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Identification of differentially expressed signature genes of PCOS under vitamin D deficiency and oxidative stress: A bioinformatics Analysis

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Abstract

Polycystic ovary syndrome (PCOS) is one of the most common hormonal disorders in women of reproductive age. However, there is a lack of genetic study of the internal mechanisms of PCOS. In this study we have identified core genes involved in the pathogenesis of PCOS by using bioinformatics analysis. For this study, the dataset GSE40400 was collected from the Gene Expression Omnibus (GEO) database. The differentially expressed genes (DEGs) were obtained by using the R package limma. We analysed the microarray dataset GSE40400 to identify DEGs of PCOS subjects with VD deficiency under oxidative stress. After applying the available numerical expression values, we 617 novel DEGS out of which 183 up-regulated and 434 were down-regulated). Out of them 144 novel DEGS related exclusively to oxidative stress (39 up-regulated and 105 down-regulated), 39 novel DEGS related exclusively to vitamin D deficiency (12 up-regulated and 27 down-regulated) and 160 novel DEGS related exclusively to oxidative stress and vitamin D deficiency (48 up-regulated and 112 down-regulated). The functional analysis was carried out by using DAVID database and software tools for the identified up-regulated and down-regulated DEGs.

Keywords: PCOS, DEGs, KEGG, Oxidative stress, GO term

Introduction

At present polycystic ovary condition (PCOS) is the most common endocrine disorder in reproductive women, with a predominance of 6–10% in general population.[**1**] It is most frequently associated with anovulatory infertility, insulin resistance (IR), hyperinsulinemia and dyslipidaemia which are all risk factors for the metabolic disorder, type 2 diabetes mellitus, and cardiovascular disease.[2]

Oxidative stress (OS) results due to imbalance caused by increased generation of free radical like reactive oxygen species (ROS: hydroperoxyl, superoxide, hydrogen peroxide, and hydroxyl radicals) above the physiological range, and their reduced clearance by the antioxidant mechanisms of cells [3]. Higher OS level is related with obesity, insulin resistance, hyperandrogenaemia, and inflammation. A lot of studies have reported that oxidative stress circulating markers like MDA, SOD, GPx etc. are significantly increased in patients with PCOS compared with the normal and are considered as a potential cause of PCOS pathogenesis. [4] OS and insulin resistance (IR) goes hand in hand. On one hand OS induces

the inflammatory responses involved in insulin resistance and on the other hand IR energizes OS since hyperglycaemia and higher levels of free unsaturated lipids leads to ROS creation [5].

Vitamin D, a steroid hormone, has a potential role in the prevention of many ailments including cancers, autoimmune disorders, hypertension, diabetes, and obesity. An inadequacy of vitamin D causes poor bone mineralization as well as has been involved in various persistent infections including diabetes, coronary heart disease, poor immunity, different malignant conditions, multiple sclerosis, rheumatoid arthritis, and hypertension. [6], is a powerful anti-oxidant and plays a physiologic role in reproduction including ovarian follicular development and luteinization through changing anti-mullerian hormone (AMH) signaling, follicle-stimulating hormone (FSH) sensitivity and progesterone production in human granulosa cells. In spite of the importance, about 67-85% of women with PCOS are suffering from vitamin D deficiency [7]. Vitamin D plays a significant role in the health and fertility for women with polycystic ovary syndrome (PCOS). The connection between VD levels and various PCOS symptoms, including insulin resistance (IR), anovulation and hirsutism has been reported in a few studies. [8] Normal VD status downregulates many of the intracellular oxidative stress-related activities. Vitamin D improves mitochondrial functions and prevents oxidative stress related protein oxidation, lipid peroxidation, and DNA damage, whereas, suboptimal concentrations of serum 25(OH)D fail to control oxidative stress conditions, augment intracellular oxidative damage and the rate of apoptosis.[9]

Bioinformatics provides different platforms to analyses the data using in silico approach to predict differential expression level of genes in various condition. Microarray analysis is one of the largescale and proficient methods to retrieve biological data. This technique can monitor genome-wide variants in levels of gene expression and discover the sequence changes of more than ten thousand genes together. [10] Apart from the mentioned conventional diagnostic method, biomarkers may serve as confirmative diagnostic method for PCOS and further add to the development of the new molecular targets for drug development. In this study, the gene expression profile (ID: GSE40400) analysis was performed to find differential gene expressions (DEGs).

