Systems-level analysis of genome wide association study results for a pilot juvenile idiopathic arthritis family study

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Genome wide association studies (GWAS) determine susceptibility profiles for complex diseases. In this study, GWAS was performed in 26 patients with oligo and rheumatoid factor negative polyarticular juvenile idiopathic artritis (JIA) and their healthy parents by Affymetrix 250K SNP arrays. Biological function and pathway enrichment analysis was done. This is the first GWAS reported for JIA families from the eastern Mediterranean population. Enrichment of FcγR-mediated phagocytosis pathway and response to various stimuli were the leading discoveries, along with the presentation of the strong interaction of JIA-associated genes with HLA cluster in the co-expression network. The co-expression network also presented the direct interaction of a gene in FcγR-mediated phagocytosis pathway, namely GAB2, with BLK, CDH13, IL4R and MICA. The systems biology approach helped us to investigate the interactions between the identified genes and biological pathways and molecular functions, expanding our understanding of JIA pathogenesis at molecular level.

Key words: Genome wide association study, juvenile idiopathic arthritis, single nucleotide polymorphism, transmission disequilibrium test, trio analysis.

Genome-wide association studies (GWAS) are considered as the major approach to unravel the link between disease susceptibility loci and associated phenotypic traits. Since the first GWAS study in 2005, there have been numerous attempts in identifying single-nucleotide polymorphisms (SNPs) associated with complex diseases[1]. Juvenile idiopathic arthritis (JIA) represents a group of heterogeneous diseases which are classified as arthritis of unknown origin and have onset before age of 16 years[2]. Although JIA is considered as the most common type of chronic rheumatic disease of childhood, lower prevalence as compared to rheumatoid arthritis and the different subgroups which probably have different pathogenesis mechanisms, limit sophisticated genetic studies and the GWAS[3]. JIA is divided into seven subgroups defined by the course at onset of the disease[4,5]. In recent years it has become apparent that systemic JIA has more pronounced autoinflammatory features[6]. Furthermore systemic onset JIA is associated with macrophage activation syndrome (MAS) and defects in natural killer cells have been identified in some of these patients[6]. Genetic studies tend to analyze systemic onset JIA patients separately. Oligoarticular and rheumatoid factor (RF) negative polyarticular subtypes which make up the majority of patients[7], have overlapping HLA associations and share a female gender bias[8]. Thus we have studied only oligoarticular and RF negative polyarticular JIA patients to maximize the homogeneity of our study group.

Rheumatoid arthritis is accepted to be an autoimmune disease. However, because of low penetrance and heterogeneous nature of the disease, little is known about the pathogenesis of JIA and probably it is more complex.

Previous attempts for the identification of the pathogenesis classify disease susceptibility genes into two categories which are human leukocyte antigen (HLA) genes and non-HLA related genes[2]. The classified HLA genes are composed of HLA class I and HLA class II allelic forms and the association suggests the role of T cells in the pathogenesis of the disease[9]. Non-HLA related genes are also associated with the disease. Majorly, these non-HLA genes are the genes coding for cytokines and a few of them are validated in independent studies so far which are *PTPN22, MIF, SLC11A6, WISP3, BLK,* and tumor necrosis factor (*TNF*)[10, 11].

Family based association design aims to avoid the potential confounding effects of population stratification by using the parents as controls for the case, which is their affected offspring. The most commonly used test is the transmission disequilibrium test, or TDT which measures the association of genetic markers in nuclear families by transmission from parent to offspring. If an allele increases the risk of having a disease then that allele is expected to be transmitted from parent to offspring more often in populations with the disease [12] . The trio study analyses the affected child and the parents. Under normal conditions each parental allele has a 50% probability for transmission. Thus, statistical departure from this probability suggests that the SNPs defined are both associated with and linked to the disease susceptibility locus[13].

In this study, a GWAS using Affymetrix Gene Chip Human Mapping 250K SNP Genotyping arrays was performed in the trio sets of 26 patients with oligo or RF negative polyarticular JIA belonging to eastern Mediterranean population.

Material and Methods

Patient Data: Families (trios) of 26 patients with oligo or RF negative polyarticular JIA who met the International League of Associations for Rheumatology (ILAR) classification criteria [5] were investigated. Clinical investigation and SNP genotyping performed by Hacettepe University were used. This study was approved by the research ethics committee of the study hospital.

