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Original Paper

Whole Transcriptome Analysis (RNA Sequencing) of Peripheral Blood Mononuclear Cells of Vitiligo Patients

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Key Words

Vitiligo · Gene expression · Transcriptome · RNA sequencing

Abstract

Vitiligo is an idiopathic disorder characterized by depigmented patches on the skin due to a loss of melanocytes. The cause of melanocyte destruction is not fully understood. The aim of this study was to detect the potential pathways involved in the vitiligo pathogenesis to further understand the causes and entity of vitiligo. For that the transcriptome of peripheral blood mononuclear cells of 4 vitiligo patients and 4 control subjects was analyzed using the SOLiD System platform and whole transcriptome RNA sequencing application. Altogether 2,470 genes were expressed differently and *GRID2IP* showed the highest deviation in patients compared to controls. Using functional analysis, altogether 993 associations between the gene groups and diseases were found. The analysis revealed associations between vitiligo and diseases such as lichen planus, limb-girdle muscular dystrophy type 2B, and facioscapulohumeral muscular dystrophy. Additionally, the gene groups with an altered expression pattern are participating in processes such as cell death, survival and signaling, inflammation, and oxidative stress. In conclusion, vitiligo is rather a systemic than a local skin disease; the findings from an enormous amount of RNA sequencing data support the previous findings about vitiligo and should be further analyzed.

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Introduction

Vitiligo is characterized by progressive disappearance of skin pigment cells, with straightforward clinical translation – white macules or white hair appear, usually without any accompanying clinical symptoms. Vitiligo occurs worldwide with an estimated prevalence of 0.5–2% in most populations. In almost half of the patients, vitiligo starts before the age of 20 years, and males and females are affected with approximately equal frequency [1, 2]. Vitiligo has been associated with the concomitant occurrence of a number of other autoimmune diseases, as well as a wide range of psychosocial difficulties significantly impacting the quality of life [3–5].

In vitiligo patients, skin melanocytes are partially or completely lost, and no melanin is synthesized in this area. The cause of the destruction of epidermal or follicular melanocytes is complex and not yet fully understood; however, there are several theories (autoimmune, neural, and biochemical hypotheses) [6, 7]. Melanocyte death may occur due to factors from inside and/or outside the cell, and many potential systems could be involved. Histological data have demonstrated that on the perilesional skin of patients with nonsegmental vitiligo an inflammatory infiltrate of low intensity made of mononuclear cells occurs in the upper dermis and the dermal-epidermal interface [8]. The initiation mechanism of this microinflammatory reaction is still unknown, but it has been suggested that various local triggers alert the skin's innate immune system and may precede adaptive immune responses targeting melanocytes [9, 10].

The genome-wide association studies account for a limited part of the heritability of the disease but may mediate a crucial part of the disease phenotype, such as progression or age at onset [11]. Most vitiligo susceptibility loci encode immunoregulatory proteins or melanocyte components that are likely to mediate immune targeting and the relationships among vitiligo, melanoma, and eye, skin, and hair coloration [12]. For example, associations have been established between vitiligo pathogenesis and polymorphisms in tyrosinase gene (*TYR*), catalase gene (*CAT*), melanocortin 1 receptor (*MC1R*), major histocompatibility complex genes (*MHC*), protein tyrosine phosphatase nonreceptor type 22 gene (*PTPN22*), NLR family pyrin domain containing 1 gene (*NLRP1*), and X-box binding protein 1 gene (*XBPI*). Both protective and susceptibility-increasing effects have been found in the case of different polymorphisms in these genes [13, 14].

There is a wide range of evidence that vitiligo is not a local but rather a systemic disease. Abnormalities in both humoral and cell-mediated immunity have been documented in vitiligo patients [7, 15]. Besides, increased local and systemic cytokine expression is observed [16–21]. Furthermore, in vitiligo skin, the elevated H₂O₂ level is the main cause of tetrahydrobiopterin (6BH₄) recycling errors; the latter is essential for melanogenesis [22]. The activity of enzymes important to metabolize H₂O₂ has impaired on the patients' skin as well as systemically – the latter has been proven in the case of CAT and glutathione peroxidase (GPX1) [23–25].

The purpose of this study was to describe potential transcriptional changes in the peripheral blood mononuclear cells (PBMCs) of vitiligo patients. We aimed to find the changes in the patients' blood, which may help to detect the potential pathways participating in the vitiligo pathogenesis and to further understand the causes and entity of vitiligo.

