

Analysis of differentially expressed genes between rheumatoid arthritis and osteoarthritis based on the gene co-expression network

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Received August 11, 2013; Accepted March 5, 2014

DOI: 10.3892/mmr.2014.2166

Abstract. The aim of the current study was to investigate disease-associated genes and related molecular mechanisms of osteoarthritis (OA) and rheumatoid arthritis (RA). Using GSE7669 datasets downloaded from Gene Expression Omnibus databases, the differentially expressed genes (DEGs) between RA and OA synovial fibroblasts (SFBs) (n=6 each) were screened. DEG-associated co-expression and topological properties were analyzed to determine the rank of disease-associated genes. Specifically, the fold change of differentially expressed genes, the clustering coefficient and the degree of differential gene co-expression were integrated to determine the disease-associated gene ranking. The underlying molecular mechanisms of these crucial disease-associated genes were investigated by gene ontology (GO) enrichment analysis. A total of 1313 DEGs, including 1068 upregulated genes and 245 downregulated genes were observed. The top 20 disease-associated genes were identified, including proteoglycan 4, inhibin β B, carboxypeptidase M, alcohol dehydrogenase 1C and integrin β 2. The major GO biological processes of these top 20 disease-associated genes were highly involved in the immune system, such as responses to stimuli, immune responses and inflammatory responses. This large-scale gene expression study observed disease-associated genes and their associated GO function in RA and OA, which may provide opportunities for biomarker development and novel insights into the molecular mechanisms of these two diseases.

Introduction

Human rheumatoid arthritis (RA), a polyarticular disease of autoimmune nature (1), is perpetuated by an invasive pannus tissue, whereas osteoarthritis (OA), as a non-inflammatory degenerative disease of the articular cartilage (2), is characterized by an increased tendency for novel blood vessel formation (3,4). Furthermore, recent studies have reported information regarding the differences in pathogenesis between RA and OA. Patients with RA present with joint destruction caused by hyperplasia of the synovial lining, infiltration of mononuclear cells into the sublining layer, stimulation of fibroblast-like synoviocytes and the increase of catabolic mediators, including interleukin (IL)-1 β tumor necrosis factor (TNF)- α and matrix metalloproteinases (MMP) (5). By contrast, in patients with OA, joint destruction is due to cartilage degradation and elevated concentrations of cartilage matrix components, which elicit the presence of synovitis. In addition, synovitis aggravates the damage of articular cartilage by releasing inflammatory cytokines and destructive proteases (6).

Although OA and RA have different modes of pathogenesis, the current treatment of these two disease is similar, including nonsteroidal anti-inflammatory drugs, applied for pain and inflammation management (7,8); disease-modifying antirheumatic drugs, which function as a classical first-line therapy to minimize or prevent joint damage (7,9); and surgical treatment performed to replace the joints (10,11). However, these approaches induce a number of adverse events and less than satisfactory clinical outcomes (12,13). Thus, disease-specific therapy requires further investigation.

Genetic factors are also critical in the pathogenesis of RA and OA. Bramlage *et al* (14) identified that bone morphogenetic protein (BMP)-4 and BMP-5 were downregulated in OA and RA compared with that expressed in normal synovial tissue, suggesting a role of distinct BMPs in joint homeostasis, which may be altered in inflammatory and degenerative joint diseases. In addition, Pohlers *et al* (15) demonstrated that upregulation of the tumor growth factor (TGF)- β pathway was observed in RA synovial fibroblasts (SFBs), resulting in significant overexpression of MMP-11 mRNA and protein in RA SFBs, but not in OA SFBs. However, differentially expressed

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Key words: differentially expressed genes, molecular mechanisms, gene ontology

genes (DEGs) and molecular mechanisms underlying RA and OA are not yet fully understood.

In the current study, comparative analysis of DEG characteristics between RA and OA profiles was performed to identify DEGs with potential pathophysiological relevance. Differential gene co-expression networks were constructed and analyzed to identify disease candidate genes.

Materials and methods

Microarray data analysis. The gene expression data was downloaded from the Gene Expression Omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/geo/>) using GEO accession no. GSE7669 (15). The database contains six RA and six OA SFBs derived from a human study using the Affymetrix Human Genome U95 version 2 (Affymetrix Inc., Santa Clara, CA, USA) platform. Background-corrected signal intensities were determined using the MAS 5.0 software (Affymetrix®). The normalization of datasets obtained on Affymetrix arrays was performed using the preprocessCore package in R (16).