SNP Genotyping: Affymetrix Gene Chip Human Mapping 250K SNP Genotyping arrays were used for the genotyping of samples from both patients and parents. SNP genotyping and quality control of data were performed with Affymetrix Genotyping Console, which provided a quick preview of data quality prior to performing a full clustering analysis.

Data Quality Controls and Pre-processing: Few mislabelings between the .ods files and JIA data file, especially with unique family numbers, have been corrected and recorded into JIA_pedfile.ods. The Affymetrix Genotyping Console revealed over 90% QC Call rate for the study data. Out of 262,264 total markers on the array, a total of 249,676 SNP markers has been included in the PLINK analysis[14] after genotyping and pruning. Total genotyping rate among the remaining individuals was 0.92. None of the remaining SNPs failed missingness test (GENO > 1) and the frequency test (MAF < 0)[14].

Statistical Methods

Family Based Association Analysis: Family based associations have been analyzed with the TDT through PLINK toolset (http://pngu.mgh.harvard.edu/~purcell/plink/) to determine JIA associated SNPs. A specificity of the TDT is that it detects genetic linkage only in the presence of genetic association. While genetic association can be caused by population structure, genetic linkage will not be affected, which makes the TDT robust to the presence of population structure. Initial quality control based filtering and preprocessing was performed by using the default thresholds of the system; Minor Allele Frequency = 0.05, SNP Missingness Rate = 0.1, Individual Missingness Rate = 0.1, Hardy Weinberg Equilibrium = 0.001[14, 15].

Annotation of Significantly Associated SNPs with SNPNexus:

SNPnexus knowledge base was used for the functional annotation of the top 1000 significantly associated SNPs after the TDT

Table I. Demographics of Patients

Diagnostic groups (n)	Gender (female/male), n	Age at diagnosis Median (range), years
Oligoarticular JIA (13)	8/5	2 (1-11)
RF negative polyarticular JİA (13)	10/3	4 (1-9)

Table II. Genes with Three or More Associated Single Nucleotide Polymorphisms (SNPs)

Associated genes	Number of SNPs
ASAP1	11
ALK	4
GRID2	4
MAST4	4
ALG6	3
FHIT	3
GAB2	3
ITGB3BP	3
KCNIP4	3
KIRREL3	3
MACROD2	3
NXPH1	3
RBFOX1	3
UBE2CBP	3
VWF	3

SNP, single nucleotide polymorphisms

analysis. dbSNP identifiers have been used for initiating the query. SNPnexus provides a list of data integrated for the list of SNP under the query such as genomic mapping and additional annotations, gene/protein consequences, effect on protein function, hapmap population data, regulatory elements conservation, phenotype and disease association and structural variations. In this study, we have retrieved phenotype and disease association data from the Genetic Association Database (GAD) through SNPNexus[16-18]. Later GAD annotations for associated diseases are classified under three groups and 1) autoimmune 2) autoinflammatory 3) other disease. Disease list under autoimmune and autoinflammatory groups is provided in Supplementary Table I.

Functional annotation and Pathway enrichment analysis with DAVID:

The Database for Annotation, Visualization, and Integrated Discovery (DAVID) is used to visualize enriched pathways in the dataset, and gene-specific functional data is extracted for all three disease groups. After gene lists were uploaded as official gene symbols to DAVID, functional annotation analysis was performed. Default options of the DAVID was cleared and following terms were selected for the pathway enrichment analysis: OMIM, all GO Terms for biological process and molecular function, and two pathway databases with highest number of query genes (KEGG, PANTHER)[19,20]. Functional annotation clusters, analysis and enriched pathways are reported.

Network analysis with GeneMANIA:

GeneMANIA is a publicly available gene network tool for predicting the function of query genes and gene sets. It finds other

Table III. Functional Annotation Chart for Top 257 Genes Associated with JIA After GWAS: **a-** Two Enriched Biological Pathways **b-** JIA Associated Genes Which Were Reported to be Included in the FcγR -Mediated Phagocytosis Pathway.