2 Materials and methods

2.1 Samples retrieval

Raw gene expression profile (GSE ID GSE40400) of endometrial cells from vitamin D deficient PCOS women vs. control cell samples under oxidative stress condition. The raw dataset was based on the platform GPL570 (Affymetrix Human Genome U133 Plus 2.0 Array) and was acquired from the GEO NCBI database [11]. The GSE40400 dataset has 3 control samples and 3 PCOS samples.

2.2 Pre-processing of samples

Using Benjamini and Hochberg algorithm, pre-processing of raw expression profile was performed to compute the adjusted p-value. Also, eluding force normalization and Limma precision weights log transformation was applied to the expression profile. The significant cut off level was adjusted to 0.05. Probe level conversion to gene-level symbols was also accomplished using GEO2R.

2.3 DEGs retrieval

We retrieve the differentially expressed genes (DEGs) from the GSE40400 dataset by using the limma package of the R programming language [12]. For obtaining the DEGs, the false discovery rate (FDR) of the Benjamini and Hochberg (BH) technique was fruitful to adjust p-values for multiple comparisons [13]. FDR is one of the most used methods to obtain

microarray data [14]. An adjusted p-value < 0.05 and the absolute log fold change (FC) > 1.40 were used as the cut-off criteria to attain DEGs.

2.4 *Functional analysis of DEGs*

For the functional studies of large-scale transcription or genomic data Gene Ontology (GO), functional experiment method is frequently used [15]. Gene Ontology (GO) analysis reveals cellular component (CC), molecular function (MF), and biological process (BP) The Kyoto encyclopedia of genes and genomes (KEGG) is widely used to understand metabolic pathways for gene annotation [16]. In the present study, GO and KEGG pathway enrichment analysis was conducted which expresses upregulated and downregulated DEGs using the Database for Annotation, Visualization, and Integrated Discovery software (DAVID; http://david. ncifcrf.gov/) [17] with adjustable p values <0.05 and log fold change values >0.1 for overexpressed and <0.1 for under-expressed genes.

3 Results

3.1 DEGs enrichment for PCOS

We analysed the microarray dataset GSE40400 to identify DEGs of PCOS subjects with VD deficiency under oxidative stress. After applying the available numerical expression values, we identified a total number of 635 DEGs with 183 up-regulated and 452 downregulated genes (Supplementary file 1). Further, comparisons of DEGs with gene list obtained from OMIM and Gene Cards leads to the identification of a 617 novel DEGS (183 up-regulated and 434 down-regulated) (Figure1). Out of them 144 novel DEGS related exclusively to oxidative stress (39 up-regulated and 105 down-regulated), 39 novel DEGS related exclusively to vitamin D deficiency (12 up-regulated and 27 down-regulated) and 160 novel DEGS related exclusively to oxidative stress and vitamin D deficiency (48 up-regulated and 112 down-regulated).

3.4 Functional analysis of DEGs

By using the DAVID database, the enrichment analysis outcomes were screened for the upregulated and downregulated DEGs of the GO analysis. The biological process, cellular component and molecular function enrichment analysis outcomes are displayed in Table 1 &2.

The KEGG pathway analysis by DAVID showed that the upregulated DEGs are mainly involved in pathways including pathways of cancer like lung cancer, viral carcinogenesis and p53 signalling whereas downregulated DEGs are involved in Insulin signalling and Insulin resistance pathways (Figure 2).

Discussion

PCOS is recognized by hyperandrogenism, ovulatory dysfunction, gonadotropic abnormalities, and chronic anovulation]. [18] However, the molecular mechanisms underlying the development of PCOS have remained unclear until now.

In the present study, DEGs between vitamin D deficient PCOS and normal samples under oxidative stress were identified, and bioinformatics analysis techniques were applied in order to determine the key genes and GO terms connected with PCOS. A total of 617 novel DEGs were identified from the GSE40400 dataset; which included 183 upregulated and 434 downregulated DEGs. the identification of a 617 novel DEGS (183 up-regulated and 434 down-regulated. Out of them 144 novel DEGS related exclusively to oxidative stress (39 up-regulated and 105 down-regulated), 39 novel DEGS related exclusively to vitamin D deficiency (12 up-regulated and 27 down-regulated) and 160 novel DEGS related exclusively to oxidative stress and vitamin D deficiency (48 up-regulated and 112 down-regulated).