Screening DEGs. Significance analysis of microarray (SAM) is widely used to detect genes on a microarray with statistically significant changes in expression (17). As an alternative to a t-test, significance SAM4.0 was employed for determination of differentially expressed genes between RA and OA SFBs and the threshold was set as \log_2 fold change ≥ 2 .

Constructing differential gene co-expression networks. Prior to the construction of co-expression networks, the expression value was determined from differentially expressed gene samples between RA and OA. Next, Pearson's coefficient was applied to calculate correlations between DEGs. The differential co-expression network was then constructed as the threshold was set as $r \geq 0.8$.

Network topological analysis. The Cytoscape plug-in Network Analyzer (18) was used for network visualization and gene co-expression analysis. For each network the number of nodes and edges was simply calculated. The clustering coefficient of a network C_n was counted as the average clustering coefficient of all of its non-singleton nodes via the formula: $C_n = 2e_n/k_n(k_n-1)$ (1); where e_n denotes the number of edges between the k_n neighbors of n and k_n is the degree of n (19).

Gene ranking. Conventionally, disease-associated gene ranking is determined by linkage analysis and gene expression profile analysis (20-22). However, these methods are mostly limited to a single statistic indicator. In the current study, the fold change of differentially expressed genes, the clustering coefficient and the degree of differential gene co-expression were integrated to determine the disease-associated gene ranking using the formula (2): $Cri_n = FC_n + degree_n + C_n$ (2); where Cri_n is an indicator of the gene ranking (the larger the value is, the higher the gene ranks), FC_n stands for the value of gene fold change, $degree_n$ and C_n denotes the average degree and the clustering coefficient of a co-expression network respectively.

Calculating GO enrichment. Biological function and candidate gene-associated biological pathways can be determined

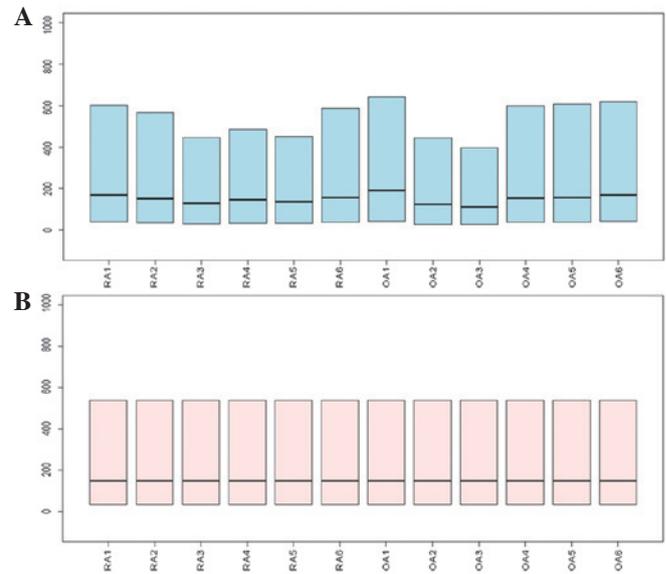


Figure 1. Normalization of samples. (A) Prior to normalization and (B) following normalization, RA1-6 = 6 RA samples, OA1-6 = 6 OA samples. RA, rheumatoid arthritis; OA, osteoarthritis.