A- Pathway records

Category	Term	Count	p-value
KEGG_PATHWAY	Fc_gamma R-mediated phagocytosis	5	4.70E-02
KEGG_PATHWAY	Vascular smooth muscle contraction	5	7.60E-02

B- Gene report

Official gene symbol	Gene name
FCGR2A	Fc fragment of IgG, low affinity IIa, receptor (CD32)
GAB2	GRB2 associated binding protein 2
AMPH	amphiphysin
DOCK2	dedicator of cytokinesis 2
PRKCE	protein kinase C, epsilon

Table IV. Non-Coding SNPs on an eQTL with a Score of 1.

rsID	Regulome score	Affected gene
rs10957999	1f	*PAG1*
rs11228521	1f	*TPCN2*
rs1703937	1f	*ERICH1*
rs1737068	1f	*HLA-A,F and H*
rs7000407	1f	*CLDN23*
rs733004	1f	*STEAP4*
rs755115	1f	*PARP16*
rs9409552	1f	-

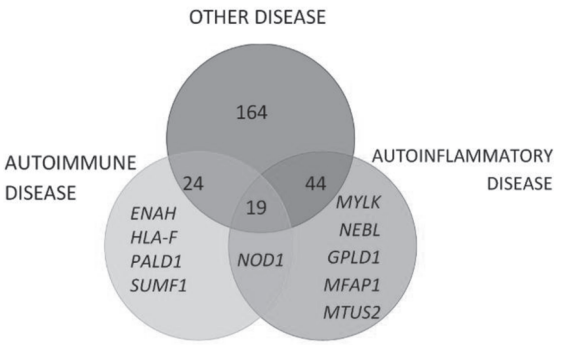

Fig. 1. Venn Diagram summarizing disease categories

genes that are in a relationship with genes in interest by hosting a very large set of functional association data comprising protein and genetic interactions, pathways, co-expression, co-localization and protein domain similarity [21]. Network analysis for autoimmune and autoinflammatory disease related genes was performed using all co-expression networks, and GO Molecular Function based network weighting. Functional categories of the network genes were ranked according to FDR and coverage for the specific functions were reported.

RegulomeDB:

The RegulomeDB[22] is a public database for the annotation and scoring of non-coding SNPs with known and predicted regulatory elements such as expression quantitative trait loci (eQTLs) (ref), regions of DNAase hypersensitivity, binding sites of transcription factors, and promoter regions. Regulome scoring scheme refers to the available datatypes for a single coordinate, and ranges from one to six, 1a being most annotated. A Regulome score varying between 1a-1f indicates presence of an expression quantitative trait loci (eQTLs), which are a genomic loci that regulate expression levels of mRNAs. Details of the Regulome scoring scheme is provided in the

