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Diabetic Retinopathy and Laser Therapy in Rats: A Protein-Protein Interaction Network Analysis

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Abstract

Introduction: Diabetic retinopathy (DR) is a serious microvascular complication of diabetes which can cause vision loss or blindness ultimately. Non enzymatic glycation of proteins leads to advanced glycation end products (AGEs) in DR. Since laser therapy is a well-established method, in this study, protein-protein interaction (PPI) network is applied for protein targets in DR disease in rats treated by laser.

Methods: In this study, we focused on articles that investigated and compared the proteome profiles of DR rats with healthy control and also DR rats before and after laser therapy. The networks of related differentially expressed proteins were explored using Cytoscape version 3.3.0, the PPI analysis methods and ClueGO.

Results: Analysis of PPI network of 37 related proteins to DR rats including 108 nodes, introduced 10 hub-bottleneck proteins and 5 concerned biochemical pathways. On the other hand, PPI analysis of related proteins to DR rats before and after laser therapy corresponded to 33 proteins and 2 biological pathways.

Discussion: Centrality and cluster screening identified hub-bottelneck genes, including Aldoa, HSPD1, Pgam2, Mapk3, SLC2A4, Ctnnb1, Ywhab, HSPA8, GAPDH and Actb for DR rats versus healthy control and ENO1, Aldoa, GAPDH for DR samples after laser therapy.

Conclusion: Gene expression analysis of the DR samples treated via laser therapy provides a molecular evidence in support of the therapeutic effect of laser.

Keywords: Diabetic retinopathy rat; Laser therapy; Protein-protein interaction network (PPI) analysis.

Introduction

Diabetic retinopathy (DR) is a serious microvascular complication of diabetes which leads to vision loss or blindness ultimately. In fact, it is one of the most frequent causes of visual impairment in the world.[1] Signs of DR are retinal vascular permeability and retinal ischemia. Since progression of DR are accompanied with biochemical, cellular and histopathological changes that are not detectable by the clinician, molecular investigation is an important requirement.[2] It is reported that in DR genetic, environmental, and immunological factors act together.[3,4] The main process affected in patients is hyperglycemia. Chronic high levels of blood glucose in diabetic patients effect destructively on retina because retina cells are sensitive to overflow of glucose. One of the complications of DR is vascular permeability changes which plays an important role in the development of visual impairment in diabetes. Non enzymatic glycation of proteins leads to advanced glycation end products (AGEs). AGEs are able to produce cross-links between proteins.[5,6] In vitro and in vivo investigation have proven the relationship between AGEs concentration and DR signs.[7] Diabetic condition induces some alterations in the expression of a diverse range of retinal proteins, so study of biochemical pathways may provide valuable insights into the pathogenesis of DR.[8] Protein complexes are known as essential components in cellular processes and analysis of the proteins in these complexes is a state of art approach.[9] Protein- protein interaction network analysis in pathophysiological conditions attracted significant attention from researchers, in order to discover an integrated biological output.[10] Human molecular

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interaction networks are not just basic science exercise for a more clear view of fundamental human biology but will transform molecular diagnosis, treatment and patients monitoring tools.[11-13] Protein networks analysis provides a scientific model that can improve understanding of the mechanisms underlying human diseases.[14-19] Pathogenesis of DR is complex, and despite a lot of research, it has not been completely elucidated. Published evidence supported clinical use of lasers for the proliferative DR macular edema.[20] The importance of early treatment of DR depends on the level of knowledge in molecular changes and cellular processes.[21,22] Identification and comparison of the protein-protein interaction (PPI) changes and involved pathways in the diabetic retina before and after laser will provide clues to the underlying therapeutic mechanism(s) involved in laser treatment as well as other diseases.[17,18,23] Laser therapy is used as an anti-VEGF treatment of DR patients but this therapeutic method may cause retinal damage and scarring.[24] Discovery of molecular interventions and understanding disease processes can be helpful in introducing less invasive therapeutic options. In this study, we want to analyze PPI network to identify critical proteins and signaling pathways related to diabetic nephropathy and also in response to laser therapy. It is aimed to reveal common molecular changes in DR pathogenesis and following laser therapy.