The GO functional analysis of upregulated DEGs revealed that the top GO terms were mainly engaged in positive regulation of cyclin-dependent protein, negative regulation of cell

migration, response to cytokine, cell chemotaxis, positive regulation of proteasomal ubiquitin, protein phosphorylation, bleb assembly, small GTPase mediated signal transduction, cell division, CXCR chemokine receptor binding, receptor signal protein serine/threonine, serine/threonine kinase activity, primary amine oxidase activity, cytoskeleton adaptor activity, kinase activity, GDP binding, trans-Golgi network, nuclear speck, focal adhesion, cis-Golgi network, microtubule organizing centre. From the functional analysis component, we determined that cell proliferation may play an important role in PCOS. It has been reported earlier that microRNA miR-324 affects cell proliferation in PCOS by targeting the Wnt2B gene [19].

The GO functional analysis of downregulated DEGs revealed that the top GO terms were mainly engaged in Wnt Signaling pathway, response to interferon-gamma, cell matrix adhesion, extracellular matrix organization, cellular response to DNA damage stimulus microvillus assembly, cytokines, sister chromatid cohesion, mitotic nuclear division, wnt-activated receptor activity, PDZ domain binding, Wnt-protein binding, phosphoprotein phosphatase activity, protein kinase binding, GTPase activity, protein binding, microtubule cytoskeleton, extracellular exosome, nucleoplasm, chromatin, cytoplasm.

The KEGG pathway analysis was applied to identify the top significant pathways for the upregulated DEGs and the analysis revealed that the gene set involved are small cell lung cancer, FoxO signaling pathway, Viral carcinogenesis, HTLV-I-infection, Wnt signaling pathway, pathways in cancer, cell cycle, p53 signaling pathway. Most of the pathways are associated with cancer pathways. Vitamin D has a role in cancer prevention. Many evidence indicates that intake or synthesis of vitamin D is associated with reduced incidence and death rates of colon, breast, prostate, and ovarian cancers.[20]

The KEGG pathway analysis was applied to identify the top significant pathways for the downregulated DEGs and the analysis revealed that the gene set involved are insulin signaling pathway, ubiquitin mediated proteolysis, insulin resistance, glycine, serine and threonine metabolism, metabolic pathways, biosynthesis of antibiotics and sphingolipid metabolism. Most of the pathways are involves in insulin resistance and insulin signalling pathways. Vitamin D deficiency has been proposed to play an important role in the development of insulin resistance and the pathogenesis of type 2 DM by affecting insulin sensitivity or/and β-cell function. [21] Vitamin D directly effects insulin action by stimulating the expression of insulin receptors and thus enhancing insulin responsiveness for glucose transport, [22] or indirectly by regulating extracellular calcium influx through cell membranes [23]. Further, Oxidative stress also impairs insulin signalling by inducing IRS-1 and IRS-2 serine phosphorylation, which in turn results in a disturbed IST [25-26]. Hence vitamin deficiency in the PCOS subjects increased insulin resistance under oxidative stress conditions.

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Table 1: GO enrichment for Downregulated DEGs

