Identification of key genes associated with the human abdominal aortic aneurysm based on the gene expression profile

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Abstract. The present study was aimed at screening the key genes associated with abdominal aortic aneurysm (AAA) in the neck, and to investigate the molecular mechanism underlying the development of AAA. The gene expression profile, GSE47472, including 14 AAA neck samples and eight donor controls, was downloaded from the Gene Expression Omnibus database. The total AAA samples were grouped into two types to avoid bias. Differentially expressed genes (DEGs) were screened in patients with AAA and subsequently compared with donor controls using linear models for microarray data, or the Limma package in R, followed by gene ontology enrichment analysis. Furthermore, a protein-protein interaction (PPI) network based on the DEGs was constructed to detect highly connected regions using a Cytoscape plugin. In total, 388 DEGs in the AAA samples were identified. These DEGs were predominantly associated with limb development, including embryonic limb development and appendage development. Nuclear receptor co-repressor 1 (NCOR1), histone 4 (H4), E2F transcription factor 4 (E2F4), and hepatocyte nuclear factor 4 α (HNF4A) were the four transcription factors associated with AAA. Furthermore, HNF4A indirectly interacted with the other three transcription factors. Additionally, six clusters were selected from the PPI network. The DEG screening process and the construction of an interaction network enabled an understanding of the mechanism of AAA to be gleaned. HNF4A may exert an important role in AAA development through its interactions with the three other transcription factors (E2F4, NCOR1 and H4), and the mechanism of this coordinated regulation of the transcription factors in AAA may provide a suitable target for the development of therapeutic intervention strategies.

Introduction

Abdominal aortic aneurysm (AAA) is characterized by permanent, localized dilations of the abdominal aorta, which are defined as having diameters 1.5 times greater than normal (or which measure >3 cm) (1). Aortic rupture is the most serious clinical condition resulting from the progression of AAA (2). Almost 80% of patients who experience aortic rupture succumb to mortality (3). Since the majority of aneurysms are usually asymptomatic until rupture occurs, diagnosis is therefore problematic, and no preventative therapies are currently available for patients to effectively limit the progression of AAA (2,4). Therefore, it is important to investigate the mechanisms of AAA initiation and progression in order to assist diagnostic applications and to develop therapeutic options.

AAA is considered to be a particular, localized form of atherothrombosis (5). It shares the usual risk factors with occlusive atherothrombosis, including an increasing age, male gender, smoking, possible genetic susceptibility and low high density lipoprotein-to-cholesterol levels (6). The pathogenesis of AAA is complicated and multifactorial. Unique hemodynamic forces, which particularly impact on the infrarenal area, and variations in the content of elastin and collagen in different parts of the aorta rendered the infrarenal part of the abdominal aorta highly susceptible to AAA development (2). Numerous animal models and clinical studies reported that the initiation of AAA involves an inflammatory response, which is often enforced by the upregulation of adhesion molecules (7-9). The degradation of the extracellular matrix, which is caused by an increased activity of matrix metalloproteinases (MMPs) and serine proteases, and smooth muscle cell apoptosis are the predominant features associated with the progression of...
AAA (10,11). Previous genome-wide studies using microarrays have investigated the pathogenesis of AAA (12-14). The involvement of the immune system in AAA formation and progression was also reported in previous studies (15,16), however, the molecular mechanisms leading to the development and progression of AAA remain to be fully elucidated.

Using the identical gene expression profile, Biros et al (17) demonstrated that immune pathways are upregulated within the undilated aorta proximal to an AAA. In the present study, the differentially expressed genes (DEGs) featured in the gene expression profile in AAA necks were analyzed. Furthermore, a function and pathway-enrichment analysis was performed on the DEGs, and a protein-protein interaction network (PPI) was constructed to identify those DEGs which have a central role in AAA. The present study also aimed to gain further insights into the molecular mechanisms underlying the development of AAA. Understanding these molecular mechanisms may assist in developing the understanding of the pathogenesis of AAA, and to translate these pathogenic activities into therapeutic applications.

Materials and methods

Microarray data and data pre-processing. The gene expression profile of GSE47472 was downloaded from the Gene Expression Omnibus ( GEO (17) in the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/geo/) based on the platform of GPL10558 (or the Illumina HumanHT-12 V4.0 expression beadchip). A total of 22 data biopsies were obtained from the AAA neck samples, comprising 14 AAA samples from patients undergoing open AAA repair and eight normal samples from beating heart organ donors following brain mortality. The original data were pre-processed using the beadarray package in R language (version 2.18.0; http://bioconductor.org/packages/release/bioc/html/beadarray.html) (18), and normalized using the quantile method (19). Boxplots of the raw and normalized data were produced.