Table 1. Characteristics of study participants

Subjects	Age, years	Sex	Onset age, years (duration)	Vitiligo stage	Koebner's phenomenon	Hair involvement	Spontaneous repigmentation	Presence of halo nevus	TPO AB positivity
Control 1	27	male	–	–	–	–	–	–	–
Control 2	29	female	–	–	–	–	–	–	–
Control 3	40	male	–	–	–	–	–	–	–
Control 4	27	female	–	–	–	–	–	–	–
Patient 1 (V105)	24	male	17 (7)	active	no	yes	yes	no	yes
Patient 2 (V142)	43	male	21 (22)	active	yes	no	no	no	no
Patient 3 (V215)	27	female	5 (22)	active	yes	yes	no	yes	yes
Patient 4 (V217)	36	female	35 (1)	active	yes	no	no	no	no

Materials and Methods

Patients and Controls

The protocols and informed consent forms used in this study were approved by the Ethics Review Committee on Human Research of the University of Tartu, Estonia. All the participants signed a written informed consent and were Caucasians living in Estonia. Unrelated patients with vitiligo from the Department of Dermatology, University of Tartu were included in the study. The diagnosis of vitiligo was based on loss of pigmentation with typical localization and white color on the skin lesions under a Wood's lamp. All of the patients included in this study had vitiligo vulgaris and none of them had received specific therapy in the previous 6 months. The controls were volunteers without a positive family history of vitiligo, and they were recruited among medical students and health care personnel. The main characteristics of the participants are presented in table 1.

Sample Collection and RNA Extraction

Blood was collected from 4 volunteers and 4 vitiligo patients between 8:00 and 12:00 in the morning to limit the effect of circadian variation of cytokine production. BD Vacutainer CPT tubes (BD, New York, N.Y., USA) were used to separate PBMCs from other blood cells. The cells were centrifuged at 1,500 *g* for 30 min at 20°C. After that, blood sera were collected from the top of the PBMCs. Phosphate-buffered saline was used to wash the isolated PBMCs twice, after which they were centrifuged at 190 *g* for 10 min at 20°C. The supernatant was collected and the cells were stored at –80°C until RNA extraction.

Total RNA was extracted applying the mirVana miRNA Isolation Kit (Ambion, Life Technologies Corp., Carlsbad, Calif., USA) according to the manufacturer's protocol. RNA quality was assessed using Agilent 2100 Bioanalyzer and the RNA 6000 Nano Kit (Agilent Technologies Inc., Calif., USA). The RNA integrity number was over 9 for all samples.

Whole Transcriptome RNA Sequencing Library Preparation

For whole transcriptome sequencing, 10 µg of total RNA was taken and treated with the RiboMinus Eukaryote Kit for RNA sequencing (Invitrogen Corp., Carlsbad, Calif., USA) to eliminate ribosomal RNA from the rest of the transcriptome. Five hundred nanograms of ribodepleted RNA and the SOLiD Total RNA-Seq Kit were used (according to the manufacturer's protocol; Life Technologies Corp.) for whole transcriptome RNA sequencing library preparation. The libraries were marked with different barcodes and pooled together for the following template preparation.

