Hypoxia on vhl-deficient cells to obtain hif related genes through bioinformatics analysis.

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Abstract

Vhl is responsible for degrading the transcription factor hif-1. Hif-1 transcription factors drive changes in hypoxic gene expression to adapt cells to exposure to hypoxic environments. This study hopes to analyze the effect of hypoxia on the vhl-deficient cells to obtain hif regulated genes through bioinformatics analysis. The top ten genes evaluated by connectivity degree in the PPI network were identified. CDK1, CTNNB1, NHP2, CCNA2, CTNNB1, MRPL16, CCND1 were down-regulated. CDK1, RPL12, RPL17, RPL27, RPS10 were up-regulated.

Introduction

Vhl is responsible for degrading the transcription factor hif-1. Hif-1 transcription factors drive changes in hypoxic gene expression to adapt cells to exposure to hypoxic environments. Hypoxia active the heterodimeric transcription factor hif-1 to trigger a synergistic transcriptional response resulting in solid tumours.³ under oxygen deprivation hif1 is an essential helix-loop-helix PAS domain transcription factor.⁴⁻⁷

Hif-1 α dimerizes with hif-1 β in the nucleus, hif-1 decreased the speed of degrading under hypoxia condition. Hypoxia response elements are the heterodimer of hif-1 binding to regulatory DNA sequences. Transcriptional co-activators Promote angiogenesis through enhancing the transcription of a lot of target genes.^{4,5,8} Vhl gene is a tumour suppressor gene, and its proteins pVHL30 and pVHL19. It has the function of tumour inhibition, and the two structures are similar, which are collectively called vhl protein(pvHL). Hypoxia-inducible factor hif gene is a target gene of vhl gene. hif is an oxygen-dependent transcriptional activator produced by cells during hypoxia, by only B subunits. Oxygen regulates hif activity primarily through hif-a. For cell growth, inhibition of hif1 expression can be promoted, causing the death of the cells under hypoxia condition. Therefore, this study hopes to analyze the effect of hypoxia on the vhl-deficient cells to obtain hif regulated genes through bioinformatics analysis.

Materials and methods

We download a database from the GEO database(<https://www.ncbi.nlm.nih.gov/geo/>) to obtain the gene expression datasets. One series(GDS1772) was selected out from the database about human, three groups including control, hypoxia and hypoxia-reoxygenation were retrieved from the database. All of those three groups contain two parts of vhl minus and vhl plus.(Table 1) GDS1772 was based on the Agilent

GPL3423: Stanford Denko EOS Human 35K GeneChip v1.1. All of the data we're freely available online. This study has not been reported by any experiment on humans and declared by any other authors.

Table 1 Dividing into three groups according to the degree of hypoxia

	First group	Second group	Third group
VHL minus	Control 1	Hypoxia 1	Hypoxia (1h or 4h) and reoxygenation 1
VHL plus	Control 2	Hypoxia 2	Hypoxia (1h or 4h) and reoxygenation 2

Data processing of DEGs

R: The R Project for Statistical Computing(<https://www.r-project.org/>) was used to detect the DEGs of three groups between vhl minus and vhl plus samples, and the adjusted P-value and $|\log FC|$ were calculated. We selected the DEGs by adjusting $P < 0.01$ and $|\log FC| \geq 2.0$.

GO and KEGG pathway analysis of DEGs

GO analysis is a widely used method for functional enrichment studied. Also, gene functions were composed of biological process (BP), molecular function (MF), and cellular component (CC) three parts. KEGG is a large-scale used database, including vast amounts of genomes, biological pathways, diseases, chemicals, and drugs. We use the Database for Annotation, Visualization, and Integrated Discovery (DAVID) tools

(<https://david.ncifcrf.gov/>) to analysis the DEGs through GO annotation analysis and KEGG pathway enrichment analysis. $P < 0.05$ was considered statistically significant.¹⁸