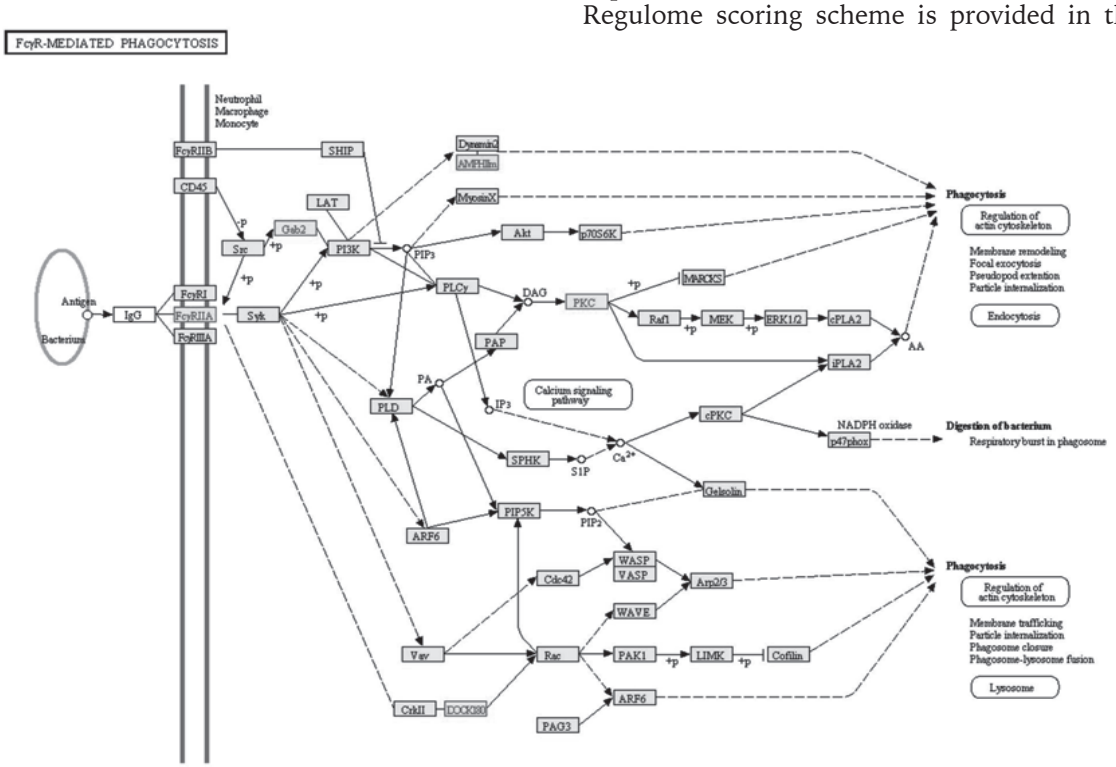

Fig. 2. Representation of FcγR -mediated phagocytosis pathway (hsa04666) in KEGG: Total of Seven SNPs that are found to be associated with JIA maps to 5 pathway genes; *FCGR2A,GAB2, AMPH, DOCK2, PRKCE* (labelled with star)

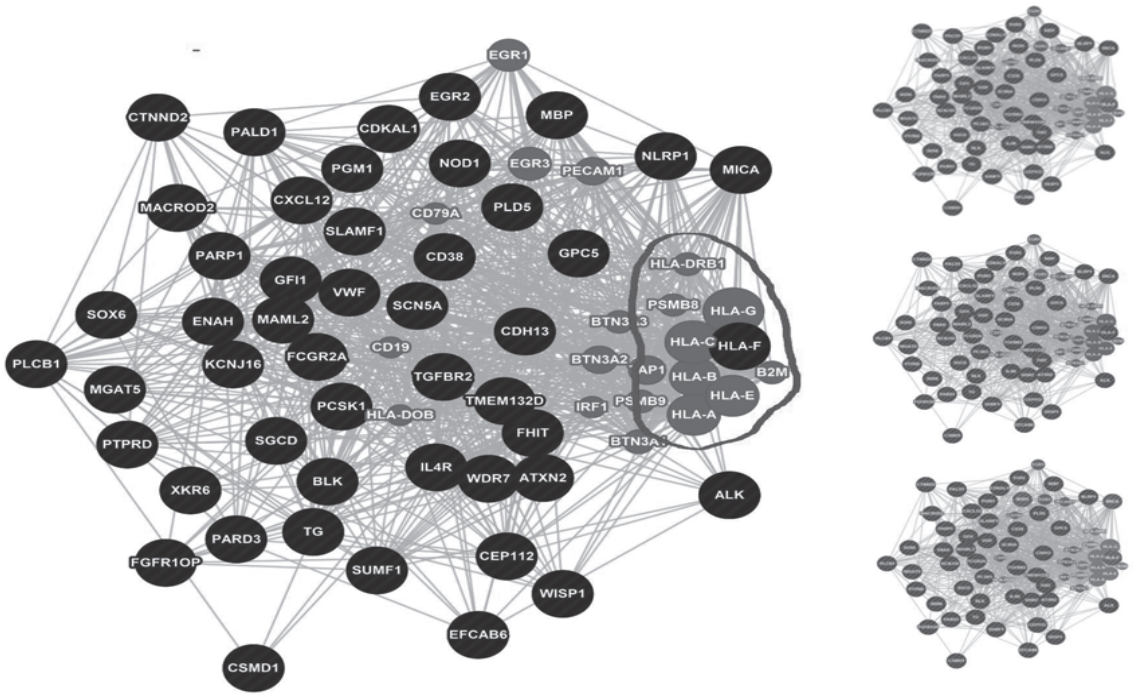

Fig. 3. Analysis of the autoimmune network: HLA cluster revealed, which is marked within the circle and functions of top three co-expression sub-networks a) Protein Antigen binding b) Antigen binding c) Antigen processing and presentation were also found to be interacting with the HLA cluster