Screening of DEGs. Multi-dimensional scaling (MDS), which was constructed with the ptoolsMDS (20) function in the linear models for microarray data (Limma) (21) package (version 3.24.15; http://www.bioconductor.org/packages/release/bioc/html/limma.html) was used to investigate the association of the samples as a measure of quality control. From the results of the MDS procedure, the AAA samples were separated into types A and B. The DEGs in the AAA sample types A and B were identified using the Limma package and were compared with the controls. The common DEGs, which featured consistent changes in their expression levels, were selected as the targets for further analysis. The false discovery rate (FDR) was calculated for multiple testing correction using the Benjamini and Hochberg method (22). The threshold for the DEGs was set as the log fold change (FC)>1 and FDR≤0.01. Pearson's correlation coefficient was used to examine the associations between these DEGs (23).

Enrichment analysis of the DEGs. The probe sets, which featured differential expression between the controls and the AAA samples, were annotated to Ensembl gene identifiers (IDs) for ID mapping using the database for annotation, visualization and integrated discovery (DAVID) tool (version 6.7; http://david.abcc.Ncifcrf.gov/) (24,25). Gene ontology (GO; http://www.geneontology.org/) (26) and Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.jp/kegg/pathway.html) analyses were performed on the selected lists of genes. The threshold was set as P≤0.05.

Constructing an interaction network and functional analysis. Following ID mapping, all selected genes were exported into Cytoscape plugin (27) using the BisoGenet module (28) to create network visualizations. The source of the interaction network database was the Biomolecular Interaction Network Database (BIND) (29). Subsequently, a cluster analysis on the resulting network was performed with the Plugin, ClusterONE (http://apps.cytoscape.org/apps/clusterone) (30) program, using a P≤0.05 as a cut-off. The significant GO categories of the DEGs in the subnetworks were analyzed using the DAVID tool.

Results

Data pre-processing. The raw data downloaded from the GEO databases were normalized (Fig. 1A). The median values of each sample were almost at the identical level, suggesting that the data were eligible for further analysis. An MDS plot was constructed as a means of visualizing the data. This was performed for all the probes available. The AAA samples were observed to cluster into two groups, types A and B (Fig. 1B).

Screening of the DEGs. Comparing the AAA type A samples with the donor controls yielded the identification of 1,584 DEGs (Fig. 1C). Comparing the AAA type B samples with the donor controls yielded 984 DEGs in total (Fig. 1D). A total of 340 genes were identified as being DEGs common to each group, as revealed by the Venn diagram (Fig. 2A), and therefore, 338 DEGs exhibited a consistent change. An assessment of the correlation of the 338 DEGs revealed a positive pattern of correlation, with a Pearson's correlation coefficient (r) of 0.974 (P<2.2×10−16). The top three upregulated and downregulated DEGs, with the highest logFC, are listed in Table 1. Subsequently, the expression pattern of the DEGs was determined. From the heat map shown in Fig. 2, it was observed that the expression pattern of the DEGs enabled the AAA samples to be distinguished from the donor control samples. Furthermore, the AAA sample types A and B were consistent.

GO and KEGG enrichment analyses. The results of the GO and KEGG enrichment analyses of the common DEGs using the DAVID tool are presented in Fig. 3. A total of 18 significantly enriched categories were identified, which comprised 15 biological processes, two molecular functions and one KEGG pathway, grouped and annotated manually. The DEGs were predominantly associated with limb development, including embryonic limb morphogenesis and appendage development. The tyrosine kinase signaling pathway was also significantly enriched.
Table I. Differentially expressed genes in Type A and Type B.

| Probe ID     | Expression change | Gene symbol | Type A          | Type B          |
|--------------|-------------------|-------------|-----------------|-----------------|
| ILMN_1899549 | Upregulated       | NA          | 3.369751        | 3.112059        |
| ILMN_1717168 | Upregulated       | PCDHG4A     | 2.753187        | 2.225494        |
| ILMN_2177965 | Upregulated       | RPS19BP1    | 2.678406        | 2.365836        |
| ILMN_2231051 | Downregulated     | TCP11L2     | -3.164650       | -1.003280       |
| ILMN_1787591 | Downregulated     | xpa         | -3.391970       | -1.162970       |
| ILMN_1717733 | Downregulated     | NA          | -4.015280       | -2.217670       |

Positive values for logFC represent upregulated and negative values represent downregulated genes, ID, identifier; FC, fold change, adj.P.Val, adjusted P-value.

