A Network Biology Approach for Assessing the Role of Pathologic Adipose Tissues in Insulin Resistance Using Meta-analysis of Microarray Datasets

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**Abstract:** Background: The role of adipose tissue in insulin resistance (IR) and Type 2 Diabetes (T2D) has well been received in the biomedical community; being a precursor of T2D, identification of the molecular basis of IR is therefore, vital to elucidate T2D-pathogenesis and meta-analysis of previously conducted microarray studies provides an inexpensive approach to achieve this end.

Methods: In this study, we have carried out a statistical meta-analysis of 157 microarray datasets from five independent studies and identified a meta-signature of 1,511 genes; their functional meaning was elucidated by integrated pathways-analysis. Further, a protein-protein interaction network was constructed and key genes along with their high confidence transcriptional- and epigenetic-mediators were identified using a network biology approach.

Results: Various inflammation- and immune system-related pathways such as TGF-β signaling, IL7 signaling, Neutrophil degranulation, and Chemokine signaling etc. were enriched in sick adipose tissues; identified transcription factors, and microRNAs were also found to regulate processes relevant to IR/T2D pathophysiology.

Conclusion: This study endorses the development of effective bioinformatics workflow and further grants an indication for the acceptance of adiposopathy as the root mechanistic pathology that poses risk for development of type 2 diabetes; concept of adiposopathy in place of metabolic syndrome will open the possibility to design drugs, those will ameliorate adipose functions and hence proved to be more effective against Type 2 Diabetes.

Keywords: Cytoscape, Insulin resistance, Microarray, Network biology, Type 2 diabetes, Pathologic adipose tissues.

1. INTRODUCTION

Insulin resistance (IR) has been considered as a precursor of Type 2 Diabetes (T2D). Clinically, it is attributed to defective insulin signaling in its responsive tissues such as adipose, liver, and muscle.

Identification of the molecular basis of IR is, therefore, vital to elucidate the mechanism of T2D pathophysiology. Obesity has long been implicated towards the development of insulin resistance with subsequent clinical manifestations of T2D. However, metabolically healthy obese (MHO) people are clinically characterized in population, delineating the fact that it is indeed incorrect fat distribution rather mere excess fat storage that sets the stage for IR and T2D etiology; recent findings unraveling the role of adipose tissue in energy homeostasis in addition to its primary function as energy reserve further corroborate this fact [1, 2].

Clinical association of IR with obesity is attributed to inflammation in adipose tissues due to macrophage infiltration, adipocyte hypertrophy, visceral deposition, and the defective process of adipogenesis. These molecular changes deregulate insulin signaling [3]. In addition to it, pathologic adipose tissue also impairs insulin secretion by releasing various non-glucose secretagogues i.e. non-esterified fatty acids, glycerol, pro-inflammatory cytokines, leptin, and adiponectin hormones. The distribution of body fat also affects the prevalence of IR. People with peripheral obesity (subcutaneous fat) are less prone to develop IR than people with central obesity (visceral fat) as secretary proteins responsible for energy production seem to predominate in visceral fat. Moreover, adipocytes present in subcutaneous and visceral regions also differ with respect to the amount of protein secretion [2].

DNA microarray is an unbiased method of investigating the genome-wide transcriptome profile in a group of cells or tissue. A number of studies have reported its use in assessing adipose biology in T2D/IR conditions like differences in gene expression across various adipose depots, between lean and obese individuals, the effect of insulin/NEFA/adipokines
on cellular physiology, different stages of adipogenesis and brown vs. white adipose tissue gene expression. In addition, it has also been used to unravel the effect of various environmental factors on these pathologies viz. drugs, toxins, diet etc.

Meta-analysis of several microarray datasets is an inexpensive means that accounts for demographic variabilities more elegantly, increases the statistical power, and hence helps in obtaining more exact gene expression differentials. Previously one meta-analysis on microarray datasets has been conducted to assess the systemic cellular pathology in IR/T2D conditions [4].

To interpret the functional meaning of gene-list obtained from microarray studies, it is necessary to analyze it in the context of known biology and network analysis approach provides a framework to achieve this end. It offers the representation of intracellular biological systems as the network of interacting molecular components by treating them as ‘nodes’ and interactions between them as ‘edges’.