PPI network construction and hub gene identification

In this study, we use the Search Tool for the Retrieval of Interacting Genes (STRING) database (<http://string-db.org/>) and GeneMANIA online database (<https://genemania.org/>) to analyze the PPI information and evaluate the potential PPI relationship. They were also used to identify the DEGs to analysis the PPI information. A combined score was set to 0.4, and then the PPI network was visualized by Cytoscape software (www.cytoscape.org/). The stability of the entire system was guaranteed by a higher degree node of connectivity. We calculated the degree of each protein node by using CytoHubba, a plugin in Cytoscape. Through those steps, we can select ten hub genes.¹⁸

Results

Identification of DEGs

Gene expression profile (GDS1772) was selected in this study. GDS1772 contained vhl minus and vhl plus samples. Based on the criteria of $P < 0.05$ and $|\log FC| \geq 1$, the first group of control without hypoxia and the third group hypoxia-reoxygenation have no differential genes with each other. The second group of hypoxia, we found that a total of 376 DEGs were identified from vhl minus samples compared with vhl plus samples, including 162 upregulated genes and 214 downregulated genes (Table 2).

Table 2 Differentially expressed genes

	Number	Differentially expressed genes
up-regulated	162	W76492 EOS26732 M31994 M26576 HDDC2 ZDHHC4 STK32B PPM1H SYNGR2 EOS22868 EOS25123 FBXO4 EOS24821 C1S C1R RAB27B CITED2 MMP7 EOS08324 M13560 SMG7 EMILIN2 TMEM41A NSMCE4A EOS25322 TSPAN15 AA393801 AES AA253217 SQRDL NBL1 DAB2 AA211385 DVL3 AA449789 ETV5 EOS22353 LGALS3BP DDOST ECH1 KIAA2022 SET NTN4 TRAPPC2 CCND2 MFGE8 BMPR1A CNN1 UPP1 R79723 ATP9A SERF2 N25921 RPS10 W38419 FPGS PPP1R1A EOS20917 SEPW1 GNS TRIM21 C10orf128 NDUFA4 CFI CFHR1 EIF3K SPRED1 COPE NEK4 DCBLD2 EOS04979 TIMP2 ADAMTS15 AA481143 M27891 PARP4 CTSA TTC28 TM4SF4 UST SULF2 ALDH2 EOS15038 C3 EOS06606 ATXN7L3B EOS06229 SFRP4 CDK1 KCTD12 RPL12 DDA1 EOS25782 SREBF1 EOS24924 RPL17 TPCN1 AA024622 DOCK10 KL SLC48A1 AA232673 C9orf16 N26713 NME3 EPHX1 AIF1L CASP4 TPK1 NIPSNAP1 SLC17A3 OSBPL6 FKBP14 EIF2A ZDHHC23 TNFRSF21 PHLDA1 AR RPL27 LOC100288911 UBE2Q1 SPINT2 HINT3 R59093 GLIS2 LOC100509635 H88207 EOS25376 CDK5R1 KRT18 EOS34082 HIF1A NDEL1 STAT1 MAL2 EOS26908 CTGF PSG4 DOCK11 PEPD POGK PEX11B AGTRAP EOS33573 COL12A1 U46499 PNPLA2 N90041 EOS07844 FN1 ABCB1 EOS33884 PRDX2 ZNF652 PPRC1 LOC100507486 THBD EEF1A2 EOS26690
down-regulated	214	EOS05245 PPARD C3orf14 STK24 EOS05860 M73547 VPS51 VPS26B EIF4EBP2 LOC100506057 EOS13004 TKT NDUFA4L2 EOS14697 LRRC41 EOS33502 PANX1 RASSF4 CUEDC2 KSR1 MRPS28 NHP2 EOS20666 ANXA6 VSTM4 C7orf10 MT1F AA421712 AA478962 N66127 POC1A DEPDC1B RAD1 EOS04509 C15orf48 STK32C LOXL4 FLJ44896 ELOVL1 NDUFAF3 SMIM12 BCCIP NDST1 PTPRE FERMT1 M84349 SCFD2 EOS33972 ZNF511 TBC1D25 FADS1 N91347 BST2 SFXN2 CAMK1 GSTO1 N24917 TWF2 NREP EOS31055 AA416627 LDHA U51010 SLIT3 GPX7 SCD B4GALT2 EOS24606 AA470074 EOS27040 N41029 EOS18999 NUDT11 IMPDH2 PDLIM1 AA412163 EOS15806 EOS26508 AA490212 CA9 EOS28736 AA347023 PHKA2 SUV39H1 GPX3 EOS05325 KIF20A PLP2 PLEKHA7 COA4 RNF130 NDUFAF2 C19orf70 CTNNB1 HNRNPFC CHCHD1 EOS05280 GEMIN8 TFDP1 H88117 PGRMC2 MRPL16 PPAPDC1A EOS20393 BAD ABLIM3 CCND1 EOS27103 YIPF1 EOS25515 PDDC1 ATP1B1 W26520 SYT13 SMS MRPL23 EOS27951 PTGR1 PCED1A TBC1D1 TRAPPC9 EOS25719 AA083373 ELMO3 PRDX5 CDKN2AIPNL LOC100506233 MINPP1 CDK16 MRPL36 WNT5B EOS25727 R3HCC1 HADH EOS35101 WDR13 GLUL U97105 CYB561A3 GPX1 C10orf10 AMACR MICAL2 PPIF PPA1 MICU1 NLGN1 VAMP5 EOS26458 RNF145 RBBP7 LAGE3 CCNA2 FAM156A ELOF1 HINT1 DYNLT3 PRAF2 FGD1 H47839 SCD5 KIAA1191 LRPAP1 MRPL37 MMAB TMEM97 N59543 KIFC1 FUND1 EOS07009 ITGB5 EOS26066 EOS07594 CA12 EOS33012 IPO13 PTTG1 EOS25548 CENPM SLC10A3 POMZP3 EOS21257 EOS26811 CMTM7 RPS6KA3 SEMA6A NSDHL EOS33879 GNG11 TMEM54 INTS5 EOS06365 HYAL1 EBP C21orf33 SRPX FBXL7 EOS06530 IFIT3 PCSK1N EOS06443 R43204 STEAP1 GLCC11 FABP6 KCTD14