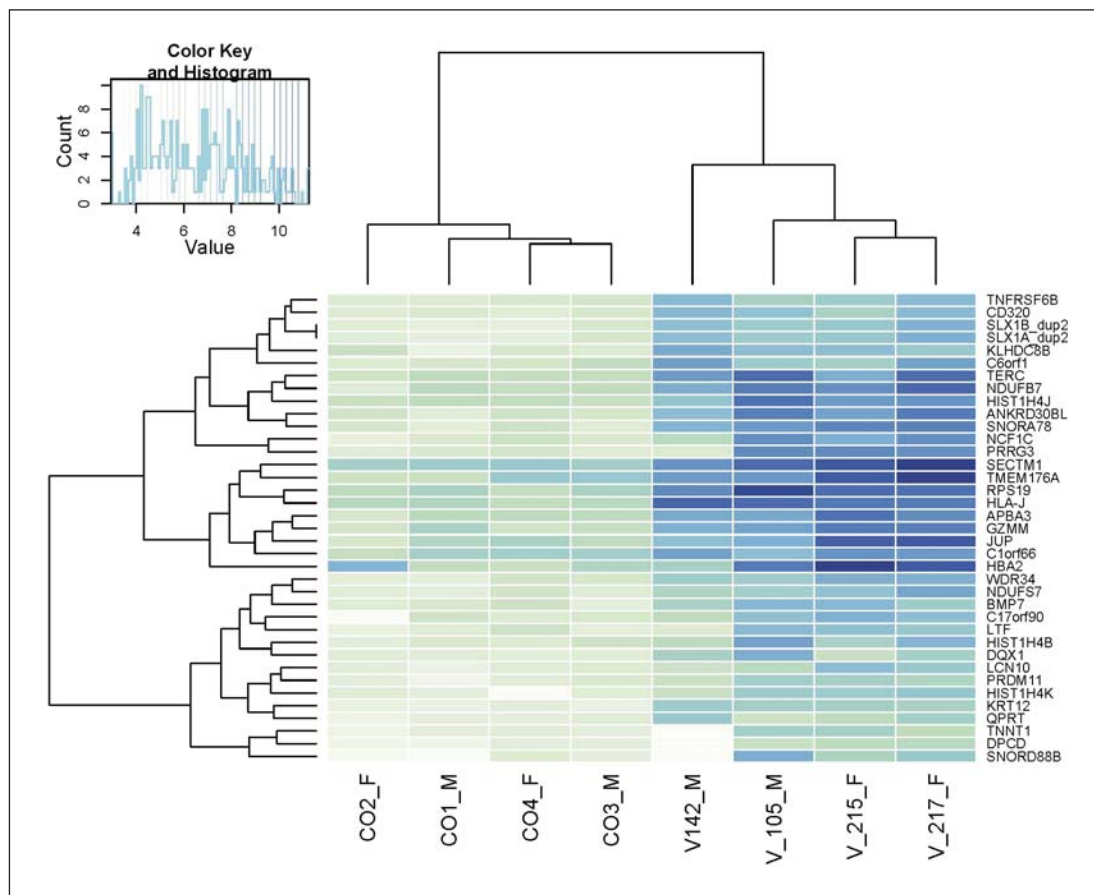


Fig. 1. Heat map. Vitiligo patients have a distinct transcriptome profile in the blood. We clustered the 30 most significantly changed genes according to the expression values. For clustering, we transformed the count matrix to get sample-to-sample distances. The clustering correctly reflects the experimental design and the differential expression pattern between the study groups.

The automated SOLiD EZ Bead System and SOLiD EZ Bead E80 System Consumables (Life Technologies Corp.) were applied for the template preparation. For sequencing the controls' samples, the SOLiD 4 System and paired-end (50 bp forward and 35 bp reverse) chemistry for RNA sequencing were used (Life Technologies Corp.). For sequencing the patients' samples, the SOLiD 5500xl System and paired-end (75 bp forward and 35 bp reverse) chemistry for RNA sequencing were applied (Life Technologies Corp.).

Statistical and Functional Analysis

Raw reads were mapped using Lifescope 2.5.1 software (Life Technologies Corp.) and the whole transcriptome analysis workflow. This workflow generates a very complex output, from gene and exon counts to the alternative splicing and fusion transcripts. For further analysis, we only focused on the gene counts because our primary question was related to the abundance of gene-targeted transcripts. For differential expression analysis, we used the R Bioconductor package edgeR [26], which implemented exact statistical methods and generalized linear models for multigroup and multifactorial experiments. A particular feature of edgeR functionality is the empirical Bayes method that permits the estimation of gene-specific biological variation, even for experiments with minimal levels of biological replication. edgeR

Table 2. First 20 genes with the most altered RNA expression levels in vitiligo patients versus controls

GeneID	Gene name	Fold change	FDR	Main function
GRID2IP	glutamate receptor, ionotropic, delta 2 (Grid2) interacting protein	7.2	1.31E-20	synaptogenesis and synaptic plasticity
MYO6	myosin VI	6.7	9.28E-20	intracellular vesicle and organelle transport
LRRC48	leucine rich repeat containing 48	-7.5	1.32E-18	cytoplasmatic protein
C3orf32	ssu-2 homolog (<i>C. elegans</i>)	4.6	5.62E-16	cytoplasmatic protein
HMMR (HRAMM)	hyaluronan-mediated motility receptor (RHAMM)	6.3	4.36E-15	involved in cell motility
SNORD78	small nucleolar RNA, C/D box 78	-5.7	4.36E-15	small nucleolar RNA
SPON1	spondin 1, extracellular matrix protein	-5.9	4.56E-15	cell adhesion
MIR103-A1	microRNA 103a-1	-5.4	6.46E-15	microRNA
NAA11	N(alpha)-acetyltransferase 11, NatA catalytic subunit	-6.4	1.01E-14	peptide alpha-N-acetyltransferase
LOC285547	uncharacterized LOC285547	-5.5	9.87E-14	miscellaneous small RNA
LOC283761	uncharacterized LOC283761	-5.8	2.65E-13	miscellaneous small RNA
SNORD23	small nucleolar RNA, C/D box 23	5.4	3.41E-13	small nucleolar RNA
ARSJ	arylsulfatase family, member J	-5.6	4.05E-13	sulfatase, hormone biosynthesis
FOLR3	folate receptor 3 (gamma)	5.4	3.77E-12	folic acid transport
SNORD81	small nucleolar RNA, C/D box 81	-4.7	8.35E-12	small nucleolar RNA
SNORA78	small nucleolar RNA, H/ACA box 78	4.8	2.30E-11	small nucleolar RNA
SPEM1	spermatid maturation 1	-4.6	2.30E-11	spermatogenesis, cell differentiation
PRRG3	proline rich Gla (G-carboxyglutamic acid) 3 (transmembrane)	5.1	2.76E-11	K-dependent transmembrane proteins
MIR181C	microRNA 181c	-6.7	3.99E-11	microRNA
SLC2A14	solute carrier family 2 (facilitated glucose transporter), member 14	-6.7	7.69E-11	glucose transporter

FDR = False discovery rate.

can be applied to examine differential expression at the gene, exon or tag level. In our study, we used model-based normalization and applied the negative binomial model. Testing for differential expression was done using the exact test.