In the present study, a statistical meta-analysis of 157 microarray datasets from five independent studies belonging to normal, and IR individuals and specific to adipose tissue was conducted and a meta-signature which comprised of 1,511 genes was identified.

As type 2 diabetes is ascribed to interaction between environmentally triggered weak insulin signaling with genetically programmed pancreatic β-cell dysfunction; our meta-analysis included adipose samples from individuals, those have been subjected to disparate environmental conditions such as treatment with insulin-sensitizing drug, fasting, and hyperinsulinemia-euglycemic clamp in an attempt to obtain a robust meta-signature of insulin-resistant state.

Our analysis has reported 22 cellular pathways implicated by the genes comprising meta-signature. A selected number of most significant proteins (genes) were further identified on the basis of two network metrics; sub-network derived from these proteins was extended to include high confidence transcription factors and microRNAs.

The role of these pathways and regulatory mediators in the overall IR pathophysiology was inferred by the literature search. Our findings underscore that adiposopathy i.e. sick fat is, in fact, a primary cause of the development of IR and T2D.

2. MATERIALS AND METHODS

We have selected five independent microarray studies from NCBI Genomic Expression Omnibus (GEO) database which have been conducted primarily to assess the transcriptional alteration in adipose tissues between normal and insulin resistant individuals (Table 1).

Hardy O.T. et al. [5] generated two microarray series (GSE15773, and GSE20950) - both comprising paired samples from subcutaneous and visceral adipose tissues from BMI-matched (BMI ≥40) normal glucose tolerant, and impaired glucose tolerant individuals. As all participants were morbidly obese, their study design allowed identification of differentially expressed genes solely related to defective insulin signaling and not simply a feature of the obese state.

Soronen J. et al. [6] conducted another microarray study (GSE26637) for measuring gene expression differentials in subcutaneous adipose tissues between lean normal glucose tolerant, and obese impaired glucose tolerant individuals. Samples were extracted both after an overnight fast as well as after hyperinsulinemic-euglycemic clamp.

Sears D.D. et al. [7] conducted a large microarray series (GSE13070) comprising 364 samples from 72 individuals to ascertain the influence of an insulin-sensitizing drug, thiazolidinedione (TZD) on the transcriptional profile of skeletal muscle, and abdominal subcutaneous tissues. Samples were collected before and after hyperinsulinemic-euglycemic clamp, at baseline and after three-month TZD treatment to unravel transcriptional alteration exclusively attributed to impaired insulin signaling and therefore discounting influence of other factors.

Keller P. et al. [8] generated a microarray series (GSE27949) comprising type 2 diabetic, normal glucose tolerant and impaired glucose tolerant individuals to assess the role of selected miRNAs, those have been reported to be involved in the process of adipogenesis.

As all studies utilized Affymetrix-hgu33Plus2 platform, therefore our meta-analysis is expected to minimize chip to chip variation across different studies.

All microarray datasets were independently preprocessed using Bioconductor package germa [9]. A non-specific filtering step was further carried out to select high sensitivity probes using package genefilter [10] and Affymetrix probe ids were collapsed to Entrez ids and official gene symbols.

Meta-analysis of normalized datasets was conducted using a web-based tool NetworkAnalyst [11] which offers various meta-analysis procedures on summary level data (P-values, gene ranks, and effect sizes etc.). Fisher’s method was used on gene-level log-transformed P-values to obtain a meta-signature of genes after adjustment of batch size (P < 0.05).

For pathway and regulatory analysis of obtained meta-signature, a network-based approach was adopted using Cytoscape [12] - a commonly used framework that is extensively used to visualize, and analyze biological data with its various apps.

Meta-genes were first imported to ClueGO app [13] and a functionally organized GO/pathway term network was constructed, considering all the three pathway databases: WikiPathways, KEGG, and Reactome (P < 0.05).

To identify most important regulatory mediators of these meta-genes, a functional interaction network for protein products of these genes was created using stringApp that provides access to STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) database [14]. Two sub-networks those comprised of top 20 genes based on their degree-centrality, and bottleneck-centrality respectively were created using cytohubba app [15] and subsequently merged to obtain a sub-network of proteins (genes). CyTargetLinker app [16] was then used to extend this sub-network with regulatory information (miRNA-targets, and TF-targets).

