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Pathway Crosstalk Analysis of mTOR Gene in Insulin Resistance Mediated Obesity Using Microarray Data

Munir A[1,2], Janbey A[2*], Al-Janaby A[2], Bakhtiar SM[3] and Sajjad B[2]

[1]School of Biosciences, University of Kent, Canterbury, United Kingdom

[2]Department of Health and Applied Sciences, The London College UCK, London, United Kingdom

[3]Department of Bioinformatics and Biosciences, Faculty of Health, and Life Sciences, Capital University of Science and Technology, Islamabad, Pakistan

[*]Corresponding author:

Alan Janbey,
Department of Health and Applied Sciences, The London College UCK, London, United Kingdom

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1. Abstract

Obesity is defined as the early deposition of adipose tissue caused by a consistent caloric intake that exceeds the individual's caloric needs. As a worldwide issue, the rising incidence of obesity is a major source of concern—not because of sickness, but because of excess. Obesity is linked to a number of ailments, the most serious of which might be type 2 diabetes, and the fundamental reason for this link is obesity's proclivity to develop insulin resistance. The GSE69039 microarray profile was taken from the Gene Expression Omnibus database, included 4 normal-weight samples, 7 slightly obese samples, and 7 moderately obese samples. To characterise differentially expressed genes (DEGs), the R Limma package was utilised. For DEGs, gene ontology and enrichment analyses were performed; moreover, the relevance of the mTOR gene in numerous enriched pathways was revealed. The protein-protein interaction networks were generated for DEGs. In addition, a crosstalk network for the mTOR-associated pathways was developed. 193 DEGs have been reported in total. The roles of DEGs have been greatly enriched in the biosynthesis process and the cellular nitrogen compound metabolic process. The cross-talk network shows the major involvement of several signalling pathways and common pathways of cancer in insulin resistance-mediated obesity. In comparison, the cross-talk network offers foundations that target several pathways together more efficiently than targeting one pathway on its own.

2. Introduction

Obesity is the premature deposition of adipose tissue that results from a constant caloric intake that exceeds the individual's caloric requirements [1]. In humans, the expansion of adipose deposits results in a depot-dependent fashion from increased numbers of individual adipocytes (hyperplasia) and adipocyte hypertrophy [2]. Importantly, the size and expandability of different adipose tissue deposits in humans vary widely at individual levels [3-5]. This factor is critically important in understanding the correlation between obesity and insulin resistance since deposit expansion is associated with increased risk, while expansion of other substances is associated with decreased risk of obesity [6-9]. The growing incidence of obesity is a major global issue, not one of illness but one of excess. In the U.S., only around one-third of adults are estimated to be of "average" weight [10], and similar patterns are found worldwide [10, 12]. Obesity is linked to several conditions, the most devastating of which may be type 2 diabetes. 171 million people were projected to have diabetes at the beginning of this century worldwide, and this is predicted to rise to 366 million by 2030. Adipose tissue produces elevated quantities of non-esterified fatty acids, glycerol, hormones, pro-inflammatory cytokines, and