Functional enrichment analyses of DEGs

GO function and KEGG pathway enrichment analysis for DEGs were performed using the Cytoscape software (Table 3). The enriched GO terms were divided into CC, BP, and MF ontologies. BPs including cartilage development, oxidoreductase activity, acting on paired donors, with the oxidation of a pair of donors resulting in the reduction of molecular oxygen to two molecules of water and acyl-CoA desaturase activity. MF including large ribosomal subunit rRNA binding. CC, including mitochondrial protein

complex. In addition, the results of KEGG pathway analysis showed that DEGs were mainly enriched in pathways in biosynthesis of unsaturated fatty acids (Table 4).

Table 3 The enrichment analysis of differentially expressed genes between vhl minus and vhl plus

Category	Term	PValue	Associated Genes Found
GOTERM_BP_DIRECT	GO:0051216~ cartilage development	0.02687*	ANXA6, BMPR1A, CCN2, CCNA2 , COL12A1, CTNNB1 , HIF1A, HYAL1, POC1A, PRDX2, SULF2, WNT5B
GOTERM_BP_DIRECT	GO:0016717~ oxidoreductase activity, acting on paired donors, with oxidation of a pair of donors resulting in the reduction of molecular oxygen to two molecules of water	0.00364*	FADS1, SCD, SCD5
GOTERM_BP_DIRECT	GO:0016215~ acyl-CoA desaturase activity	0.00148*	FADS1, SCD, SCD5
GOTERM_CC_DIRECT	GO:0098798~ mitochondrial protein complex	0.02403*	C15orf48, CHCHD1, HADH, MICOS13, MICU1, MRPL16 , MRPL23, MRPL36, MRPL37, MRPS28, NDUFA4, NDUFA4L2, PPIF
GOTERM_MF_DIRECT	GO:0070180~ large ribosomal subunit rRNA binding	0.01969*	MRPL23, RPL12 , RPL17

*Pvalue<0.05

Table 4 The KEGG pathway enrichment analysis of DEG differentially expressed genes between vhl minus and vhl plus

Term	Pvalue	Associated Genes
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		Found
KEGG:01040~ Biosynthesis of unsaturated fatty acids	0.03303*	ELOVL1, FADS1, SCD, SCD5