Highly significant top 10 miRNAs and all transcription factors were selected considering the degree-centrality of each node using NetworkAnalyzer [17].
3. RESULTS

NetworkAnalyst identified 17 and 546 differentially expressed (DE) genes \((P<0.05)\) in GSE26637 (G1) and GSE26637 (G2) respectively, indicating there exist large transcriptional differences between insulin sensitive-lean and insulin resistant-obese phenotypes, whereas other studies did not report any DE genes.

The subsequent analysis reported a meta-signature of 1,511 genes on the basis of combined \(P\)-value and \(t\)-statistics of differential expression across all datasets (Supplementary Table S1).

Protein-protein interaction network of these meta-genes using stringApp connected 1,335 of these genes with 11 additional linker proteins.

We further identified twenty-seven hub proteins in this network by selecting two topological properties - degree-centrality, and bottleneck-centrality; both are highly correlated. However, proteins with high degree-centrality tend to be ‘party hubs’ and connect similar biological processes whereas proteins with high bottleneck-centrality tend to be ‘date hubs’ and connect diverse processes and therefore have a strong tendency to be products of essential genes [18].

The 27 proteins identified were: LRRK2, MMP9, FGF2, PTPRC, MYC, TLR4, CTNNB1, RAC2, JUN, PIK3CG, ACACB, IL8, VEGFA, ITGAM, EHMT1, SYK, IL1B, LYN, MAPK1, FOS, ESR1, PIK3C3, PLEK, ACTA2, OASL, XPO1 and KI.

A Regulatory Interaction threshold of 2 was selected in CyTargetLinker app while extending this sub-network to include high confidence miRNAs and transcription factors that resulted in the identification of 43miRNAs and 3 transcription factors (Table 2; Fig. 1).

An elaborate literature searching for assessing the implication of pathways and regulatory agents have proved fruitful in elucidating some aspects of pathologic adipose tissue induced insulin resistance.

3.1. Pathways Analysis

ClueGO pathway analysis enriched 299 pathways (Supplementary Table S2) represented by highly suggestive 22 term-leading pathways (Table 3).

Enrichment of various immune system related pathways such as ‘B Cell Receptor Signaling Pathway’, ‘Chemokine signaling pathway’, ‘IL1 and megakaryocytes in obesity’, ‘Interleukin-7 signaling’, ‘Neutrophil degranulation’, ‘HTLV-I infection’, ‘Leishmaniasis’, ‘Pertussis’, and ‘Staphylococcus aureus infection’ indicates that adipose tissue is indeed inflamed and that it may likely contributes toward the development and progression of IR-phenotype.

The most significant pathway term representing 146 pathways and also consistent with IR-phenotype was ‘IRS-mediated signaling’. Another pathway term ‘MAPK Signaling Pathway’ has also well studied downstream of insulin receptor that mediates most of the anabolic effects of insulin signaling such as cell proliferation, differentiation, and gene expression. These phenotype suggestive pathways indicated fruitfulness of our bioinformatics approach.

| S. No. | GSE          | Adipose Site     | Normal: IGT                      |
|-------|--------------|------------------|---------------------------------|
| 1     | GSE15773 (G1)| subcutaneous     | 4:5 (obese)                     |
| 2     | GSE15773 (G2)| visceral         | 5:5 (obese)                     |
| 3     | GSE20950 (G1)| subcutaneous     | 10:9 (obese)                    |
| 4     | GSE20950 (G2)| visceral         | 10:10 (obese)                   |
| 5     | GSE26637(G1) | Subcutaneous     | 5:5 (lean: obese)               |
| 6     | GSE26637(G2) | Subcutaneous     | 5:5 (lean: obese)               |
| 7     | GSE13070     | Subcutaneous     | 28:29 (thiazolidinedione-treated with hyperinsulinemic-euglycemic state) |
| 8     | GSE27949     | subcutaneous     | 11:11                           |
|       | Total        |                  | 157                             |

Table 1. Details of microarray sample used in meta-analysis.
‘Neutrophil degranulation’ is considered the hallmark of adipose inflammation as these are one of the first cells recruited to the site of inflammation. Neutrophils release elastase which is a protease that degrades Insulin Receptor Substrate (IRS), and adaptor proteins, leading to attenuation of insulin signaling [19].