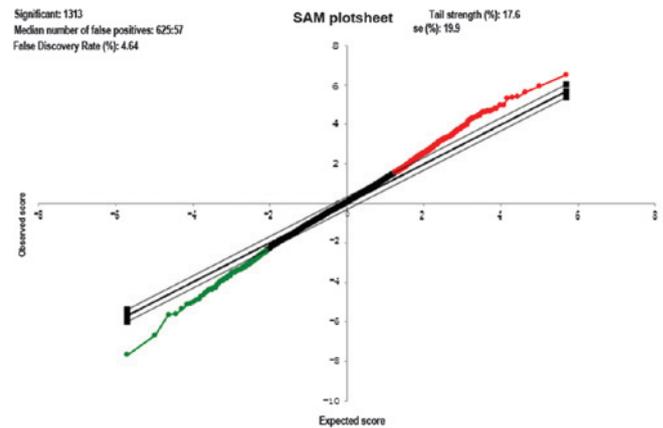


Figure 2. SAM plotsheet outputs. The red, green and black dots indicate upregulated, downregulated and insignificant genes, respectively. The upper and lower 45° lines indicate the Δ threshold boundaries. Tail length (17.6%) and standard error (se) rate (19.9%) is shown at the upper right corner of the plotsheet. The number of significant genes (1313), median number of false positives (625.57) and false discovery rate (4.64%) are shown at the upper left corner of the plotsheet. SAM, significance analysis of microarray.

by GO (<http://www.geneontology.org>). The open access software DAVID (23) was used to access the GO enrichment of candidate genes, which is a slightly modified Fisher's exact test, identical to the EASE score (24).

Results

Differentially gene expression. Gene expression in six RA SFBs was compared with that in six OA SFBs. The R preprocessCore was used to normalize and preprocess the presented data (\log_2 fold change ≥ 2) (Fig. 1). A total of 1313 differentially expressed genes were observed, in which 1068 genes were upregulated and 245 genes were downregulated in OA SFBs compared with RA SFBs (Fig. 2).

Table I. genes with the highest node degrees in the co-expression network.

No.	Gene ID	Degree	Clustering coefficient
1	TBCD	190	0.3267613
2	ABCA3	169	0.3709495
3	DDAH2	164	0.3754302
4	SLC29A1	164	0.3787221
5	ARL2	162	0.3647726
6	MAPKAPK3	161	0.3473602
7	GUK1	160	0.3463050
8	GNB5	159	0.3894594
9	C16orf45	154	0.3922417
10	PPP1R7	152	0.3580515

TBCD, tubulin folding cofactor D; ABCA3, ATP-binding cassette sub-family A member 3; DDAH2, dimethylarginine dimethylaminohydrolase 2; SLC29A1, solute carrier family 29; ARL2, ADP-ribosylation factor-like 2; MAPKAPK3, mitogen-activated protein kinase-activated protein kinase 3; GUK1, guanylate kinase 1; GNB5, guanine nucleotide binding protein; C16orf45, chromosome 16 open reading frame 45 [Homo sapiens (human)]; PPP1R7, protein phosphatase 1, regulatory subunit 7.

Differential co-expression network. To construct a differential gene co-expression network, data from a total 1313 DEGs were extracted from the expression profile and Pearson's coefficient was applied to calculate correlations between these DEGs (the threshold as $r \geq 0.8$). A total 1302 nodes and 20372 edges were identified among the differential gene co-expression network, which is comprised of two closely connected sub-networks (Fig. 3)

Top 10 genes with the highest node degrees in the co-expression network. Clustering coefficient and degree were analyzed to detect the importance of disease-associated genes from differential gene co-expression networks. In the current study, the top 10 degree of corresponding genes are shown in Table I. Briefly, tubulin folding cofactor, ATP-binding cassette sub-family A member 3 and dimethylarginine dimethylaminohydrolase 2 were the top three genes of the list, with a considerably higher degree in the co-expression network.

Top 20 disease candidate genes. To determine the association between DEGs and the two diseases, OA and RA, disease candidate genes were ranked according to the Cri_n value using the formula $Cri_n = FC_n + degree_n + C_n$. As presented in Table II, the top 5 of 20 disease candidate genes were inhibin β B (INHBB), carboxypeptidase M, alcohol dehydrogenase 1A, integrin β 2 (ITGB2) and collagen, type XI, α 1 (COL11A1), respectively.

Functional annotation of candidate genes. The analysis of GO enrichment was used to detect the association between the top 20 disease-associated candidate genes and associated biological processes and pathways ($P < 0.05$). Overall, the majority of the top 20 disease-associated candidate genes, including INHBB,

Table II. Top 20 disease candidate genes.