Methods

Selection of Differentially Expressed Genes

The PubMed database and Google Scholar were cross-searched (on June 2015) for the terms "diabetic retinopathy" ,"proteomic", "laser", and "treatment" (i.e., title, abstract, keyword). A total of 25 research papers were collected which surveyed tissues, serum, plasma, saliva and urine. Among them, studies with focus on same organism and same cell-position before and after treatment were selected. Finally, a study that had surveyed proteome map of normal rat retina in comparison with the proteome of diabetic rat retina was selected[25] (Table 1) and another one focusing on protein changes in rats after laser treatment[26] (Table 2).

Table 1. Thirty-Seven Changed Expression Proteins in Retina of Rats With Diabetic Retinopathy in Comparison With Healthy Control Rats

Protein Name	Gene Name
Enolase 2 gamma	ENO2
Heat shock protein 70 1A	HSPA1A
Heat shock protein 8	HSPA8
Aldehyde reductase 1	AKR1B1
Triose phosphate isomerase	TPI1
Phosducin	PDC
14-3-3 protein	Ywhab
Crystallin B1	CRYBB1
Beta catenin complex B	Ctnnb1
Creatine kinase B	CKB
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH
Enolase1	ENO1
Beta actin	Actb
Malate dehydrogenase	Mdh
Dimethylarginine dimethlaminohydrolase	Ddah1
Calreticulin	Calr
Proteasome alpha 6 subunit	Psma6
Phosphoglycerate mutase	Pgam
Aminoacyclase 1	Acy1a
Succinyl CoA ligase ADP forming subunit	Suclg1
Dystrophin-related protein 2	DRP 2
Inositol 1 monophosphate	Impa1
Triosephosphate isomerase	Tpi1
Chaperonin subunit 2	Hspd1
ATP synthase B subunit	Atp5b
Tubulin beta	TUBB3
Histone 2B	Hist1h2ba
ATP-ase, vesicle fusing	Nsf
Pyridoxine kinase	Pdxk
Calbindin	Calb1
Pyruvate dehydrogenase	Pdk2
Crystatin B	Cstb
Profilin 2	Pfn2
Glutamate ammonia ligase	Glul
Fructose bisphosphate aldolase A	Aldoa
Superoxide dismutase	Sod1
Isovaleryl coenzyme A dehydrogenase	Ivd

Construction and Topological Analysis of PPI Networks

PPI networks were generated using Cytoscape version 3.3.0.[27] The advantage of Cytoscape is that users can analyze and visualize networks of genes and compounds, and identify enriched pathways from expression profiling data. Two centrality parameters such as degree and betweenness were considered to determine the hub and bottleneck proteins. First, we prepared the list of proteins which were hub in PPI network of DR versus controls in the PPI network. The nodes with high degree defined as hub proteins and the nodes with high betweenness defined as bottleneck proteins, which both play pivotal roles in the network.[28] The results indicate that some hub nodes play a role as bottleneck, these proteins were introduced as hub-bottleneck. ClueGO was used for data analysis. The degree of functional enrichment for a given cluster was quantitatively assessed (statistical significance was calculated) using the ClueGO tool.[29] ClueGO integrates gene ontology (GO) terms and creates a functionally organized GO/pathway term network. It can analyze genes and comprehensively visualize functionally grouped terms.[29] A pack of gene annotations (e.g. functions, processes) can help identify interesting features, but due to the huge size of the sets, selecting statistically significant trends from large datasets is impractical. Thus, a method is required for the routine analysis of such datasets. We used GO as a common vocabulary for annotation as it

Table 2. A Number of Changed Expression Proteins in Retina of Rats With Diabetic Retinopathy Before and After Laser Therapy