Category	Term	P Value
GOTERM_CC_DIRECT	GO:0005737~cytoplasm	0.001599
GOTERM_CC_DIRECT	GO:0000785~chromatin	0.001808
GOTERM_CC_DIRECT	GO:0005829~cytosol	0.002406
GOTERM_CC_DIRECT	GO:0005654~nucleoplasm	0.003167
GOTERM_CC_DIRECT	GO:0070062~extracellular exosome	0.008083
GOTERM_CC_DIRECT	GO:0015630~microtubule cytoskeleton	0.010731
GOTERM_CC_DIRECT	GO:0016020~membrane	0.015275
GOTERM_CC_DIRECT	GO:0005881~cytoplasmic microtubule	0.020891
GOTERM_CC_DIRECT	GO:0031965~nuclear membrane	0.027994
GOTERM_CC_DIRECT	GO:0032580~Golgi cisterna membrane	0.031113
GOTERM_CC_DIRECT	GO:0016324~apical plasma membrane	0.040011
GOTERM_CC_DIRECT	GO:0005694~chromosome	0.045896
GOTERM_MF_DIRECT	GO:0005515~protein binding	9.78E-07
GOTERM_MF_DIRECT	GO:0005525~GTP binding	9.07E-05
GOTERM_MF_DIRECT	GO:0016301~kinase activity	0.004161
GOTERM_MF_DIRECT	GO:0034237~protein kinase A regulatory subunit binding	0.012834
GOTERM_MF_DIRECT	GO:0004721~phosphoprotein phosphatase activity	0.013199
GOTERM_MF_DIRECT	GO:0030165~PDZ domain binding	0.018759
GOTERM_MF_DIRECT	GO:0042813~Wnt-activated receptor activity	0.03071
GOTERM_MF_DIRECT	GO:0045296~cadherin binding	0.034512
GOTERM_MF_DIRECT	GO:0004672~protein kinase activity	0.035908
GOTERM_BP_DIRECT	GO:0007067~mitotic nuclear division	4.74E-06
GOTERM_BP_DIRECT	GO:0007062~sister chromatid cohesion	4.38E-04
GOTERM_BP_DIRECT	GO:0010628~positive regulation of gene expression	0.001908
GOTERM_BP_DIRECT	GO:0000910~cytokinesis	0.004009
GOTERM_BP_DIRECT	GO:0043392~negative regulation of DNA binding	0.011369
GOTERM_BP_DIRECT	GO:0032436~positive regulation of proteasomal ubiquitin-dependent protein catabolic process	0.016016
GOTERM_BP_DIRECT	GO:0060326~cell chemotaxis	0.017185
GOTERM_BP_DIRECT	GO:0001932~regulation of protein phosphorylation	0.022182
GOTERM_BP_DIRECT	GO:0034097~response to cytokine	0.024488
GOTERM_BP_DIRECT	GO:0009308~amine metabolic process	0.024975
GOTERM_BP_DIRECT	GO:0030336~negative regulation of cell migration	0.031987
GOTERM_BP_DIRECT	GO:0071407~cellular response to organic cyclic compound	0.039451

GOTERM_BP_DIRECT	GO:0045737~positive regulation of cyclin-dependent protein serine/threonine kinase activity	0.039615
GOTERM_BP_DIRECT	GO:0072661~protein targeting to plasma membrane	0.048584