Rank	Symbol	Regulation	Cri_n
1	INHBB	OA (down)	10.10852052
2	CPM	OA (down)	8.60116868
3	ADH1C	OA (up)	6.83154569
4	ITGB2	OA (down)	6.62193398
5	COL11A1	OA (down)	6.28105739
6	ADH1A	OA (up)	6.23549718
7	C10orf116	OA (up)	5.89913165
8	SLC29A1	OA (up)	5.84062491
9	PRG4	OA (up)	5.57878155
10	CHL1	OA (up)	5.54248666
11	RARRES2	OA (down)	5.40151339
12	MS4A2	OA (down)	5.36385476
13	CCR3	OA (up)	5.05797001
14	IFIT2	OA (up)	4.88706770
15	RSAD2	OA (up)	4.77719664
16	CLIC5	OA (up)	4.77298747
17	MYRIP	OA (up)	4.77081124
18	SPARCL1	OA (up)	4.70336130
19	ABCA3	OA (up)	4.51342883
20	THBS1	OA (down)	4.29227205

FC_n , C_n and degree were transformed by Z core. Data of gene regulation was observed by using SAM4.0 (RA vs. OA). The threshold was set as \log |fold change| ≥ 2 . RA, rheumatoid arthritis, OA, osteoarthritis; INHBB; inhibin β B; CPM, carboxypeptidase M; ADH1C, alcohol dehydrogenase 1C; ITGB2, integrin β 2; COL11A1, collagen, type XI, α 1; ADH1A, alcohol dehydrogenase 1A; C10orf116, chromosome 16 open reading frame 45 [Homo sapiens (human)]; SLC29A1, solute carrier family 29; PRG4, proteoglycan 4; CHL1, close homolog of L1; RARRES2, retinoic acid receptor responder protein 2; MS4A2, membrane-spanning 4-domains, subfamily A, member 2; CCR3, IFIT2, interferon-induced protein with tetratricopeptide repeats 2; RSAD2, radical S-adenosyl methionine domain containing 2; CLIC5, chloride intracellular channel 5; MYRIP, myosin VIIA and Rab interacting protein; SPARCL1, secreted protein, acidic and rich in cysteines-like 1; ABCA3, ATP-binding cassette, sub-family A, member 3; THBS1, thrombospondin 1.

CCR3, radical S-adenosyl methionine domain containing 2, thrombospondin 1 (THBS1) and membrane-spanning 4-domains, subfamily A, member 2, were intensively enriched in immune-associated biological process terms, including defense, inflammatory, immune responses, immune system process and response to wounding. In addition, ITGB2, THBS1, COL11A1, and close homolog of L1 were closely involved in cell adhesion and biological adhesion (Table III).

Discussion

Gene expression studies have been widely used to allow improved diagnosis and identify novel pathways implicated in the pathogenesis of autoimmune diseases. In the current study, DEGs in OA SFBs compared with RA SFBs were identified based on gene expression profiling, 1068 upregulated genes

Table III. Top 20 disease candidate genes and related BP terms.

Rank	BP term	N	Genes
1	Response to stimulus	9	INHBB, PRG4, CCR3, RSAD2, MS4A2, ITGB2, THBS1, COL11A1, ABCA3
2	Localization	8	SLC29A1, MYRIP, CLIC5, MS4A2, ITGB2, THBS1, ABCA3, DNM1
3	Defense response	6	INHBB, CCR3, RSAD2, MS4A2, ITGB2, THBS1
4	Response to stress	6	INHBB, CCR3, RSAD2, MS4A2, ITGB2, THBS120
5	Response to external stimulus	6	INHBB, CCR3, MS4A2, ITGB2, THBS1, COL11A1
6	Immune system process	5	PRG4, RSAD2, MS4A2, ITGB2, THBS1
7	Cell adhesion	5	CCR3, ITGB2, THBS1, COL11A1, CHL1
8	Biological adhesion	5	CCR3, ITGB2, THBS1, COL11A1, CHL1
9	Immune response	4	PRG4, RSAD2, MS4A2, THBS1
10	Inflammatory response	4	CCR3, MS4A2, ITGB2, THBS1
11	Response to wounding	4	CCR3, MS4A2, ITGB2, THBS1
12	Inner ear morphogenesis	2	CLIC5, COL11A1

Rank, the rank of BP term determined by the number of the 20 disease-related genes enriched in the BP term. N, number of the 20 disease-related genes involved in the BP term. BP, biological process; INHBB, inhibin β B; PRG4, proteoglycan 4; RSAD2, radical S-adenosyl methionine domain containing 2; MS4A2, membrane-spanning 4-domains, subfamily A, member 2; ITGB2, integrin β 2; THBS1, thrombospondin 1; COL11A1, collagen, type XI, α 1; ABCA3, ATP-binding cassette sub-family A member 3; SLC29A1, solute carrier family 29; MYRIP, myosin-VIIa- and Rab-interacting protein; CLIC5, chloride intracellular channel 5; DNM1, dynamin 1; THBS120, thrombospondin 120.

