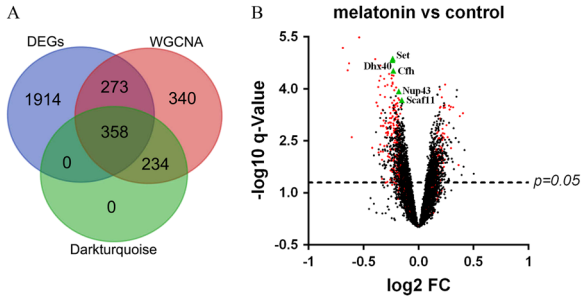

Fig. 2 Venn diagram for weighted gene co-expression network analysis (WGCNA) and differentially expressed gene (DEG) analysis (Limma) results and volcano plot of all probe signals. **a** Venn diagram. The number of genes screened by WCGNA and Limma and the overlap between them are shown in the Venn diagram. **b** Volcano plot. *x* axis represents $\log_2$ fold change and *y* axis indicates $\log_{10}$ *q*-value. Each dot represents one probe that had detectable expression in both groups. Red dots represent probes in darkturquoise module. Green solid triangles represent identified hub genes

pathway analysis. Results revealed that genes in darkturquoise module were significantly related to several enriched terms. We listed the top ten enriched terms in Supplementary Fig. 4. GO:0051984 (positive regulation of chromosome segregation) was the most abundant GO BP ($p =$ 3.87555E-08); GO:0016605 (PML body) was the most significant GO CC ($p = 2.97552E-06$); GO MF was found to mainly focus on GO:0008094 (DNA-dependent ATPase activity) ($p = 0.002812957$, data not shown); the KEGG pathway associated with most genes was mmu05161 (Pathway Hepatitis B) ($p = 0.000415322$), followed by mmu03040 (Spliceosome) ($p = 0.001352011$, data not shown). Interestingly, from the enrichment analysis we found that these DEGs in darkturquoise module were mostly enriched in terms related to physiological processes of genetic materials (chromosome, DNA, and RNA). These results might suggest an important role of melatonin in regulating the biological function of cardiomyocytes.

Identification of hub genes associated with melatonin treatment

We used GS value to identify hub genes. GS is a WGCNA parameter defined by the correlations between individual genes and clinical traits (here it is "melatonin treatment"). When combined limma and WGCNA results, those DEGs with their GS value >0.9 were defined as hub genes. Through this method, we identified five hub genes tightly related to melatonin treatment in cardiomyocytes (Table 1 and Fig. 2b). Among these genes, Set, Scaf11, Cfh, and Nup43 fell into darkturquoise module while Dhx40 was in red module. Notably, Dhx40 and Set were the two most significant differentially expressed genes with the highest fold change value within hub genes. Notably, the five genes were all negatively correlated with melatonin treatment in the co-expression network (Table 1). There were also positively correlated DEGs in the identified modules (Supplementary Tables 3 and 4). They might be involved in the effect of melatonin on cardiovascular system as well.

Discussion

In this study, we used WGCNA and DEG analysis to analyse the whole-genome expression data obtained from melatonin-treated mouse myocardium. WGCNA is a systems biology method for revealing the higher-order relationships of genes. Six gene co-expressed modules significantly correlated with melatonin treatment were identified. We selected the most significant module, the darkturquoise module, for further analysis. Darkturquoise module contained 357 DEGs. These DEGs were mostly enriched in GO:0051984 (positive regulation of chromosome segregation), GO:0016605 (PML body), and GO:0006281 (DNA repair). Several hub genes (Set, Dhx40, Scaf11, Cfh, and Nup43) were identified according to GS ranking. The results offered novel insights into the potential molecular basis underlying the cardioprotective effect of melatonin.