Another enriched pathway, ‘Chemokine signaling’ has also been reported to be associated with insulin resistance and obesity. Over nutrition-induced hypertrophic preadipocytes and adipocytes secrete a large amount of a chemokine Monocyte Chemoattractant Proteins (MCP)-1/CCL2 [20]. CCL2 causes the infiltration of monocytes via binding to their C-C motif Chemokine Receptor 2 (CCR2) into obese adipose tissue wherein they differentiate into macrophages and induce the process of inflammation by expressing pro-inflammatory cytokines and thereby lead to insulin resistance state.

‘IL1 and megakaryocytes in obesity’, and ‘Interleukin-7 signaling’ further point towards the link between IR and pro-inflammatory signaling. One study has demonstrated enhanced glucose homeostasis and insulin sensitivity in IL-7 receptor (IL-7R) KO mice [21].

Pro-inflammatory signaling is mediated by IKK/NF-κB kinases. It is interesting to note that insulin signaling also activates these kinases through PI3K/Akt pathway. Therefore, it has been argued that inflammatory signaling actually constitutes a negative feedback loop in insulin signaling that may lead to IR phenotype particularly in conditions such as tissue inflammation [22].

Another pathway ‘TGF-β signaling pathway’ is also central in regulating glucose and energy homeostasis. It inhibits proliferation of pancreatic β-Cell by controlling the expression of p27, a master regulator of cell cycle [23]. It also suppresses IRS-1 phosphorylation, [24] and insulin/insulin-like growth factor-1 mediated adipogenesis process. Experimental inhibition of TGF-β1 signaling has been reported to protect mice from obesity, Insulin resistance, and diabetes, conferring it an attractive target for therapeutic interventions [24].

### 3.2. microRNA Analysis

MicroRNAs are the molecular regulators that fine-tune the expression of protein-encoding genes as per the need of the organism. Many metabolic diseases including type 1 and type 2 diabetes, [25] cancer, and autoimmune disorders have been reported to be associated with changes in microRNA expression.

All the four members of miR-181 a, b, c, and d were present in top 10 miRNAs. miR-181c is reported to regulate Hippo signaling pathway which controls cell proliferation and apoptosis and have been implicated in a wide variety of cancer including pancreatic cancer [26]. In addition to glucose and lipid metabolism shows mitogenic effects through MAPK/ERK pathway and increased insulin production to counteract diminished insulin signaling is a characteristic feature of IR phenotype and may be linked to miR-181c.

miR-181a has been reported to negatively correlate with high levels of IL6, macrophage infiltration, or metabolic conditions such as type 2 diabetes [27] and therefore, might be considered an important therapeutic target. It has been reported that insulin itself down-regulates expression of some miRNAs including miR-181d as its target genes were supposed to involve in insulin signaling [28]. Another miRNA, miR-181b has also been reported to ameliorate insulin-mediated glucose homeostasis by regulating endothelial function in adipose tissue [29].

### Table 2. MicroRNA-targets and TF-targets reported in CyTargetLinker analysis.

| S. No. | Name           | Biological Type       | Degree | Betweenness Centrality |
|-------|----------------|-----------------------|--------|------------------------|
| 1     | hsa-miR-181c   | Micro-RNAs            | 22     | 0.001665               |
| 2     | hsa-miR-181a   | Transcription Factor  | 21     | 0.002106               |
| 3     | hsa-miR-181d   |                       | 21     | 0.002078               |
| 4     | hsa-miR-338-5p |                       | 20     | 0.002038               |
| 5     | hsa-miR-181b   |                       | 20     | 0.001665               |
| 6     | hsa-miR-302a   |                       | 19     | 0.002023               |
| 7     | hsa-miR-302b   |                       | 19     | 0.002023               |
| 8     | hsa-miR-302c   |                       | 19     | 0.002023               |
| 9     | hsa-miR-520a-3p|                       | 19     | 0.002023               |
| 10    | hsa-miR-520b   | Micro-RNAs            | 19     | 0.002023               |
| 1     | Spi-1/PU.1     |                       | 11     | 3.2421E-4              |
| 2     | ALDOA          |                       | 6      | 1.4985E-4              |
| 3     | SRF            |                       | 5      | 0                      |
MicroRNA, miR-338-5p is also involved in adipose biology by regulating the expression of three proteins - Peroxisome proliferator-activated receptor-γ, Peroxisome proliferator-activated receptor-γ coactivator (PGC)-1α, and Myogenic factor5α, those are involved in the browning of white adipose tissue [30].

miR-302a has been reported to be a negative regulator of peroxisome proliferator-activated receptor γ-mediated adipogenesis; [31] miR-302b inhibits insulin-like growth factor signaling [32] and therefore, both have potential to diminish insulin signaling mediated glucose homeostasis.