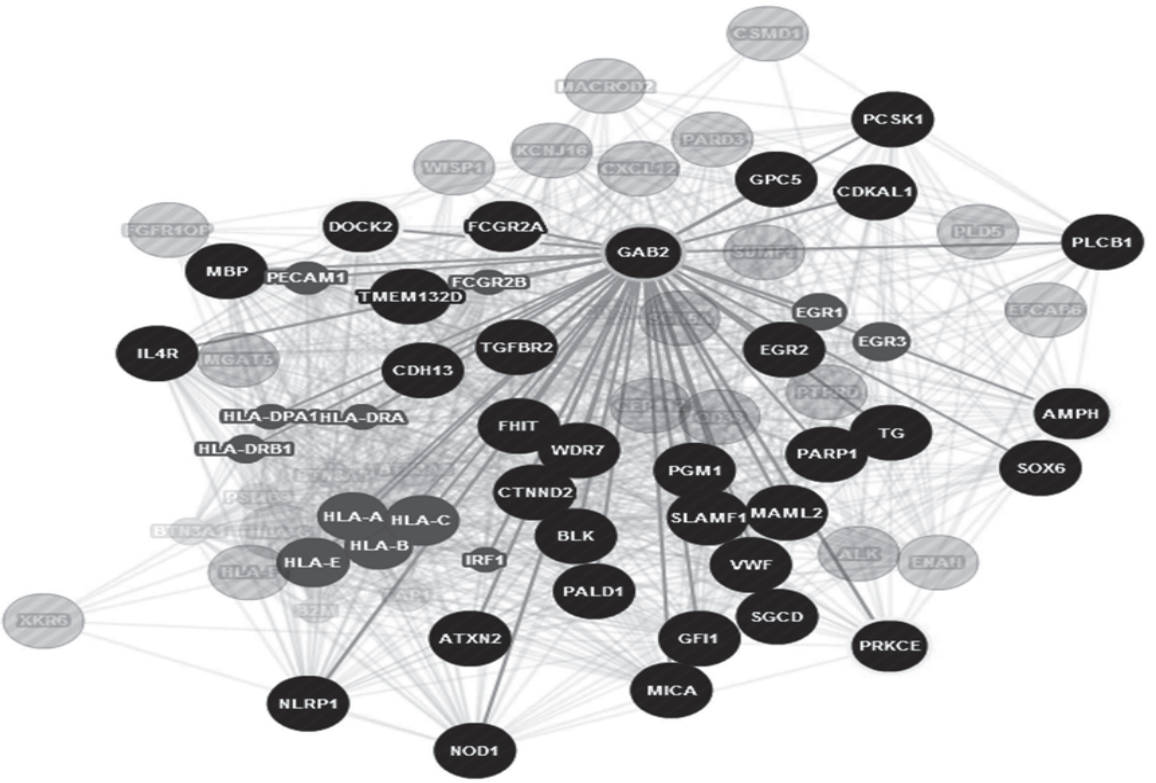

Fig. 4. Association of *GAB2* with the autoimmune disease genes: Co-expression network with FcγR-mediated phagocytosis pathway genes *FCGR2A*, *GAB2*, *AMPH*, *DOCK2*, *PRKCE* (labeled with star) and autoimmune disease genes identified to be associated with JIA is presented with a focus on *GAB2*'s connectivity.

Supplementary Table I. Diseases in Autoimmune and Autoinflammatory Groups

Autoimmune	Autoinflammatory
Addison's disease	Behcet syndrome
Anti neutrophil cytoplasmic antibody (ANCA)-associated vasculitis	Diabetes mellitus, type II
Anti-GBM disease	Inflammatory bowel disease
Antiphospholipid syndrome	Metabolic syndrome X
Atopic dermatitis	Obesity
Autoimmune hepatitis	Psoriasis
Autoimmune thyroiditis (Graves and Hashimoto)	Sarcoidosis
Celiac disease	Still's Disease, adult-onset
Diabetes mellitus, type I	
Guillain-Barre syndrome	
Heparin-induced thrombocytopenia	
Idiopathic thrombocytopenic purpura	
Lichen planus	
Multiple sclerosis	
Myasthenia gravis	
Pemphigus	
Rheumatoid arthritis	
Scleroderma	
Systemic lupus erythematosus	
Vitiligo	

Supplementary Table II.