A heat map was generated with the DESeq package in R. For the functional network analysis, the Ingenuity Pathway Analysis (IPA, www.ingenuity.com) software was used.

Results

To observe possible changes in the RNA expression pattern in vitiligo patients' blood cells (PBMCs) compared to the controls' cells, the whole transcriptome was sequenced.

RNA Expression Pattern in PBMCs

We found altogether 2,470 genes to be expressed differently ($p < 0.05$) in vitiligo patients compared to controls, after applying the false discovery rate correction (fig. 1). The gene with the most altered gene expression is glutamate receptor, ionotropic, delta 2 interacting protein (*GRID2IP*), and its expression has increased 7.2 times ($p = 1.31 \times 10^{-20}$) in vitiligo patients' PBMCs compared to controls' cells. *GRID2IP* is associated with synaptogenesis and synaptic plasticity; furthermore, the genes with an altered expression belong to different functional groups and participate in various processes. In table 2, the first 20 genes with the most altered RNA expression levels in patients versus controls are presented.

Table 3. First 20 diseases which are associated with genes with an altered expression pattern in vitiligo patients' PBMCs

Functions annotation	Category	p value	Molecules
Lichen planus	Dermatological diseases and conditions Immunological disease Inflammatory disease	2,24E-09	24
Limb-girdle muscular dystrophy type 2B	Hereditary disorder Skeletal and muscular disorders	3,26E-08	14
Facioscapulohumeral muscular dystrophy	Hereditary disorder Skeletal and muscular disorders Developmental disorder	5,61E-08	12
Idiopathic pulmonary fibrosis	Inflammatory disease Connective tissue disorders Inflammatory response Organismal injury and abnormalities Respiratory disease	5,67E-08	21
Miyoshi myopathy	Hereditary disorder Skeletal and muscular disorders	6,61E-08	13
Limb-girdle muscular dystrophy type 2A	Hereditary disorder Skeletal and muscular disorders	1,39E-07	13
Cell death	Cell death and survival	4,07E-07	287
Congenital myopathy	Hereditary disorder Skeletal and muscular disorders Developmental disorder	6,12E-07	18
Nemaline myopathy	Hereditary disorder Skeletal and muscular disorders Developmental disorder	9,53E-07	13
Viral infection	Infectious disease	1,66E-06	169
Limb-girdle muscular dystrophy	Hereditary disorder Skeletal and muscular disorders	1,89E-06	15
Apoptosis	Cell death and survival	2,27E-06	234
Necrosis	Cell death and survival	2,99E-06	220
Function of blood cells	Cellular function and maintenance	3,96E-06	68
Pneumonia	Inflammatory response Respiratory disease	4,72E-06	23
Proliferation of cells	Cellular growth and proliferation	5,99E-06	301
Function of leukocytes	Cellular function and maintenance	6,03E-06	64
Cancer	Cancer	1,84E-05	330
Production of reactive oxygen species	Free radical scavenging	3,65E-05	38
Breast cancer	Cancer	3,92E-05	103

Functional Analysis

Additionally, taken from RNA expression data, we demonstrated associations between the genes with an altered expression pattern and diseases (table 3). Using IPA software, altogether 993 associations between the gene groups and diseases were found. Among the first 20 diseases, the terms 'dermatological', 'immunological' and 'inflammation' are found, which are all related to vitiligo [27, 28].