*Pvalue<0.05

PPI network construction and hub gene identification

Protein interactions among the DEGs were predicted with STRING tools and GeneMANIA online database. A total of 268 nodes and 428 edges were involved in the PPI network, as presented in Figure 1. The top ten genes evaluated by connectivity degree in the PPI network were identified. The results showed that ribosomal protein L12(RPL12) was the most outstanding gene with connectivity degree=12, followed by ribosomal protein L27(RPL27; degree=13), ribosomal protein L17(RPL17; degree=10), ribosomal protein S10(RPS10; degree=11), NHP2 ribonucleoprotein(NHP2; degree=10), cyclin D1(CCND1;degree=21), cyclin A2(CCNA2; degree=14), mitochondrial ribosomal protein L16(MRPL16; degree=10), cyclin-dependent kinase 1(CDK1; degree=15), catenin beta 1(CTNNB1; degree=20). All of these hub genes were upregulated in vhl plus.

Figure 1 Based on STRING and GeneMANIA online database, protein-protein interaction networks of the differentially expressed genes were constructed and modular analyses

Figure 2 The PPI network was visualized by Cytoscape software and the top ten genes was evaluated by connectivity degree in the PPI network

Discussion

In recent years, with the rapid development of modern biotechnology, such as biochip and high-throughput sequencing, bioinformatics has attracted more and more attention. Through big data analysis, it is an important application of bioinformatics to explore relevant genes that play a leading role in the development of diseases and provide ideas for the study of the mechanism of diseases. This study hopes to analyze the effect of hypoxia on vhl-deficient cells to obtain hif regulated genes through bioinformatics analysis. The first group of control without hypoxia and the third group hypoxia reoxygenation have no differential genes with each other. The second group of hypoxia, we found that a total of 376 DEGs were identified from vhl minus samples compared with vhl plus samples, including 162 up-regulate genes and 214 downregulated genes. The enriched GO terms were divided into CC, BP, and MF ontologies. BPs including cartilage development, oxidoreductase activity, acting on paired donors, with oxidation of a pair of donors resulting in the reduction of molecular oxygen to two molecules of water and acyl-CoA desaturase activity. MF including large ribosomal subunit rRNA binding.

CC, including mitochondrial protein complex. In addition, the results of KEGG pathway analysis showed that DEGs were mainly enriched in pathways in biosynthesis of unsaturated fatty acids. The top ten genes evaluated by connectivity degree in the PPI network were RPL12, RPL27, RPL17, RPS10, NHP2, CCND1, CCNA2, MRPL16, CDK1, CTNNB1. NHP2, CCNA2, CTNNB1, MRPL16, CCND1 were down-regulated. CDK1, RPL12, RPL17, RPL27, RPS10 were up-regulate. According to the enrichment analysis of DEG between vhl minus and vhl plus, CCNA2 and CTNNB1 were related to cartilage development. MRPL16 had corresponded with a mitochondrial protein complex. RPL12 and RPL17 involved in large ribosomal subunit rRNA binding.

CCNA2, also called cyclin A2. The protein encoded by CCNA2 is part of the Highly conservative cyclin family. Cyclin family members operate as regulating the cell cycle, which can bind and active cyclin-dependent kinase2 to promote the transition through G1/S and G2/M. Deng H, et al. find that overexpressed CyclinA2 improve the cardiomyocytes proliferation under hypoxia impair. Other articles find that in the neonatal rat hearts, the proliferation of cardiomyocytes was inhibited under hypoxia. However, overexpressed CyclinA2 weaken the proliferation of cardiomyocytes suggesting that CyclinA2 is an essential part of regulating cardiomyocytes growth and increasing protection of cardiomyocytes from disability of proliferation in hypoxic conditions.⁹ A few days after birth, the proliferative function of mammals' cardiomyocytes almost lost because of the sharp down-regulation of CyclinA2 in the heart.¹⁰ Previous studies indicated that in the adult heart CyclinA2 almost have no function. Increasing the content of CyclinA2 in adult animals' heart can make a difference in restoration of cardiac proliferation.^{10,11} Also, Schulze A, et al. find that in

the primary neonatal rat cardiomyocytes overexpressed CyclinA2 increase proliferation. After exposure to hypoxia for 12h, CyclinA2 down-regulate and marked decrease the cardiomyocyte proliferation. In conclusion, overexpressed CyclinA2 have beneficial to cardiomyocyte growth under impair of hypoxia.¹² In summary, decrease the expression of vhl can up-regulate CyclinA2 and also increase hif. There was no other organs protection related studied about CyclinA2 protection under hypoxia.