Results

The demographics of patients were summarized in Table I. Family based association study of 26 patients with oligo or RF negative polyarticular JIA with TDT revealed 843 SNPs associated with JIA with a p-value of <0.005, and 140 of these SNPs had an odds ratio (OR) of >3.5.

After TDT analysis with PLINK, among top 1000 SNPs (p-value <0.0067), there were 444 coding SNPs and 573 non-coding SNPs, according to SNPNexus annotation. Coding SNPs were mapping to 343 unique ENTREZ genes. The genes with three or more associated SNPs are summarized in Table II.

After TDT, top 1003 associated SNPs with the p-value <0.0067 were analyzed based on the previously associated disease groups with SNPNexus analysis. All the GAD disease titles were classified under three categories; autoimmune, auto inflammatory and other diseases are explained in the methods. Under the autoimmune and autoinflammatory categories, there were 96 unique genes, where JIA associated SNPs were mapping, whereas 164 genes were related to other diseases. Distribution of the number of unique genes under each group is presented in the Figure 1 (Venn Diagram). *NOD1*, also known as *CARD4,* is found to be the only gene involved in the intersection of autoimmune and autoinflammatory diseases.

Enrichment analysis has been done in order to gain more insight to the JIA etiology at molecular and biological pathway level. Functional Annotation Clustering for all 257 JIA associated genes revealed enrichment in protein tyrosine kinase activity and neuronal development related molecular functions in the top two clusters.

When the Functional Annotation Clustering was performed for exclusively on the 48 autoimmune disease related gene which were found to be associated with JIA in the GWAS, the top two clusters were found to be involved in regulation of biological process and response to stimulus. And further investigation of GO Terms for biological processes showed enrichment in regulation of immune system process, response to stimulus such as endogenous, hormonal, bacterial and organic substances. No known biological pathway including the query genes is found with the default DAVID parameters.

As a result of Functional Annotation Analysis for top 257 Genes associated with JIA after GWAS, two known biological pathways are

found to be enriched: 1) Fcγ Receptor (FcγR) -mediated phagocytosis and 2) vascular smooth muscle contraction (Table III A). Five genes associated with JIA in this study (*FCGR2A, GAB2, AMPH F, DOCK2, PRKCE*) were reported to be included in the FcγR -mediated phagocytosis (Table III B). KEGG pathway for FcγR -mediated phagocytosis is shown in Figure 2 with genes which were found to be associated with JIA through our GWAS.

Functional analysis of the co-expression network genes revealed four functional categories in relation with the HLA cluster: antigen binding (*SLAMF1, MICA, HLA-F*), antigen processing (*NOD1, MICA, HLA-F*), immune response regulating cell surface receptor signaling pathway (*ENAH, BLK, FCGR2A, CD38),* and response to bacterium (*GFI1, NOD1, NLRP1, MICA*) (Fig. 3).

GeneMANIA network of autoimmune disease genes presented functional categories of "immune response regulating cell surface receptor signaling pathway", "response to bacteria", and " antigen binding and processing" supporting DAVID annotation results.

Unlike the autoimmune network, analysis of the co-expression network for the genes under the autoinflammatory category did not present a consensus at the functional level. Top three functions and genes engaged in these functions were 1) Extracellular matrix (*GPLD1, MFAP1, THBS4, USH2A, VMF) 2)* Secretary granule (*SRI, ABCC4, CD36, PCSK1, VMF*) 3) Platelet activation (*ABCC4, CD36, VMF, DGKB, DGK1G, PRKG*).