Protein Name	Gene Name
Alpha-A crystalline	Cryaa
Recoverin	Rcvrn
Isovaleryl coenzyme A dehydrogenase	Ivd
Claudin-12	Cldn12
Enolase 1 alpha	Eno1
Germinal histone H4	Hist1h4b
Glyceraldehyde 3 phosphate dehydrogenase	Gapdh
Aldolase A	Aldoa
Wnt-5 beta	Wnt5a
LEK-1	Cenpf
Dismutase	Sod
Calretinin	Calb2

allows identification of semantically related genes and gene products.[30] Kappa statistic ≥0.4, enrichment and Bonferroni step down method were used for probability value correction.[31]

Laser Parameters

The laser (20 spots of 100-mW power, 200-µm diameter, 0.1 ms) which has been used in rat DR studies was Argon green with a wavelength of 514 nm slit-lamp laser (HGM Medical Laser Systems Inc., USA). The one eye of the rat DR samples was treated by laser under anaesthesia condition.[26]

Results

Topological Analysis of PPI Networks for Diabetic Retinopathy vs. Normal Rats

Cytoscape analysis revealed a great number of close interconnections (see Figure 1). The PPI network properties are tabulated in Table 3. The top 10% proteins in terms of degree and betweenness (2) were defined as

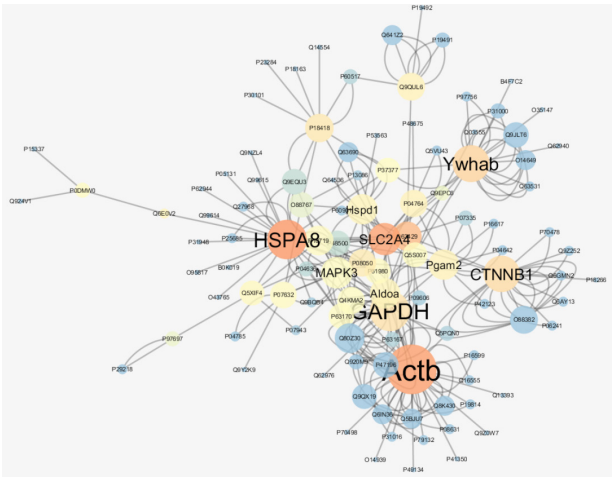

Figure 1. PPI network which consists of 108 nodes and 56 multi-edge node pairs for diabetic retinopathy versus normal rats. The larger circles correspond to the higher degree and blue to brown color refers to increment of betweenness value

Table 3. Properties of protein-protein interaction Network of Diabetic Retinopathy Versus Normal Rats

Topological Parameters	Values
Number of nodes	108
Multi-edge node pairs	56
Average degree	4.981
Average betweenness	0.023
Average closeness centrality	0.309
Network density	0.029

Table 4. The Hub-Bottleneck Nodes in the Protein Network of Diabetic Retinopathy Versus Normal Rats

Gene Name	Degree	Betweenness	Uniprot Code
Aldoa	14	0.055213	P05065
HSPD1	14	0.055388	P63039
Pgam2	14	0.060204	P16290
Mapk3	16	0.030572	P21708
SLC2A4	17	0.297383	P19357
Ctnnb1	24	0.133282	Q9WU82
Ywhab	25	0.153595	P35213
HSPA8	29	0.31212	P63018
GAPDH	30	0.11042	P04797
Actb	46	0.30541	P60711

hubs and bottlenecks respectively. These key proteins are tabulated in Table 4. As it is depicted in Table 4 all hub proteins play a role as bottlenecks too; so we introduced them as hub-bottleneck proteins. The GO pathway clusters enriched by crucial proteins in DR rats versus healthy controls are shown in Table 5.

PPI Network Analysis for Diabetic Retinopathy Rats Before and After Laser Therapy

The results of network analysis in subpart 3.1 and the results of PPI network analysis related to DR rats before and after laser therapy are shown in Figure 2 and Tables 6-8.