So far, few studies have reported the function of the five novel hub genes in melatonin's effect on cardiovascular system. The hub gene Nup43 is a nucleoporin gene. Nup43 protein is part of the Nup107–160 complex [7], which is one of nuclear pore complexes that mediate the bidirectional transport of macromolecules between the cytoplasm and nucleus in eukaryotes. More recently, Nup43 mutations have been found to implicate in cardiac disease [8]. Decrease of Nup43 gene expression led to cardiac abnormalities in zebrafish, including pericardial oedema and heart failure [8]. Set was identified as another important hub gene. Set was the most significant DEG in the darkturquoise module (Table 1). Set, also known as I2PP2A, is an endogenous inhibitory protein of protein phosphatase 2A (PP2A) [9]. Set can directly bind to PP2A and suppresses its phosphatase activity. The function of PP2A in heart and cardiovascular diseases is well established [10]. PP2A is a central cardiac phosphatase that regulates diverse cardiomyocyte functions through plenty of its target molecules [11]. Meanwhile, the therapeutic effect of melatonin on Alzheimer's disease [12], cerebral ischaemia [13], and other various diseases [14] has been found to be associated with PP2A activity. Thus it would be reasonable to speculate that Set may be involved in the regulation melatonin on PP2A activity. It would also be interesting to investigate the role of Set in the effects of melatonin on cardiovascular system. Cfh is another identified hub gene. Several reports have shown that Cfh polymorphism was inversely associated with susceptibility to coronary heart disease [15, 16]. Cfh

Table1 Five hub genes identified by GS value

Gene	RefSeq	Module	GS.melatonin treatment	*p*.GS. melatonin treatment	Log_2FC	*p* value
Set	NM_023871	Darkturquoise	−0.925650996	1.57774E−05	−0.234765927	0.0000134
Dhx40	NM_026191	Red	−0.921829031	2.0139E−05	−0.237315974	0.0000148
Scaf11	NM_028148	Darkturquoise	−0.918186998	2.51307E−05	−0.151562215	0.00021277
Cfh	NM_009888	Darkturquoise	−0.908028725	4.43354E−05	−0.228992461	0.0000299
Nup43	NM_145706	Darkturquoise	−0.902211922	5.96419E−05	−0.179353476	0.000118517

GS Gene Significance, Log_2FC $\log_2$(fold change)

belongs to the regulator of complement activation gene cluster and encodes two proteins, Factor H (FH) and Factor H-like protein 1 (FHL-1), via alternative splicing [17]. FH is the major soluble inhibitor of complement in human blood and has been found recently to be associated with the risk of atherosclerosis [18]. The role of the melatonin-sensitive gene Dhx40 and Scaf11 in cardiovascular system remains unknown. Actually, only a few studies reported the biological function of the two genes in mammals. Dhx40 and Scaf11 are both involved in RNA metabolism [19, 20]. Since melatonin exerts important effects in the process of material metabolism and energy metabolism [21], the sensitivity of Dhx40 and Scaf11 expression to melatonin treatment probably exists in multiple tissues instead of only in the myocardium. However, melatonin may influence RNA metabolism in myocardial cells through the two genes and then achieve its cardioprotective effect.

The main weakness of this study was the lack of experimental evidence to validate the important role of the five key genes in the cardioprotective effect of melatonin. The current study was primarily focussed on data analysis. Nevertheless, compared with a lot of previous usual DEG analysis, the present study using WGCNA has several advantages. WGCNA will not miss genes with low fold change and allows the topology and dynamics of the co-expression network itself to be explored [22]. As a systems biology method, WGCNA has already been successfully applied in various diseases and biological contexts to identify candidate biomarkers or therapeutic targets in the past few years [23].

In conclusion, by combining WGCNA with DEG analysis, we revealed Set, Dhx40, Scaf11, Cfh, and Nup43 as potential hub genes in mouse myocardium after melatonin treatment. The five genes are probably related to the myocardial benefits of melatonin. Further functional explorations on these hub genes are needed to promote the understanding of the mechanism underlying the influence of melatonin on the cardiovascular system.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with animals performed by any of the authors.

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