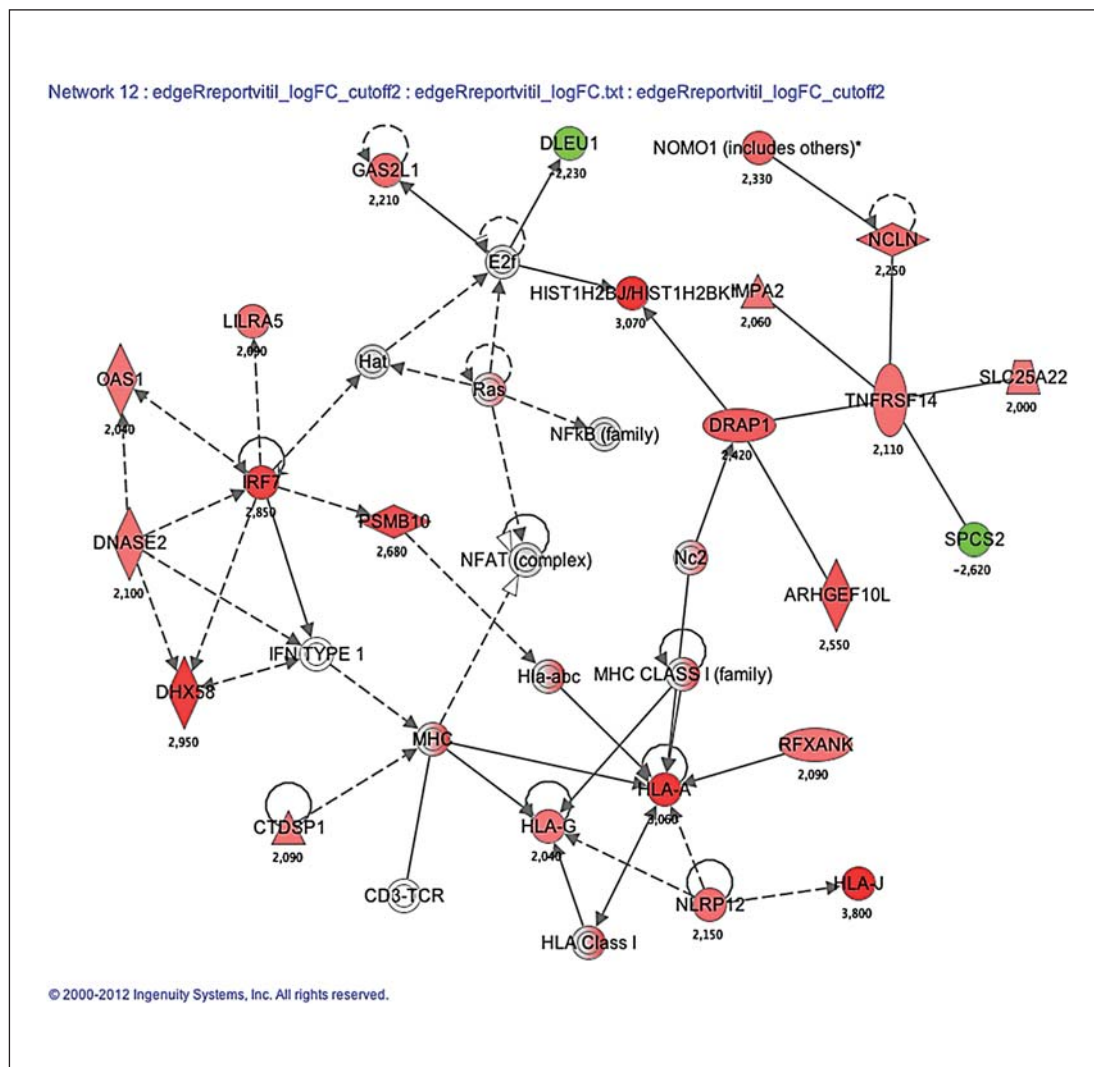


Fig. 2. The illustration of genes in a pathway associated with dermatological diseases and conditions, immunological disease and inflammatory disease (table 4) (filter set to $p < 0.05$ after false discovery rate correction) in vitiligo patients compared to controls. Gene expression profiles were uploaded to the Ingenuity Pathway Analysis software and based on the differential expression of these genes, the most relevant biochemical network with functional links is found. Green: decreased expression level in patients vs. controls; red: increased expression level; gray: similar expression level; white: no associations between vitiligo and the gene were found. The numbers reflect the t value of the statistical comparison with the Bayes moderated t test. The figure was generated through the use of the Ingenuity Pathway Analysis (IPA, Ingenuity Systems, <http://www.ingenuity.com>).

We also performed an analysis showing the top functions of the genes with an altered expression pattern in vitiligo patients' PBMCs (table 4). The genes are participating in processes such as cell death, survival and signaling, inflammation, and oxidative stress among others, which are all associated with vitiligo pathogenesis [27–29]. Furthermore, with this analysis, a group of 23 genes associated with dermatological diseases and conditions, immunological disease and inflammatory disease was found (fig. 2).