other factors that lead to the production of insulin resistance in obese people [11]. For decades, the correlation between obesity and type 2 diabetes has been known, and the main reason for this relationship is obesity's capability to develop insulin resistance. Insulin resistance is a central feature of type 2 diabetes etiology and is often correlated with a broad range of other pathophysiological features, including obesity, hyperlipidemia, atherosclerosis, and polycystic ovary disorders [12]. Biliopancreatic diversion prompts normalisation of insulin sensitivity in diabetic obese patients, which lasts up to two years [13]. Insulin resistance is a common phenomenon in obesity [14], as demonstrated by low rates of whole-body glucose consumption during clamping [15]. It has been shown that weight loss improves the insulin-mediated storage of glucose by improving both the oxidation and glucose storage in the skeletal muscle [16]. Insulin lowers the blood glucose level by promoting the release of glucose into insulin-sensitive tissues such as skeletal muscle, fat, the abdominal area, and the heart. Insulin frequently controls the development of glucose in the liver, kidney, blood, and small intestine. Insulin resistance happens as the insulin-tolerant tissue deficit responds to insulin [17]. Insulin resistance has shown several features in the insulin sensitivity assays: fasting hyperinsulinemia and hyperglycemia, elevated glycosylated haemoglobin (HbA1c), postprandial hyperglycemia, hyperlipidemia, reduced glucose tolerance, impaired insulin tolerance, decreased rate of glucose infusion, increased development of hepatic glucose, lack of first-step insulin secretion, hypoadiponectinemia, and elevated rates of inflammatory plasma markers [18]. In obesity, retinol-binding protein-4 (RBP4) promotes insulin tolerance by decreasing phosphatidylinositol-3-OH kinase (PI3 K) signalling in the muscle and increasing production of the phosphoenolpyruvate carboxykinase gluconeogenic enzyme in the liver via a retinol-dependent mechanism [19]. By comparison, adiponectin functions as an insulin sensitizer, inducing fatty acid oxidation in an AMP-activated protein kinase (AMPK) and receptor-dependent peroxisome proliferator (PPAR-) [20]. Furthermore, decreased tumour necrosis factor-(TNF-), interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), several macrophage products, and other adipose tissue-populating cells also play a role in insulin resistance development [20]. Similarly, the pathways of the c-Jun amino terminal kinase (JNK) and IB kinase (IKK)/nuclear factorB (NFB) also result in the up-regulation of possible inflammatory mediators that can contribute to insulin resistance. Pathways involving suppression of cytokine signalling (SOCS) proteins and inducible nitric oxide synthase (iNOS) may also prompt insulin resistance caused by cytokines. [21, 22]. The activation of the mammalian rapamycin complex (mTORC) plays a crucial function in insulin tolerance, and thus, insulin does not inhibit gluconeogenesis. This also promotes the production of fatty acids [23]. The stage at which insulin signalling is compromised in obesity causes downstream insulin receptor activation, as well as other downstream pathways of serine-threonine protein kinase Akt2 [24] that may be responsible for the disconnection of glucose and lipid metabolism in the insulin signalling pathway [25]. Recently, crosstalk between various signalling pathways has been shown to play a crucial role in insulin resistance-mediated obesity. Therefore, the purpose of this study is to use microarray data to identify differentially expressed genes (DEGs) in obesity and the generation of crosstalk networks to check the presence of the mTOR gene in enriched pathways to confirm its role as a major culprit in insulin resistance-mediated obesity. This study enlightens further understanding of the molecular mechanisms of obesity caused by insulin resistance. In the meantime, this can also give insight into the new therapies.

3. Material and Methods

3.1. Data Collection

The mRNA expression profiles of GSE69039 last updated in NCBI by Kyungpook National University, Korea, on Feb 22, 2019, were generated by the Illumina HumanHT-12 V4.0 expression bead chip and downloaded from the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/). There were a total of 18 mRNA chips, including 4 normal-weight samples, 7 samples of mildly obese subjects, and 7 of moderately obese subjects. The raw data files and their probe annotation files were retrieved and used for further analysis.

3.2. Data Preprocessing

The probe IDs were translated into gene symbols using GEO2R. Probe samples with defective or negative values were excluded from the study. The data sets were normalised using the min-max normalisation formula (Equation 1). The expression values of all probe sets corresponding to a given gene were decreased for each sample and summed up to a single value lying in the range of 0-1. The data was then preprocessed in R Studio [26], utilising the Preprocess Core package of the R language.

(a-min)/(max-min) eq.1

a shows the observed values in the data, and min and max represent the minimum and maximum given values in the data.

3.3. Identification of Differentially Expressed Genes

The Limma package [27] in R language was employed on the preprocessed data to distinguish DEGs between the normal weight, slightly obese, and moderately obese groups. The cutoff condition for DEGs was set as the p-value between 0.01 – 0.09, the fold shift (F) > 10 to <32 and FDR<0.05. Depending on the Benjamini & the Hochberg method [28], the raw p values were adjusted to appropriate p values.