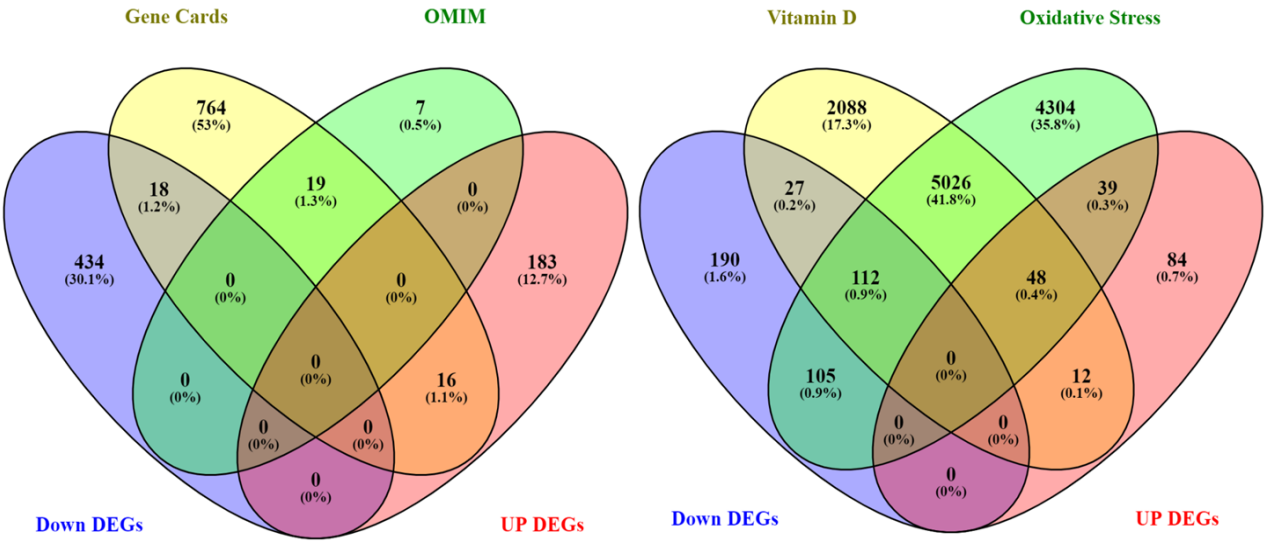
Category	**Term**	**PValue**
GOTERM_CC_DIRECT	GO:0005815~microtubule organizing center	0.001021
GOTERM_CC_DIRECT	GO:0005801~cis-Golgi network	0.001803
GOTERM_CC_DIRECT	GO:0000775~chromosome, centromeric region	0.001887
GOTERM_CC_DIRECT	GO:0005925~focal adhesion	0.002812
GOTERM_CC_DIRECT	GO:0016607~nuclear speck	0.004292
GOTERM_CC_DIRECT	GO:0005802~trans-Golgi network	0.010223
GOTERM_CC_DIRECT	GO:0000139~Golgi membrane	0.011283
GOTERM_CC_DIRECT	GO:0015629~actin cytoskeleton	0.019525
GOTERM_CC_DIRECT	GO:0030175~filopodium	0.023172
GOTERM_CC_DIRECT	GO:0045177~apical part of cell	0.0294
GOTERM_CC_DIRECT	GO:0005794~Golgi apparatus	0.031352
GOTERM_CC_DIRECT	GO:0032993~protein-DNA complex	0.04165
GOTERM_MF_DIRECT	GO:0019003~GDP binding	4.30E-05
GOTERM_MF_DIRECT	GO:0003924~GTPase activity	0.001235
GOTERM_MF_DIRECT	GO:0019901~protein kinase binding	0.008191
GOTERM_MF_DIRECT	GO:0008093~cytoskeletal adaptor activity	0.012834
GOTERM_MF_DIRECT	GO:0008131~primary amine oxidase activity	0.013669
GOTERM_MF_DIRECT	GO:0004674~protein serine/threonine kinase activity	0.015806
GOTERM_MF_DIRECT	GO:0004702~receptor signaling protein serine/threonine kinase activity	0.025295
GOTERM_MF_DIRECT	GO:0045236~CXCR chemokine receptor binding	0.030813
GOTERM_MF_DIRECT	GO:0008134~transcription factor binding	0.035756
GOTERM_BP_DIRECT	GO:0051301~cell division	2.78E-06
GOTERM_BP_DIRECT	GO:0007264~small GTPase mediated signal transduction	3.50E-04
GOTERM_BP_DIRECT	GO:0015031~protein transport	9.13E-04
GOTERM_BP_DIRECT	GO:0032060~bleb assembly	0.003264
GOTERM_BP_DIRECT	GO:0006468~protein phosphorylation	0.006237
GOTERM_BP_DIRECT	GO:0030033~microvillus assembly	0.015675
GOTERM_BP_DIRECT	GO:0006974~cellular response to DNA damage stimulus	0.016107
GOTERM_BP_DIRECT	GO:0006470~protein dephosphorylation	0.019458
GOTERM_BP_DIRECT	GO:0030198~extracellular matrix organization	0.023393
GOTERM_BP_DIRECT	GO:0007160~cell-matrix adhesion	0.024716
GOTERM_BP_DIRECT	GO:0006509~membrane protein ectodomain proteolysis	0.031578

GOTERM_BP_DIRECT	GO:0034976~response to endoplasmic reticulum stress	0.032216
GOTERM_BP_DIRECT	GO:0034341~response to interferon-gamma	0.039615
GOTERM_BP_DIRECT	GO:0007420~brain development	0.04123
GOTERM_BP_DIRECT	GO:0060070~canonical Wnt signaling pathway	0.04902

Figure Legends:

Figure 1. Reported and novel DEGs. Venn diagram: All DEGs were compared with reported gene list obtained from the Gene Cards and OMIM to reveal the novel DEGs. B. Out of total 617 novel DEGS identified 183 up-regulated and 434 were down-regulated.

Figure 2. KEGG Pathway analysis for DEGs.