Association of GAB2 with the autoimmune disease genes

GAB2 was associated with JIA in our study. Furthermore, we have shown that *GAB2* was

Supplementary Table II. Regulome Scoring Scheme

Chromosome	Start	End	rsID	Regulome score	Affected gene	Bound protein
chr8	82034310	82034311	rs10957999	1f	PAG1	
chr11	68915044	68915045	rs11228521	1f	TPCN2	
chr8	606451	606452	rs1703937	1f	ERICH1	
chr6	29730922	29730923	rs1737068	1f	HLA-F, HLA-A-H, KIT, FLJ35429, NDUFS1	NFKB1
chr8	8385335	8385336	rs7000407	1f	CLDN23	
chr7	87997383	87997384	rs733004	1f	STEAP4	CTCF
chr15	65526800	65526801	rs755115	1f	PARP16	
chr9	97228917	97228918	rs9409552	1f	-	SETDB1
chr9	117370195	117370196	rs10115585	2a	-	MEF2A
chr2	64619246	64619247	rs1426707	2a	-	CDX2
chr1	201236238	201236239	rs10920161	2b	-	FOS
chr5	141922620	141922621	rs152447	2b	-	CTCF
chr5	135064488	135064489	rs4976326	2b	-	NFKB1
chr12	52480181	52480182	rs7313454	2b	-	SPDEF
chr6	166671717	166671718	rs9364840	2b	-	FOXA1-2
chr16	89270728	89270729	rs1112462	3a		
chr5	94632165	94632166	rs154058	3a		
chr15	67111714	67111715	rs17206452	3a		
chr9	117370537	117370538	rs4978600	3a		
chr3	103333603	103333604	rs9870354	3a		
chr2	211982806	211982807	rs7589906	3b		

Chr, kromozom; rsID, regulome score identification number; PAG1, phosphoprotein associated with glycosphingolipid-enriched microdomains 1; TPCN2, two-pore segment channel 2; HLA, human leukocyte antigen; KIT, proto-oncogene receptor tyrosine kinase; FLJ35429, full length long japan 35429; NDUFS1, NADH-ubiquinone oxireductase Fe-S protein 1; CLDN 23, claudin 23; STEAP4, six-transmembrane epithelial antigen of prostate 4; PARP16, poly (ADP-ribose) polymerase family member 16; NFKB1, nuclear factor kappa B 1; FOS, Finkel Biskis Jinkis murine osteosarcoma viral oncogene homolog; CTCF, CCCTC-binding factor; SETDB1, set domain protein bifurcated 1; MEF2A, mads box transcription enhancer factor 2, polypeptide A; CDX2, caudal-type homeobox transcription factor 2

interacting with *BLK, CDH13, IL4R* and *MICA* through co-expression network analysis with GeneMANIA. Association of *GAB2* with the autoimmune disease genes identified in the study is presented in Figure 4. *GAB2* also takes role in the FcγR-mediated phagocytosis pathway with four other JIA associated genes (*FCGR2A, AMPHF, DOCK2, PRKCE*) (Fig. 2). Network with FcγR-mediated phagocytosis pathway genes and autoimmune disease genes with focus on *GAB2* is shown in Figure 4.

Analysis of Non-Coding SNPs

When 573 non-coding SNPs within the top 1000 according to their p-values have been analyzed by regulomeDB, 7 non-coding SNPs with a score of 1f (eQTL + TF binding / DNase peak) were revealed. Also there were 13 other non-coding SNPs with no eQTL but those have transcription factor binding sides with a motif (score between 2a and 3b). The non-coding SNPs on an eQTL with a score of 1 and their affected genes are listed in the Table IV.

Discussion

This work represents, to our knowledge, the first case-parent trio study in JIA. The patient population was the Caucasian eastern Mediterranean population. Since this is a family study, it is not biased for genetic background. Infante-Rivard et al[13] has compared the yield of case trio studies to the classical case-control studies. Although they both define predisposition to the disease in complex genetic trait diseases; the case-parent trio design has the major advantage of being robust to population structure bias because estimation of allele transmission within families, conditioning on parental genotypes, cannot be biased due to a different genetic background[13].

As expected, *HLA* association was significant within the autoimmune disease related SNPs in this analysis. Along with HLA type I and F, other genes interacting with the HLA cluster in the co-expression network that are identified as JIA associated genes have been revealed (*BLK, CD38, ENAH, FCGR2A, GFI1, MICA, NLRP1, NOD1,* and *SLAMF1*). Twenty one genes which were previously shown to be associated with both autoimmune and autoinflammatory diseases were identified through JIA associated SNPs mapping onto them (*BLK, CD38, CDH13, CDKAL1, CXCL12, FCGR2A, FHIT, GPC5, IL4R, MICA ,NLRP1, PARD3, PARP1, PCSK1, PTPRD, SCN5A, SOX6, TGFBR2, TMEM132D, VWF,* and *NOD1*). Three of these genes namely *BLK, IL4R, and MICA* have already been shown to be associated with JIA[23-25] while six genes (*CDH13, CD38, CXCL12, FCGR2A, NLRP1, PARP1*, and *TGFBR2*) have been previously shown to take role in rheumatoid arthritis[26-32].