CDK1, also called cyclin dependent kinase 1. The protein encoded by CDK1 is belonging to the Ser/Thr protein kinase family. It is a catalytic subunit of M-phase promoting factor(MPF), and have an essential function in the transition of the eukaryotic cell cycle for G1/S and G2/M phase. This protein is related to mitotic cyclins stably, which has a function to regulate subunits. The content of cyclin and the phosphorylation and dephosphorylation of this protein in the cell cycle can regulate the kinase activity of this protein. This gene has spliced transcript variants which encodes various isoforms. The protein encoded by CDK1 belongs to the Ser/Thr protein kinase family and also is a catalytic submit of M-phase promoting factor(MPF). MPF is important for the transition of G1/S and G2/M in the eukaryote cell cycle. This protein is regulatory subunit related to Mirotic cycling stable. Therefore, no matter whether hypoxia, CDK1 regulate the phosphorylation of hif-1a, which works for the steady-state levels of hif1a. Inhibitors of CDK1 have been suggested using for the treatment of many kinds of cancers and that it can increase the sensitization of the TRAIL-induced apoptosis.¹⁵⁻¹⁷ Inhibiting CDK1 can decrease hif-1a expression, and active transcription and decrease surviving phosphorylation so that increasing the sensitivity of cancer cells apoptosis. Recently, Herrera MC, et al find that regulating RNA polymerase III activity affects cdk1 gates cell

cycle-dependent tRNA synthesis.¹⁹ Jones MC, et al. find cdk1 also related to cell adhesion.²⁰ Wang Z, et al. find that during the cell cycle G2/M Progression, Cdk1 coordinates Mitochondrial Respiration.²²

So that, vhl can increase the expression of CDK1, and CDK1 can regulate and activate the expression of hif-1a under the condition of hypoxia. CDK1 was mainly studied in the regulation of cancer cells apoptosis at present.

CTNNB1, also called catenin beta 1. The protein encoded by CTNNB1 belongs to the constitute adherents junctions (AJs), which can regulate cell growth and adhere between cells to create and maintain epithelial cell layers. This protein works on acting cytoskeleton and transmitting the contact inhibition signal to stop dividing once the epithelium is formed. A kind of cancer can be caused by a mutation of this gene. We suppose that this gene inhibits the dividing of cancer cells.^{1,24} Hirata H, et al. find that β -catenin promotes cell survival and adapt to hypoxia by strengthening hif-1 mediated transcription. So that, we find that vhl down-regulate the expression of CTNNB1 same as the result of Hirata H's findings and CTNNB1 can protect cells from hypoxia.¹

NHP2、 CCNA2、 CTNNB1、 MRPL16、 CCND1 were down-regulated by vhl. NHP2 has an effect on the rRNA production and rRNA pseudouridylation. MRPL16 the protein encoded by MRPL16 comprises the mitoribosome. Mutations, amplification and overexpression of CCND1 alters cell cycle progression, which is observed in various tumors and conduces to tumorigenesis. Recent article find that targeting USP22 and CDK inhibitors have a benefit on cancer patients induced by CCND1.²¹ Xu P, et al. find that CCND1 has the disadvantage of early-stage lung adenocarcinoma patients.²³ CDK1,

RPL12, RPL17, RPL27, RPS10 were up-regulated by vhl. The expression of RPS10 was related to colorectal cancers. RPL12, RPL17 and RPL27 are related to encoding the ribosomal protein. In the condition of hypoxia, other genes affecting the expression of hif have not been reported, which would be the direction of our follow-up study.

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Figure 1