Total DEGs	635 (UP + Down=183+452)
Total Novel DEGs	617 (UP + Down=183+434)
Novel DEGs related exclusively to Oxidative stress	144 (UP + Down=39+105)
Novel DEGs related exclusively to Vitamin D	39 (UP + Down=12+27)
Novel DEGs related to oxidative stress and vitamin D both	160 (UP + Down=48+112)

Figure 1. Venn diagram

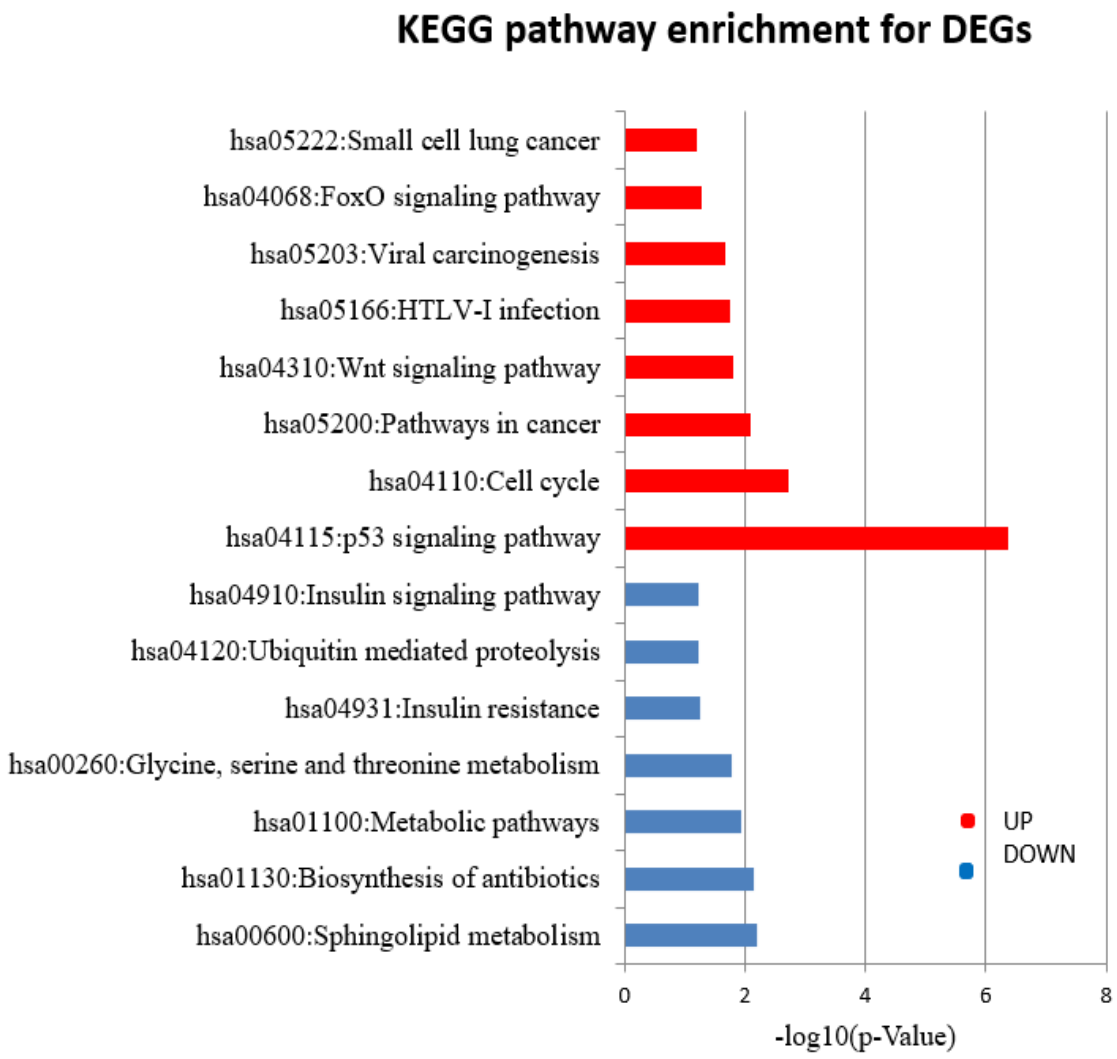


Figure2. KEGG pathway enrichment for DEGs