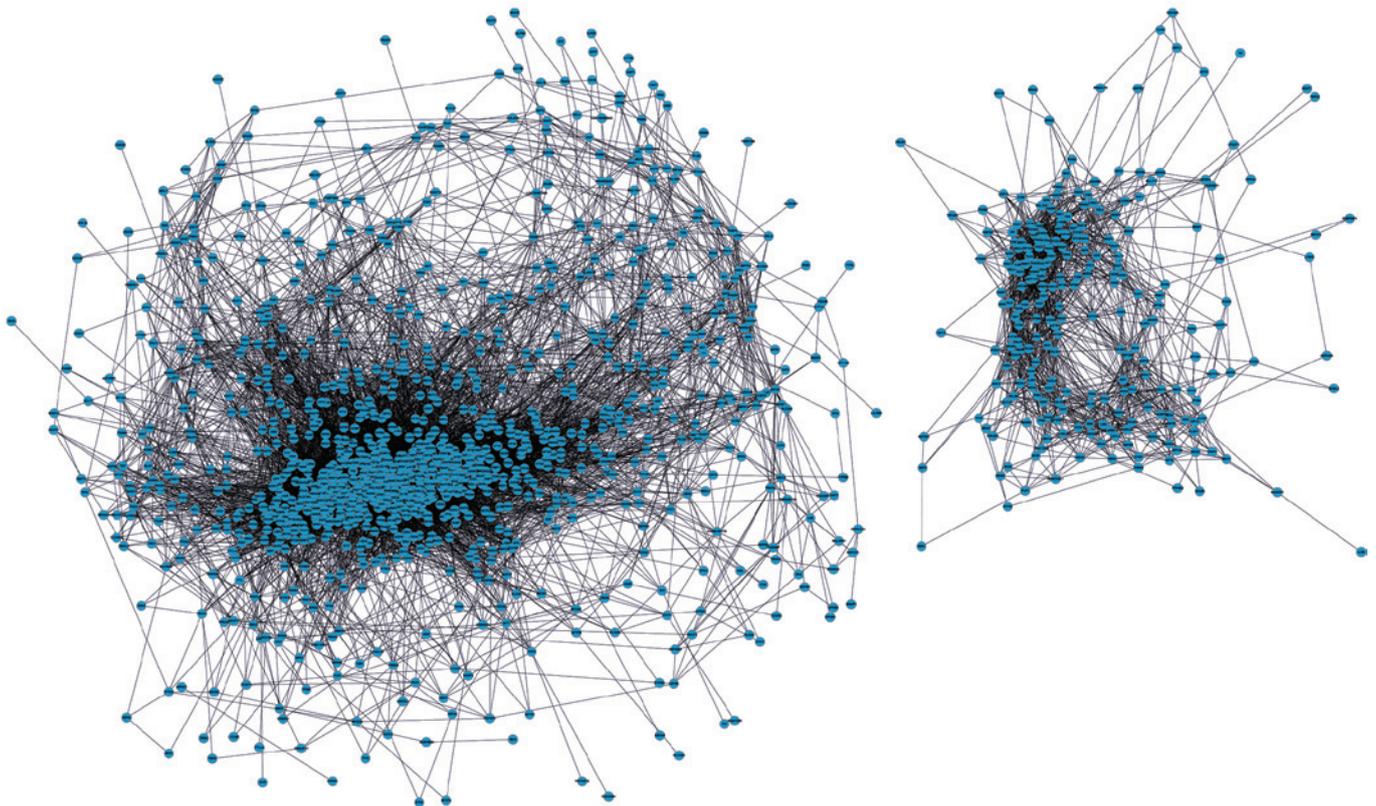


Figure 3. Differential gene co-expression network. A node represents one DEG (RA vs. OA) and is interconnected in the network. Co-expressed genes are linked by edges. DEGs, differentially expressed genes; RA, rheumatoid arthritis; OA, osteoarthritis.