Table 4. The top functions of genes with an altered expression pattern in vitiligo patients' PBMCs

Molecules in network	Score	Focus molecules	Top functions
ADRM1, AKT1S1, Ant, CORO1A, CYC1, DOK3, EDA, INO80E, KSR2, MAGEC1, MIB2, MICAL1, NCF1C, NFkB (complex), NFKBID, NMRAL1, PAM16 (includes EG:100042179), PIDD, PKN3, RAB1B, RUSC2, RUVBL2, SLC2A6, SPATA20, ST18, ST6GALNAC4, TEO2, TIMM13, TIMM17B, TNFRSF10C, TSPO, UQCRC1, VDAC2, WDR34, ZNHIT2	50	33	Cell cycle, connective tissue disorders, dental disease
ABTB1, APBA3, ATP13A2, C1QA, C1QB, CA1, CCS, CD320, CFD, CLEC10A, creatine kinase, CTSD, DBF4 (includes EG:10926), DDAH2, DYSF, EGLN2, Ferritin, FTL, HBA1/HBA2, HBB, hemoglobin, LRWD1, MCM2, MYL4, MZF1, NFE2, PAPP, PRDX2, SCAND1, SERPINF2, SIGLEC1, TBKBP1, TMSB10/TMSB4X, TNFRSF6B, Vegf	43	31	Protein synthesis, hematological disease, hereditary disorder
AES, AGTRAP, ASGR1, BCAT2, CSK, Eif4g, ERK, ETS, FAU, FERMT3, GP9, GP1BB, HK3, IL23R, ITGA2B, LIME1, Mac1, MATK, MBD3 (includes EG:17192), MVP, Ribosomal 40s subunit, RNF181, Rnr, RPS9, RPS10, RPS11, RPS16, RPS19, RPS26, RPS15A, SRCIN1, TRAF, USF2, ZBTB17, ZGPAT	38	28	Developmental disorder, hematological disease, hereditary disorder
ADAM15, ARHGAP4, c-Src, CAMK1, CLCF1, CYBA, Cyba-Ncf1c-Ncf2-Nox-Ncf4, EFN2, ERK1/2, G6PC3, HMMR, IgG2b, IgG2c, KLRC1, MTUS1, NCF1, NCF4, NOXA1, Phox, RAB4B, Rap1, Rap1-gp91-p22 phox-p40 phox-p47 phox-p67 phox, RASGRP4, RGS14, RIN1, RRAS, SCRIB, SHARPIN, SIPA1, SORBS2, SYTL1, TMEM176B, TXN2, TYMP, VEGFB	35	27	Hereditary disorder, inflammatory disease, free radical scavenging
Akt, C12orf44, CD19, CD22, CD79A, CD79B, CDON, COL6A2, COLEC12, Collagen Type VI, FCGRT, HCST, IgD, IgG1, Igg3, IgG2a, INPP5E, KLKB1, LGALS3BP, MARCO, mir-26, mir-29, mir-221, MIR17HG, PFKL, Ppp2c, PPP2R5B, PVRL2, PVRL3, SCAVENGER RECEPTOR CLASS A, SPHK2, Sphk, TCL1A, TNFRSF13C, TYROBP	32	26	Cell death and survival, cellular development, hematological system development and function
ABCD1, adenosine-tetraphosphatase, APEX2, ATP5D, ATP5G1, ATP5I, ATP6V0D1, ATPase, BLVRB, CARNS1, CD40, DQX1, EVI5L, FBL, FCER2, FCRLA, H+-transporting two-sector ATPase, Iga, IgG, Igm, IL12 (complex), Immunoglobulin, JINK1/2, LILRB3, LST1, MHC Class II (complex), NAT14, NINL, NUDT18, RABAC1, SMOX, SYP, TCIRG1, TRAF2, UBXN11	31	25	Energy production, nucleic acid metabolism, small molecule biochemistry
Actin, ADAM8, Alpha Actinin, Alpha catenin, Cadherin, Calcineurin protein(s), Calmodulin, CAMK2B, CBWD2, CDC42EP2, CORO6, CORO2B, CPNE5, DCTN6, DVL1, F Actin, FKBP8, Gamma tubulin, KCNN4, KLF5, LYPLA2, MARK4, MYL9, Myosin, ORAI1, OSCAR, PARVB, PDLIM7, PITPNM1, PLEC, PPP1R12C, RHOC, Rock, S100A9, TMOD1	31	25	Cell morphology, cellular assembly and organization, cellular function and maintenance
Acox, Alp, AMPK, CAPN10, CCDC99, CDC34, Cdk, CORO7/CORO7-PAM16, Cyclin A, Cyclin D, Cyclin E, EIF4E, FADS2, FGF9, FRMD6, GADD45GIP1, Insulin, mir-30, mir-130, mir-181, mir-368, MTORC1, PNKP, POLD1, POP7 (includes EG:100004610), RASSF8, Rb, RPPH1, SLC30A10, SLC6A8, STARD10, STK11, TFEB, TOB1, TUBE1	31	25	Endocrine system disorders, reproductive system disease connective tissue disorders
AATK, Adaptor protein 1, alcohol group acceptor phosphotransferase, Casein, Caspase 3/7, CDC37, CDK5, DIXDC1, ECSIT, GCDH, GRK6, GZMH, Ikk (family), IL-1R, IL1R2, IRAK1, IRAK, IRF, IRS, Jnk, LLGL2, LONP1, MAP2K2, MAP3K10, MAP3K11, MAP3K, mir-199, NEFH, NEFL, PAWR, PKN1, PPP5C, SIGIRR, SPSB2, TRAF7	28	24	Cell-to-cell signaling and interaction, cellular assembly and organization, cellular function and maintenance
ABCC9, ACD, ARL4A, BATF, Cbp/p300, CDA, COBRA1, EDF1, FKBP14, GAPDH, HDAC10, HIST1H3A (includes others), HIST1H4A (includes others), HISTONE, Histone h4, Holo RNA polymerase II, IFI35, IFI27L2, IFITM2, IFN ALPHA RECEPTOR, IFN alpha/beta, Ifnar, Importin alpha, Interferon-alpha induced, MED16, mediator, MX1, POLR2E, PPARG, PRMT1, RNA polymerase II, SSBP4, TERC, THAP7, ZBTB7B	28	24	Cellular assembly and organization, cardiovascular system development and function, cellular function and maintenance
60S ribosomal subunit, ADAMTS1, Ap1, C1q, Cg, CK1, Creb, DSCC1, FGF2, Fibrinogen, GAS1 (includes EG:14451), glutathione peroxidase, glutathione transferase, GPX1 (includes EG:14775), GST, GSTM3, GSTP1, GSTZ1, KLHL4, KPTN, Laminin, Mek, mir-23, NKG7, PTX3, RPL3, RPL8, RPL18, RPL23, RPL24, RPL18A, RPL37A, RPL7A, SPI1 (includes EG:20375), TNFRSF8	28	23	Drug metabolism, protein synthesis, cardiovascular system development and function
ARHGEF10L, CD3-TCR, CTDSP1, DHX58, DLEU1, DNASE2, DRAP1, E2f, GAS2L1, Hat, HIST1H2BJ/HIST1H2BK, HLA Class I, HLA-A, Hla-abc, HLA-G, HLA-J, IFN TYPE 1, IMPA2, IRF7, LILRA5, MHC, MHC CLASS I (family), Nc2, NCLN, NFAT (complex), NFkB (family), NLRP12, NOMO1 (includes others), OAS1, PSMB10, Ras, RFXANK, SLC25A22, SPCS2, TNFRSF14	27	23	Dermatological diseases and conditions, immunological disease, inflammatory disease