3.4. Gene Ontology Analysis of DEGs

Gene Ontology (GO) annotation was conducted to evaluate the roles of DEGs between standard normal-weight samples and mildly and moderately obese samples. GO analysis has been frequently

used in large-scale gene functional enrichment studies [29]. The GO studies for biological processes were conducted using the GO Term Mapper [30]. The molecular processes of DEGs were retrieved through EnrichNet [31].

3.5. Pathways Enrichment Analysis of DEGs

The enriched genes in pathways were found by GenCLiP 2.0, which is a web-based text-mining platform for gene clustering and molecular network construction [32]. The cutoff criteria for GO categories were set as p = 0.05 and a hit = 15. The gene list obtained through GenCLiP 2.0 was then submitted to the KEGG Pathways database to identify enriched pathways. For this purpose, the p-value was set to 0.05 as the cutoff value. The enriched pathways obtained through KEGG were further cross-verified using a network analyst [33] with a cutoff p-value of 0.01 to 0.09.

3.6. Construction of Protein-Protein Interaction Networks and Functional Analysis

PICKLE (Protein Interaction Knowledge Base) 2.0 [34] was used to extract human protein-protein interactions (PPIs). It is a database of proteins accessible via the internet. From PICKLE, a total of 1564 PPI pairs were retrieved. The PPI network was built using the Gephi [35] software. The edges and nodes of the created PPI network were so complicated that more analysis was required to show the PPI network's enriched functional modules, which were done using Network Analyst [33]. Finally, an analysis of the modules for GO and pathway enrichment was performed.

3.7. Significance Analysis of the mTOR Gene as a Common Attractor among Enriched Pathways

All human pathways were obtained from the KEGG database and the Network Analyst tool. The pathways involving mTOR proteins were screened as major pathways in insulin resistance-mediated obesity. The candidate pathways were retrieved with at least some mTOR protein overlap between any given pair of pathways. The Pearson correlation coefficient was used to measure gene expression similarity by weight. Then, the nodes and edges in the networks were measured. The following formula [36] was performed to assess the statistical significance of functional interactions, and a heat map was generated.

$$S(e)= f(\text{diff}(x),\text{corr}(x,y),\text{diff}(y))== -2 +\sum_{i=1} \log_e f_0(p_i) \quad \text{eq. 2}$$

Where diff(x) and diff(y) show the quantitative calculation of gene x and gene y differential expressions, corr(xy) shows the association frequency between gene x and gene y depending on the rates of expression, and f demonstrates a generic form of data integration taking into consideration various data sets through a range of statistical resources.

3.8. Disease Ontology Annotation of mTOR Gene

To identify the direct link between the mTOR gene, obesity, and insulin resistance-mediated obesity, the DiseaseGeNet tool was used [37]. Additionally, the role of mTOR in several cancer-related and other metabolic pathways and diseases was also determined.

4. Results and Discussions

The incidence of obesity is growing at an unprecedented rate across the globe. Insulin resistance is a major risk factor for the development of type 2 diabetes caused by the target tissue's inability to respond properly to insulin and contributes to obesity [38]. In the current study, we used bioinformatics methods to explore the molecular mechanisms of obesity. The results showed that 250 DEGs, including 193 genes with known probe annotations and 57 genes without known probe annotation names, were identified after preprocessing. The zinc finger E-box binding homeobox 2 (ZEB2) had the lowest p-value of 0.0264 and had the highest fold changes (F = 31.6), and proteolipid protein 1 (PLP1) had the highest p-value of 0.0894 and the lowest fold changes (F = 11.4), respectively, among all the DEGs. Among the 193 genes, 12 identifiers were duplicated: SYTL2, DNAJB14, ATXN7L3B, PTPRC, CCR2, TMEM185B, VPS13B, SLFN11, DNMT3A, CCR2, ASUN, and SNHG15. 1 identifier was found to be ambiguous: TMSB15B; 17 identifiers were un-annotated: SNHG15, RUNX1-IT1, CCDC149, C19orf48, SNORD68, LOC153684, LINC00997, TCP11L2, SEP02, TARP, SNORA32, LINC00930, SNORA61, SBDSP1, LOC146880, MIR330, SNORA20; and 4 identifiers had non-root annotations: ATXN7L3B, RHBDD2, AMMECR1 ZNF330. So a total of 161 genes were selected as DEGs and further used for analysis. The GO terms for biological processes obtained through GO Term Mapper showed that the cellular nitrogen compound metabolic process contains the maximum number of genes (71 out of 163), showing 43.56% of the DEGs, and the secondary metabolic process involves only one gene (ZEB2, 0.61% of total DEGs). The heat map generated for the DEGs is shown in Figure 1.