3.3. Transcription Factor Analyses

Transcription Factors (TF) are the single largest protein family that regulates gene expression. The TF-analyses reported Spi-1/PU.1 with highest degree-centrality in our network. Expression of this TF was reported in mature adipocytes, it induces expression of NADPH oxidase, and pro-inflammatory cytokines (TNFα, IL-1β, and IL-6). NADPH oxidase activates Reactive Oxygen Species (ROS); ROS cause oxidative stress leading to Insulin resistance and Type 2 diabetes [33].

Another TF, SRF has been reported to associate with insulin resistance, and might contribute to T2D pathogenesis; a high level of SRF gene expression in human and mouse skeletal muscle is a signature of insulin resistance. Its pharmacological inhibition has been reported improved glucose uptake and tolerance in insulin-resistant mice in vivo. Thus, it might also be an attractive therapeutic target [34].
Our analysis also reflects the effectiveness of the proposed bioinformatics approach in elucidating the pathophysiology of IR, in particular, and of any medical condition, in general. We have identified a meta-signature of genes across five microarray studies; these proteins enriched highly suggestive pathways. Extension of these proteins with their regulatory players further clued their probable role in overall pathology.

CONCLUSION

The present study reported effective bioinformatics workflows identified molecular processes responsible for the derangement of adipose functions and also identified 27 key genes based on their topological properties in the protein-protein interaction network that was derived from meta-signature. These hub proteins may have the potential to act as IR Biomarkers or drug targets for the development of therapeutics.

Our study reinforces the notion that adiposopathy lies in the root mechanistic pathology that poses risk for development of type 2 diabetes, cardiovascular complications and dyslipidemia; [35] appreciation of this fact will direct scientific efforts towards designing of therapeutics those not only reduce fat mass (adiposity) but will also correct fat dysfunctions. In addition, this adipocentric (fat tissue as a central cause) paradigm of metabolic disease will also underscore the significance of lifestyle modification to reduce excess body fat for curing metabolic disorders.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher’s website along with the published article.

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Table 3. Term-leading pathways from CluGO analysis.

| S. No. | Pathways                              |
|-------|---------------------------------------|
| 1     | B Cell Receptor Signaling Pathway     |
| 2     | Chemokine signaling pathway           |
| 3     | Degradation of the extracellular matrix |
| 4     | Estrogen Receptor Pathway             |
| 5     | Estrogen signaling pathway            |
| 6     | GPVI-mediated activation cascade      |
| 7     | Generic Transcription Pathway         |
| 8     | HTLV-I infection                      |
| 9     | Hemostasis                            |
| 10    | IL1 and megakaryocytes in obesity     |
| 11    | IRS-mediated signalling               |
| 12    | Interleukin-7 signaling               |
| 13    | Leishmaniasis                         |
| 14    | Ligand-dependent caspase activation   |
| 15    | MAPK Signaling Pathway                |
| 16    | Neutrophil degranulation              |
| 17    | Pertussis                             |
| 18    | Platelet activation                   |
| 19    | Proteoglycans in cancer               |
| 20    | Spinal Cord Injury                    |
| 21    | Staphylococcus aureus infection       |
| 22    | TGF-beta Signaling Pathway            |

The concept of adiposopathy is gaining incremental acceptance in scientific community due to the appreciation of endocrine and immune potential of adipose tissue. It is an insulin-responsive organ, at the same time it also secretes fat-derived metabolites, hormones, enzymes, cytokines and other factors that have an effect on insulin activity in other organs and may even affect insulin secretion by the pancreatic β-cells.

Our analysis reported enrichment of microRNAs - regulating lipid metabolism and insulin secretion processes, transcription factors - promoting inflammatory processes, and molecular pathways - attenuating insulin signaling, insulin secretion, adipocyte differentiation, and promoting macrophage-macrophage infiltration in adipose tissue leading to tissue inflammation, thus, endorsing the concept of adiposopathy as root mechanistic cause of IR.
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