The first 12 functional groups are presented, altogether 21 groups were found.

Discussion

The theory that vitiligo is a systemic disease affecting the whole organism rather than a local disorder of certain skin areas is best supported by the autoimmune nature of vitiligo. Additionally, other autoimmune diseases may appear in vitiligo patients, such as autoimmune thyroid disease, psoriasis, rheumatoid arthritis, pernicious anemia, and alopecia areata [30–33].

In the present study, whole transcriptome RNA sequencing was performed using 4 vitiligo patients' and 4 control individuals' PBMC samples to observe the possible systemic changes at the transcriptional level. The primary results demonstrated that among 2,470 differentially expressed genes ($p < 0.05$), the first 20 genes, whose mRNA expression has statistically altered most significantly, have distinctive functions. When looking more thoroughly at some of these genes, *GRID2IP* is the one with the most altered expression level if comparing vitiligo patients and controls, i.e. the level is higher in patients. *GRID2IP* is predominantly expressed at parallel fiber-Purkinje cell postsynapses, where it may control GRIP2 signaling in Purkinje cells [34, 35]. Vitiligo has been demonstrated to be present in addition to several neuropathologies (spastic paraplegia, spastic paraparesis, and peripheral neuropathy), which are associated for example with myelin sheet defects, neuron damages, and scarring [36, 37]. Thus, we see additional signs that the mechanisms participating in these diseases may be associated.

The expression of *MYO6* (myosin VI) has significantly increased in vitiligo patients' blood cells. It has been demonstrated to be associated with lysosome-related organelles such as melanosomes – it regulates the production of melanin and the size of maturing pigmented melanosomes. Depletion of *MYO6* accumulates TYRP1 and increases the melanin content in enlarged premature melanosomes [38]. Thus, overexpression of *MYO6* in patients may have a controversial effect and inhibit melanogenesis; however, this also needs to be observed on the skin.

The glycosaminoglycan hyaluronic acid is a ubiquitous component of the extracellular matrix and the level of hyaluronic acid synthesis is increased in highly metastatic melanoma cell lines [39]. The capacity of hyaluronic acid to induce cell growth, differentiation and motility has been shown in a number of experimental systems [40, 41]. Hyaluronan-mediated motility receptor is a cell surface receptor, and its expression has increased during melanoma progression [42]. We also found it to be overexpressed in vitiligo patients' blood cells, which correlates with an increased level of cytotoxic T cells in patients [43, 44]. However, associations between vitiligo and hyaluronan-mediated motility receptor have not been demonstrated before, and it needs to be further analyzed to understand the possible effect.