The mining of 163 genes was performed to obtain gene clusters and their molecular networks using Gene Clip 2.0. The p-value was set to 0.05 and hits to 15 as the cutoff criteria for GO categories. About 19 genes were identified through Gene Clip 2.0 for network construction, as shown in Table 1. Enriched pathways were obtained from network analysts with p 0.05 for the enriched DEGs. The maximum number of DEGs (35 DEGs) was substantially enriched in processes such as the cGMP-PKG signalling pathway (p = 3.97e-5). Cyclic AMP responsive element binding protein 1 (CREB1), myocyte enhancer factor 2C (MEF2C), solute carrier family 8 member A1 (sodium/calcium exchanger SLC8A1), inhibitor of nuclear factor kappa B kinase subunit beta (IKBKB), MAPK14 (mitogen-activated protein kinase 14), C-C motif chemokine receptor 2 (CCR2), protein tyrosine phosphatase receptor type C (PTPRC), ABL proto-oncogene 1, non-receptor tyrosine kinase (ABL1), and MEF2C (MADS-box transcription enhancer factor 2C) appeared as highly enriched genes among all the pathways. 1. The NOD-like receptor signalling pathway was retrieved as at least enriched with a p = 1.01e-10.

This study focuses on the generation of crosstalk networks to check the presence of the mTOR gene in enriched pathways and confirm it as a major culprit in insulin resistance-mediated obesity [39]. Therefore, its presence in enriched pathways was identified using the KEGG pathways mapper. The mTOR gene was found to be present in 19 pathways among the 120 enriched pathways; detailed information about enriched pathways containing the mTOR gene is shown in Table 2.

However, the mTOR gene is also linked to several genes in other enriched pathways in which it is not directly present. About 31 clustered networks and 7 associated diseases were obtained from the KEGG database for the DEGs obtained through Gene Clip 2.0. (Tables 3 and 4).

The relationship between DEGs and specific functional modules was defined by using PICKLE 2.0 to acquire the DEGs-PPI network. 1564 protein-protein interactions were retrieved; the network was so complex (Figure 2) and difficult to analyze that it was divided into several functional modules. Figure 3 shows the PPI network of highly enriched DEGs, while Figure 4 shows the tissue disease-gene PPI network, respectively.

(Small module networks are given in Supplementary Figures 1-2). A statistical method on the pathway level was used to identify the significant pathways that were altered in obesity associated with insulin resistance as a cause of mTOR gene mutations [40]. The analysis of the significance of crosstalk effects in pathways was based on the pathways obtained through Network Analyst and KEGG. The DisGenet showed the involvement of mTOR in 960 diseases, among which mTOR is responsible for obesity, having a score rate of 0.3 with one SNP, and a score of 0.1 with a single SNP was observed in insulin resistance-associated obesity. The molecular crosstalk between host and nominee pathways has shown several signalling pathways, pathways of cancer, and transcription pathways to be important (Figure 5).

The findings of Figure 5 showed the link between the mTOR gene and insulin resistance-mediated obesity and even revealed cancer's commonality.