BLK gene, encodes a member of Src kinase family involved in signal transduction downstream of the B cell receptor. Thus, it may influence the proliferation and differentiation of B cells[33]. It was found to be associated with JIA in a previous study investigating the RA susceptibility loci in JIA[23]. Our study also supported the role for *BLK* in JIA pathogenesis.

MICA is a gene from the family of MHC Class I chain related genes (MIC). *MICA* is in high linkage disequilibrium with MHC Class I genes and it was suggested as a candidate gene for HLA Class I associated diseases [34]. Expression of *MICA* on the cell surface leads to recognition by γζ T cells and natural killer (NK) cells, which may lead to cell lysis[34]. In a study on Latvian patients with JIA, it was indicated that *MICA* genetically contributed to the disease pathogenesis[24].

Interleukin 4 (IL-4) is a cytokine that regulates immunoglobulin class switching to immunoglobulin (Ig) E and the development of T helper 2 (Th2) cells[25]. The disease-limiting role of IL-4 has been documented both for RA and JIA previously[35-37]. It has also been shown that changes in IL-4 signaling due to functional polymorphisms in the *IL-4* and *IL-4R* genes may be associated as risk factors with RA and JIA[25].

CDH13 encodes a unique cadherin which is likely involved in signal transduction and not cell-cell adhesion as it lacks transmembrane and cytosolic domains of a classical cadherin[38]. It affects cellular migration and angiogenesis[39,40]. In a previous study by Briggs et al[26], SNP variants within *CDH13* showed significant evidence for interaction with *PTPN22*. *PTPN22* has been unequivocally confirmed as RA and JIA susceptibility gene in separate studies from multiple populations, as well as in GWAS[8,41]. It encodes lymphoid tyrosine phosphatase which negatively regulates the T cell receptor signaling, and this makes *PTPN22* a strong candidate gene confirming risk for multiple autoimmune diseases[42,43].

GAB2 was among the highly significant genes in our study with three different associated SNPs mapping onto it and due to its key

position in the co-expression network and the FcγR-mediated phagocytosis pathway. Batliwalla et al.[44] have shown the significance of *GAB2* expression among the RA patients they studied: The authors used gene expression profiling of the PBMCs in order to define transcriptional patterns that differentiate RA patients. *GAB2* is of interest since it is involved in signaling for cytokines and antigen receptors and may have a role in regulating activities of phosphatases, such as PTPN22[44]. *PTPN22* gene was previously shown to be associated with both RA[45, 46] and JIA[47]. *GAB2* was also on the FcγR-mediated phagocytosis pathway, which was highlighted in our analysis. Four more genes (*FCGR2A, AMPHF, DOCK2, PRKCE*) in this pathway were also associated with JIA. FcγRs on macrophages bind to the Fc portion of IgG molecules and start the phagocytosis of IgG-coated particles[48]. Cross-linking of FcγRs also triggers the secretion of reactive oxygen species and inflammatory cytokines[48]. It is tempting to speculate that the significance of this pathway implicates phagocytosis of the innate immune system in the pathogenesis of JIA.

In this study, the small sample size limited the power to detect some causal variants. Nonetheless, with the advantage of case-parent trio design, we have confirmed the association of certain SNPs that are potential susceptibility markers for JIA.

Rheumatoid arthritis is a clearly autoimmune disease whereas the role of autoimmunity is much less evident especially in some groups of JIA. When we analyze the network of the top SNPs, the leading pathway is that of FcγR-mediated phagocytosis although autoimmune networks were also evident. In fact, the strongest implicated pathways were responses to stimuli, including phagocytosis in JIA. It is tempting to speculate that exaggerated immune response is a main driving force in JIA pathogenesis.

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