Additionally, there are several small nucleolar, miscellaneous and microRNA genes, whose expression has systemically changed in vitiligo patients. Previously, using microRNA expression profiling possible serum biomarkers for vitiligo has been found [45]. microRNAs possibly derived from melanocytes, immune cells and other related cells were observed in nonsegmental vitiligo patients and controls. As Shi et al. [45] presented in their 2013 study, there were microRNAs with a higher and lower expression level in blood sera, and *miR-16*, *miR-19b* and *miR-720* were suggested as potential biomarkers for vitiligo. Also, associations between intergenic SNP sequences, where small transregulatory RNAs bind, and vitiligo pathogenesis have been demonstrated [46]. The present study provides an enormous amount of data on the expression of small RNA genes in patients, which should be further analyzed.

The present study showed that genes with an altered mRNA expression level in vitiligo patients are also associated with other diseases. The functional analysis revealed the gene groups to be linked to dermatological conditions, immunological and inflammatory diseases as well as cell survival, which all fit the vitiligo profile [27, 28]. However, the genes which gave

the highest statistical significance in the analysis are also associated with lichen planus. The latter is a mucocutaneous disease, which affects the skin, but also oral mucosa. The etiology is unknown; however, similarly to vitiligo, it is possibly associated with oxidative stress and autoimmunity [47, 48] as well as inflammatory processes [49]. Furthermore, these two diseases have demonstrated to be present at the same time together with other autoimmune diseases [50–52]. Thus, lichen planus and vitiligo both seem to be rather systemic than local diseases, and they may have similar onset and progress causes.

Additionally, the genes expressed differently in vitiligo patients are also associated with limb-girdle muscular dystrophy type 2B, which is an autosomal recessive disease caused by the diminution or absence of dysferlin. The patients may have perivascular and endomysial inflammatory infiltrate on muscle biopsy [53]. In addition, limb-girdle muscular dystrophy type 2B patients may have other autoimmune diseases [54]. The facioscapulohumeral muscular dystrophy is another example; it is an autosomal dominant disease caused by integral deletion of D4Z4 repeats on the 4q35, and it has also been associated with autoimmune diseases and inflammatory processes [55–57]. There are no direct associations demonstrated between these two muscular dystrophies and vitiligo, and in contrast to vitiligo, these dystrophies are inherited diseases. Still, the processes which are activated during the pathogeneses are similar to vitiligo and possibly use the same pathways to some extent.

The functional analysis revealed that the genes with an altered mRNA expression pattern in vitiligo patients participate in processes associated with cell cycle, signaling, death and survival. For example, it has been proven that during the vitiligo pathogenesis, in addition to melanocytes, keratinocytes are also demonstrated to be affected – apoptotic keratinocytes are not able to produce enough supportive factors essential for melanocyte survival [58]. The inflammatory processes are common to vitiligo – the level of inflammatory cytokines has increased both locally and systemically [16–21]. The level of cytotoxic T cells has also increased [44]. The pathways for free radical scavenging have been altered in patients, and the activity of enzymes important to metabolize H_2O_2 has been impaired [23–25]. Additionally, genes associated with nervous system functioning appeared on the list. This may be related to the neural hypothesis regarding vitiligo – the amount of neuro-mediators such as dopamine and norepinephrine has altered in the blood and skin of vitiligo patients [59–65].

Additionally, functional analysis revealed that in vitiligo patients' blood, most of the 23 molecules associated with dermatological diseases and conditions, immunological and inflammatory diseases were upregulated. These data again refer to the activated processes in patients, which may both cause and try to compensate the deviation from local and systemic homeostasis. Some genes in this pathway, like major histocompatibility complex (HLA), class I genes, interferon (IFN) type I genes, nuclear factor of activated T-cells (NFAT), nuclear factor of kappa B (NF- κ B), and NLR family, pyrin domain containing 12 (NLRP12), have already been demonstrated to be associated with vitiligo pathogenesis in previous studies [66–76]. Taken altogether, the results from the functional analysis rather support the previous findings about vitiligo pathogenesis.

In conclusion, the present work provided supportive evidence that vitiligo is rather a systemic than a local disease. The RNA expression pattern in vitiligo patients' blood cells relates to dermatological, immune, inflammatory, and cell survival-associated processes, which all support the previous findings about vitiligo. Additionally, the present work points out possible links between different diseases, such as lichen planus, limb-girdle muscular dystrophy type 2B, facioscapulohumeral muscular dystrophy and vitiligo. Furthermore, the study gives an enormous amount of data, which should be used for more thorough analyses, e.g. the behavior of different types of small RNA genes in vitiligo pathogenesis.

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Disclosure Statement

The authors declare that there is no conflict of interest in this paper.

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