Table 1: List of genes identified through Gene Clip 2.0 for network construction.

Gene Names	KEGG Identifiers	Co genes	Total
MAPK14	K19603	7	18496
OGT	K09667	4	1098
BCR	K08878	3	4027
ABL1	K06619	3	6570
PTPRC	K06478	3	9773
CD44	K06256	3	12376
CREB1	K05870	3	7600
IKBKB	K07209	2	1416
HNRNPL	K13159	2	125
SETD1A	K11422	1	296
PSMC3	K03065	1	85
CCR2	K04177	1	2854
BAP1	K08588	1	356
ERC1	K16072	1	64
SLC8A1	K05849	1	1891
DDX17	K13178	1	83
PSMD8	K03031	1	86
ZC3HAV1	K13092	1	65
MEF2C	K04454	1	720

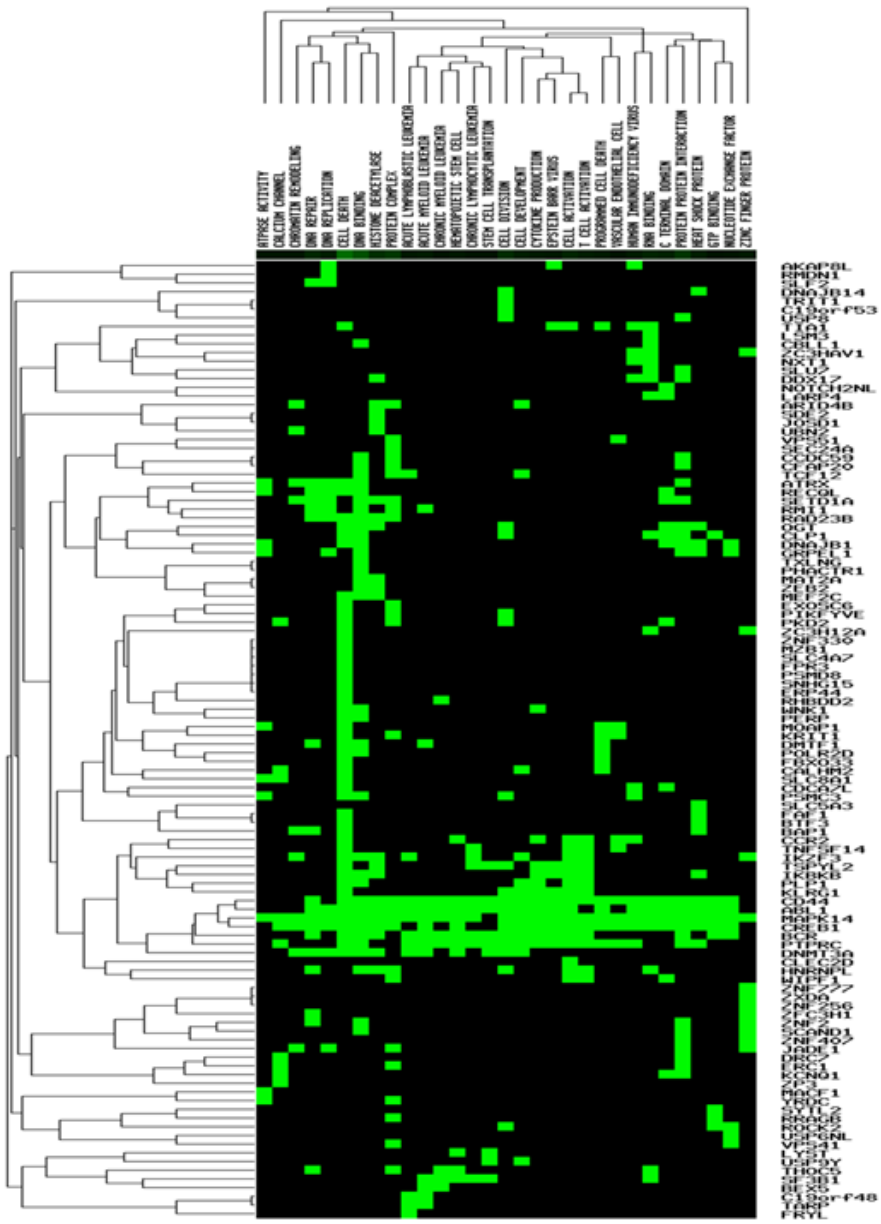

Figure 1: Heat map of DEGs between normal, mildly obese, and moderately obese samples Each row represents the relative levels of expression of a single gene across all samples, and each column represents the levels of expression for a single sample. The black colour represents the corresponding gene-term association positively reported, and the green colour shows the corresponding gene-term association not reported yet.

Table 2: The Enriched pathways obtained through crosstalk networks and statistical analysis involving the role of activated mTOR gene linked with obesity.

Name	Hits	P Value	Adjusted p Value
Pathways in cancer	146/530	5.54E-33	3.52E-31
ErbB signaling pathway	46/85	1.77E-24	5.12E-23
PI3K-Akt signalling pathway	95/354	1.88E-20	3.52E-19
NF-kappa B signalling pathway	44/100	7.28E-19	1.06E-17
Ras signaling pathway	71/232	7.36E-19	1.06E-17
EGFR tyrosine kinase inhibitor resistance	37/79	3.64E-17	3.74E-16
IL-17 signaling pathway	39/93	5.49E-16	4.85E-15
Insulin signalling pathway	45/137	1.39E-13	9.63E-13

Estrogen signaling pathway	43/138	3.73E-12	2.24E-11
Insulin resistance	37/108	5.17E-12	2.93E-11
Transcriptional misregulation in cancer	51/186	8.44E-12	4.71E-11
MicroRNAs in cancer	67/299	9.99E-11	4.97E-10