Figure 1. Data preprocessing and screening of the DEGs (A) The normalized expressed value data are shown. The black line featured in each of the colored boxes represents the median of each set of data, which determines the degree of standardization of the data through its distribution. Following normalization, the black lines in the boxes are almost in a straight line, indicating a good degree of standardization. (B) An MDS plot of the summarized microarray data following normalization. The array weights, calculated with the design matrix, reflect the association between the samples. Volcano plots are shown of the log₁₀ false discovery rate against the logFC for each gene of (C) AAA type A, vs. control and (D) AAA type B, vs. control. The FC and the statistical significance were plotted on the x- and y-axes, respectively. The genes, which are statistically significantly upregulated, are shown in red and those, which are statistically significantly downregulated, are shown in green. MDS, multidimensional scaling; FC, fold change; DEG, differentially expressed gene; AAA, abdominal aortic aneurysm.
Figure 2. Identification of the DEGs as the analysis target. (A) A Venn diagram demonstrating a comparison of the AAA type A and type B samples, vs. the control. Overlapping probes are indicated as DEGs in types A and B. (B) The correlation of the selected probe sets with a correlation coefficient of $r=0.974$ and $P<2.2\times10^{-16}$. (C) Heat maps of the genes, which are significantly differentially expressed in types A and B. DEG, differentially expressed gene; AAA, abdominal aortic aneurysm.

Figure 3. Enriched GO terms of the differentially expressed genes. A total of 15 biological processes, two molecular functions and a Kyoto Encyclopedia of Genes and Genomes pathway were enriched. GO, gene ontology.
Interaction network and functional analysis. The 338 probe sets, which were retained, were mapped, in total, to 297 gene symbols. Interactions between the genes were searched for in BIND and the results are presented in Fig. 4. The transcriptional factors nuclear receptor corepressor 1 (NCOR1), histone 4 (H4), E2F transcription factor 4 (E2F4) and hepatocyte nuclear factor 4α (HNF4A) were identified in the network to regulate other DEGs. Six noteworthy clusters emerged from the clustering analysis (Table II) and the subnetwork diagram is presented in Fig. 5.

Table II. Noteworthy clusters in the network.

| Cluster | GO ID   | Nodes | Density | Quality | P-value | Description                        | Genes in cluster         |
|---------|---------|-------|---------|---------|---------|------------------------------------|--------------------------|
| 1       | 0043565 | 6     | 0.733   | 0.917   | 0.003   | Sequence‑specific DNA binding      | BACH2, JUN, MAKF, MAFG,  |
|         |         |       |         |         |         |                                    | ATF7, MAFB               |
| 2       | 0045665 | 4     | 0.667   | 1       | 0.011   | Negative regulation of neuron     | PBX1, PAX6, IPO13, HXB   |
|         |         |       |         |         |         | differentiation                    |                          |
| 3       | 0031143 | 4     | 0.667   | 1       | 0.011   | Pseudopodium                      | ACTN2, MYOZ2, MYOZ1,     |
|         |         |       |         |         |         |                                    | LDB3                     |
| 4       | 0007242 | 3     | 0.667   | 1       | 0.026   | Intracellular signaling cascade    | NDKB, ITGB1BP1, KRIT1    |
| 5       | 0030117 | 3     | 0.667   | 1       | 0.026   | Membrane coat                      | AP3D1, AP3S2, AP3S1      |
| 6       | 0001759 | 3     | 0.667   | 1       | 0.026   | Induction of an organ              | FGR1, FGF1, FGF2         |

GO, gene ontology; ID, identifier.