and 245 downregulated genes were observed. Similarly, a previous study has reported different biological properties between RA and OA SFBs. Higher levels of specific cytokines

were found in RA SFBs compared with OA SFBs, including epidermal growth factor, basic fibroblast growth factor, TGF- β 1, granulocyte-macrophage colony-stimulating factor,

IL-1 β and IL-6. In addition, by contrast with the OA SFBs, RA SFBs were observed to stimulate [³H]thymidine incorporation in the murine fibroblast cell line (25). Therefore, the current results support the hypothesis that RA and OA may result in the alterations of gene expression in the SFBs.

The development of an RA and OA gene co-expression network based on the topological analysis is critical, since it may provide visualized structural information regarding the connectivity of genes, compared with the traditional clustering analysis (26). Notably, the clustering coefficient and degree are two of the most important features of the network model. In the present study, the top 10 clustering coefficient C_{ri_n} -associated genes were identified, which indicates a highly significant association between these genes and disease status. Notably, to the best of our knowledge the present study was the first to use the formula: $C_{ri_n} = FC_n + degree_n + C_n$, which is comprised of fold change, average degree and clustering coefficient of co-expression gene network to calculate the top 20 disease-associated candidate genes.

For OA disease, Martin *et al* (27) reported that the single nucleotide polymorphism, rs2615977, located in intron 31 of COL11A1 (5th gene at ranking) is highly associated with OA. In addition, COL11A1 has been used as a significant target for musculoskeletal disease research (28). In the present study, COL11A1 was observed to be downregulated in OA samples compared with RA samples, which further supports the results of a previous study that suggested COL11A1 may have been associated with OA (29). In addition, the inhibition of cartilage hyperplasia appears to be another favorable approach to relieve symptoms of OA disease. Ruan *et al* (30) demonstrated that PRG4 (9th gene at ranking) inhibits cartilage metabolism and proliferation by upregulating hypoxia inducible factor-3 α . In the current study, PRG4 was found to be upregulated in the OA group but downregulated in the RA group, which indicates that the PRG4 gene may have a potential association with the pathogenesis of RA and OA.

In RA, Rinaldi *et al* (31) suggested that β 1 integrins contribute to the tight binding of RA SFBs to the matrix and regulate extracellular matrix remodeling in the RA disease process *in vivo*. The function of β 1 integrins is similar to that of the ITGB2 identified in this study (4th gene at ranking). Notably, ITGB2 was identified to be upregulated in RA rather than OA, which further supports the hypothesis that ITGB2 may be critical in facilitating the RA disease process. INHBA and INHBB (1st gene at ranking) are two subunits of inhibin. El-Gendi *et al* (32) has demonstrated that the serum level of inhibin β A was significantly higher in 60 patients with RA compared with 20 normal patients. In the present study, the expression level of INHBB was identified to be higher in RA compared with OA, indicating that INHBB may have a similar function to INHBA and may serve as a novel biological marker in the diagnosis of RA disease.

Furthermore, functional enrichment analysis of the top 20 disease-associated genes was performed to demonstrate the possible biological mechanisms underlying AR and OA, including response to stimulus, localization, response to stress, response to external stimulus and process involved in the development of the immune system. This finding is consistent with the results of previous studies, suggesting that the immune system is pivotal in auto-inflammatory and

non-auto-inflammatory arthritis. The immune system regulates the alteration of cell osmosis and the inflammatory response caused by periostitis (33-36). The majority of the other top 20 disease-associated genes were enriched in terms of cell and biological adhesion. Notably, cell adhesion is also highly correlated with the pathogenesis of AR and OA. Karatay *et al* (37) stated that the decreased intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 levels following intra-articular hyaluronic acid (HA) injection may aid in explaining the anti-inflammatory effects of HA therapy in OA of the knee.

In conclusion, the present study offers significant information that may aid in understanding the molecular mechanisms underlying OA and RA. The top 20 disease-associated genes and associated BP terms were observed in this study, which may facilitate the design of targeted therapy for OA and RA in the near future.

Acknowledgments

This study was supported by the Shanghai Medical Key Subject Construction Project (ZK2012A28) and National Clinical Key Specialty Construction Project.

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