VEGF signaling pathway	24/59	5.27E-10	2.46E-09
Jak-STAT signalling pathway	41/162	1.30E-08	5.09E-08
p53 signaling pathway	24/72	5.43E-08	2.03E-07
mTOR signaling pathway	37/153	2.36E-07	8.42E-07
Autophagy - animal	32/128	6.99E-07	2.42E-06
Wnt signaling pathway	36/158	1.60E-06	5.07E-06
AMPK signaling pathway	26/120	1.09E-04	2.97E-04

Table 3: The list of clustered networks formed by DEGs

KEGG Identifier	Gene Involved	Pathway Name
06210	ABL1	ERK signalling
06214	ABL1, IKBKB	PI3K signaling
06211	MAPK14	MAPK signaling
06219	ABL1	JAK-STAT signalling
06223	IKBKB	TNF signaling
06240	MEF2C	Transcription
06263	IKBKB, MAPK14, CREB1	Hepatocellular carcinoma
06276	ABL1	Chronic myeloid leukemia
06461	CREB1	Huntington disease
06310	CREB1	CRH-ACTH-cortisol signaling
06360	CREB1	Cushing syndrome
06316	CREB1	Angiotensin-adosterone signaling
06322	CREB1	TRH-TSH-TH signaling
06324	CREB1	GHRH-GH-IGF signalling
06110	MAPK14	MAPK signalling (viruses)
06114	IKBKB	PI3K signaling (viruses)
06121	IKBKB, MAPK14	TLR signalling (viruses and bacteria)
06139	IKBKB	NLR signalling (viruses and bacteria)
06133	IKBKB	RIG-I signaling (viruses)
06123	IKBKB, MAPK14, CREB1	TNF signalling (viruses and bacteria)
06124	IKBKB, MAPK14, CREB1, CCR2	Chemokine signalling (viruses)
06160	IKBKB	Human T-cell leukemia virus 1 (HTLV-1)
06161	IKBKB, MAPK14, CREB1	Human immunodeficiency virus type 1 (HIV-1)
06169	IKBKB	Measles virus (MV)
06170	IKBKB	Influenza A virus (IAV)
06162	IKBKB, MAPK14, CREB1	Hepatitis B virus (HBV)
06163	IKBKB	Hepatitis C virus (HCV)
06168	IKBKB	Herpes simplex virus 1 (HSV-1)
06167	IKBKB, MAPK14, CREB1, CCR2	Human cytomegalovirus (HCMV)
06164	MAPK14, IKBKB	Kaposi sarcoma-associated herpesvirus (KSHV)
06165	IKBKB	Epstein-Barr virus (EBV)