Figure 4. Interaction network of the DEGs. The nodes shown in pink represent the DEGs and those in blue represent the proteins interacting with the DEGs. The red arrows indicate interactions between DNA and protein, and the blue lines denote interactions between proteins. DEG, differentially expressed gene.
Discussion

AAA is a complex disease, of which the pathobiology remains to be fully elucidated (2). Previous studies have revealed that the disease has a marked genetic component (12,14,31). Assessing gene expression profiling in cases of disease may reveal the underlying changes in gene activity, which contribute to the disease and enable targets for therapeutic intervention to be identified. In the present study, the gene expression profile downloaded from the GEO database was used to examine the mechanism of AAA development. A total of 340 genes were identified to be commonly DEGs in AAA samples of type A compared with donor controls and AAA samples of type B, among which 388 genes exhibited consistent changes. The analysis of the DEGs indicated that they were predominantly involved in limb development. The PPI network analysis revealed that four transcription factors regulated these DEGs in the network, and that NCOR1, H4 and E2F4 were clustered across the network in association with HNF4A. Furthermore, c-jun proto-oncogene (JUN) and its downstream components, which are regulated upstream in the pathway by HNF4A, were enriched in cluster 1.

NCOR1, which mediates transcriptional repression by certain nuclear receptors, is a part of a complex, which promotes histone deacetylation and the formation of repressive chromatin structures, and which may impede the access of basal transcription factors (32). MMPs exert a significant role in the degradation of the extracellular matrix of the vessel wall, which gradually leads to the formation of AAA (33). Mannello et al (34) demonstrated that MMP-3 may degrade NCOR1 to prevent transcriptional repression of the connective tissue growth factor promoter. In line with previous studies, NCOR1 may exert a critical role in the development of AAA, based on the PPI network analysis.

H4 is a core histone of the nucleosome, which functions in nucleosomal wrapping and occupies a central role in transcriptional regulation, DNA repair, DNA replication and chromosomal stability (35). Santos-Rosa et al (36) reported that the histone code set up by post-translational histone modifications also respond to DNA damage, which is frequently observed in cancer cells. HNF4A is a transcriptionally controlled transcription factor, and it may be essential for the development of the kidney, liver and intestine (37,38). A previous study revealed that phospholipase A2 g10-deficient mice were protected from angiotensin-II-induced aortic aneurysms (39). Guan et al (40) demonstrated that the transcription levels of Pla2g12b were regulated by the transcription factor HNF4A and its co-activator. Furthermore, Soutoglou et al (41) reported that HNF4A may interact with histone acetyltransferases, a process which is dependent on the acetylation status of the histones. In this context, and based on the results of the present study, it was postulated that there may be an interaction between the expression of H4 and HNF4A in the development of AAA.

E2F4 is a transcriptional activator, which binds DNA co-operatively with differentially regulated transcription factor proteins, whose products are involved in DNA replication and in cell cycle regulation (42). The transcription factor DRTF1/E2F complex functions in the control of cell-cycle progression from G1 to S phase (43). Tung et al (44) identified genes, which are involved in cell cycle regulation in AAA, using a membrane-based complementary DNA expression array. Talianidis et al (45) demonstrated that complex interactions between HNF4 bound to the proximal promoter and SP1 bound to multiple distal regulatory sites may lead to transcriptional activation of liver-specific human apolipoprotein CIII gene. SP1 is a cellular transcription factor involved in a wide variety of processes, and it has been determined.
to bind to different promoters to regulate transcription (46). In addition, Price et al (47) determined that differences in allelic expression of the MMP-2 gene, which exerts a major role in AAA formation, were attributed to the elimination of SP1 binding. Furthermore, the expression levels of HNF4A as a hepatocyte marker for hepatic differentiation were increased in the early G1 phase in human embryonic stem cells during their cell cycle (48). Therefore, an important interaction may occur between HNF4A and E2F4 in the pathogenesis of AAA.

A previous study revealed that HNF4A may be a key upstream mediator of JUN, whereas JUN was intimately associated with BTB and CNC homology 1, basic leucine zipper transcription factor 2 (BACH2), which is involved in the lymphocyte signaling pathway in cluster 1 (49). Henderson et al (50) demonstrated the death of smooth muscle cells and the expression of mediators of apoptosis by T lymphocytes in human AAA. A previous study revealed that the inhibition of c-Jun N-terminal kinase caused regression of AAA. In this context, it is surmised that JUN may interact with BACH2 in the progression of AAA.

In conclusion, genes which are critical in the development of AAA have been assessed based on the microarray data. HNF4A exerts an important role in the development of AAA through the interactions made with three other transcription factors (E2F4, NCO1 and H4), and the coordinated regulation of these transcription factors may represent potential novel targets for the mechanism underlying the development of AAA. The present study offers novel insights into the pathobiology of AAA. Further studies are required to confirm these intriguing results in terms of the possible associations with the transcriptional factors.

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