Table 4: The list of diseases associated with DEGs used for cluster networks

KEGG Identifier	Disease	Gene Name
H00001	B-cell acute lymphoblastic leukemia	ABL1
H00004	Chronic myeloid leukemia	ABL1
H01360	Allergic rhinitis	CCR2
H00091	T-B+Severe combined immunodeficiency	PTPRC
H00093	Combined immunodeficiency	IKKB
H01223	Mental retardation-stereotypic movements-epilepsy and/or cerebral malformations	MEF2C
H00773	Autosomal dominant mental retardation	MEF2C

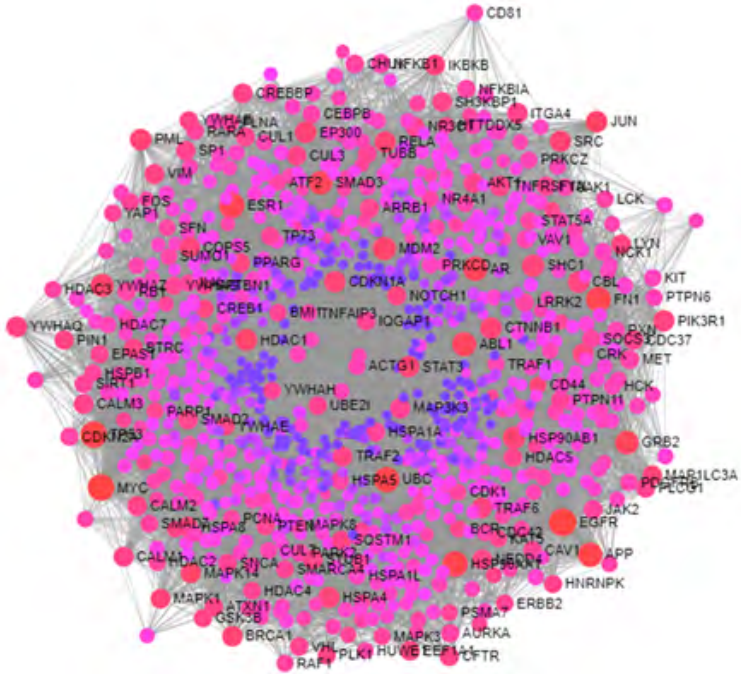

Figure 2: Protein-protein interaction (PPI) network of DEGs The red nodes represent the modules of the PPI network. Purple nodes represent up-regulated DEGs, and pink nodes show down-regulated DEGs outside the module. In the module, there were 1534 nodes and 2713 edges. The density of the module was 0.584.

Figure 3: The interaction network of highly enriched genes obtained through the Gene Clip 2.0 server. The weight of the edges is written in green, and the most significant DEGs are highlighted by purple boundaries.

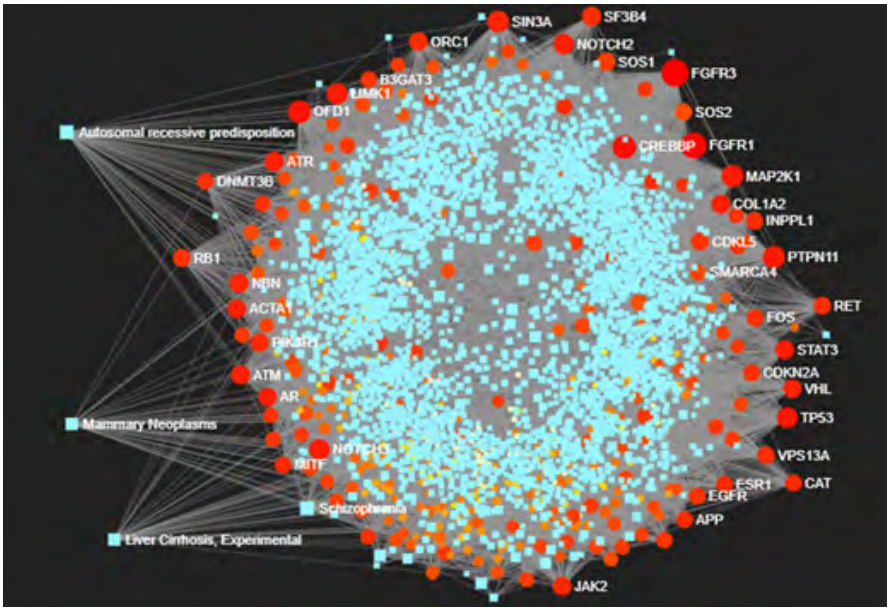

Figure 4: The disease gene interaction network of DEGs. Blue squares represent associated diseases of DEGs, and red nodes show DEGs. DEGs for which no disease association was observed are shown by a light orange colour.

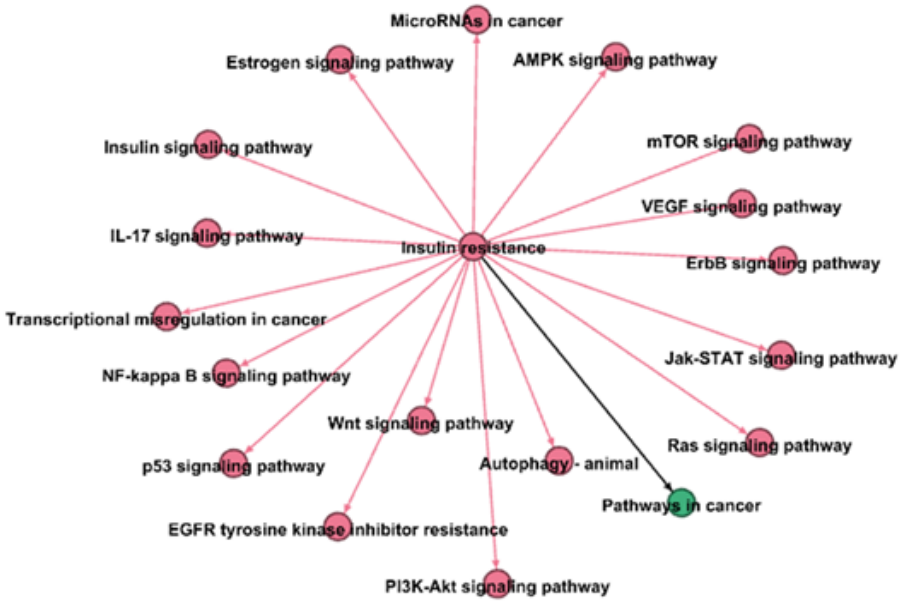

Figure 5: The Crosstalk network generated for pathways consisting of mTOR Gene as a common culprit of Obesity and Insulin resistance.

5. Conclusion

In conclusion, the identified DEGs, especially ABL, CREB1, PTPRC, IKBKB, CCR2, MEF2C, and MAPK14, may be key genes for obesity, and these genes, which are linked with metabolic processes such as biosynthesis and apoptosis, may be useful markers for predicting cancers due to obesity and act as therapeutic targets for the treatment of insulin resistance-mediated obesity in obese patients. Moreover, several signalling pathways, transcription-related pathways, and common pathways of cancer were found to play important roles in the incidence of obesity due to the activation of the mTOR gene in the crosstalk network among obesity-related pathways. Our studies shed new light on the mechanisms and treatment of obesity. However, in the future, as described above, not only genes but also pathways associated with obesity can be evaluated and verified jointly through animal and clinical experiments.

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