Discussion

As mentioned in Methods, 37 differentially expressed proteins are associated with DR rats (disease state). However, 12 proteins differentiate the treated samples from DR rats (treated state). The information is tabulated in Tables 1 and 2. Furthermore, networks corresponding to disease and treated states are analyzed and presented in Figures 1 and 2. As illustrated in these figures, disease state network consists of 108 nodes while treated state network includes 33 nodes. Both networks are about three folds wider than the initial proteins. In addition, about half of the nodes of the 2 networks are multi-edges. At the first glance, as presented in Tables 3 and 6, on average the 2 networks are 40% different. However, based on average betweenness, the networks are 5 folds dissimilar. Other

Table 5. The Concerned Pathways of Differentially Expressed Proteins in Diabetic Retinopathy Rats Versus Normal Group ($P<0.05$)

R	GO ID	GO Term	No.	Associated Gene Found
1	GO:0010226	Response to lithium ion	3	Calr, impa1, ctnnb1
2	GO:0046686	Response to cadmium ion	3	Sod1, hspa8, ctnnb1
3	GO:0050821	Protein stabilization	4	Calr,pfn2, hspa1b, gapdh
4	GO:0009408	Response to heat	4	Hsp1, hspa8, calr, hspa1b
5	GO:0072524	Pyridine-containing compound metabolic process	13	Impa1, hspa1b, akr1b1, atp5b, pdk2, gapdh, eno2, aldoa, pgam2, tpi1, eno1, mdh2, qdxk

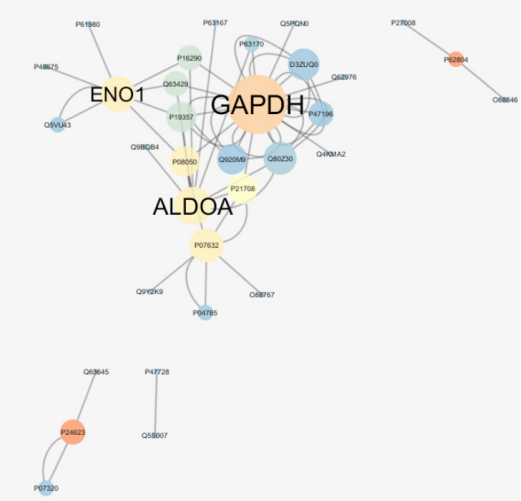

Figure 2. PPI Network With 33 Nodes and 14 Multi-Edge Node Pairs in Diabetic Retinopathy Rats Before and After Laser Therapy. The larger circles correspond to the higher degree and blue to brown color refers to increment of betweenness value.

Table 6. Properties of PPI Network of Diabetic Retinopathy Rats Before and After Laser Therapy

Topological Parameters	Values
Number of nodes	33
Multi-edge node pairs	14
Average degree	3.43
Average betweenness	0.104
Average closeness centrality	0.497
Network density	0.074

Table 7. The Key Nodes (Hub-Bottleneck Proteins) of PPI Network of Diabetic Retinopathy Rats Before and After Laser Therapy

Gene Name	Degree	Betweenness Centrally	Uniprot Code
ENO1	9	0.266403	P04764
ALDOA	14	0.264471	P05065
GAPDH	30	0.489767	P04797

Table 8. Pathways of Differentially Expressed Proteins in Diabetic Retinopathy Rat Before and After Laser.

R	GO Term	No.	Associate Genes Found
1	Columnar/cuboidal epithelial cell development	2	Wnt5a, sod1
2	Pyruvate metabolic process	3	Gapdh, Endo1, Aldoa

topological parameters of these networks, such as average closeness centrality and average shortest path length were calculated and shown in Tables 3 and 6. Graph centrality properties include essential parameters like degree, betweenness and closeness centrality in a PPI network.[32] One of the important properties of a network is the degree centrality that is related to the node connectivity (the number of connections of a node). A node with high degree value is known as a hub node. Another important component in network analysis is betweenness, which is one of the most important topological properties of a network. Bottlenecks that are key connector proteins have been introduced as proteins with a high betweenness centrality (nodes that many "shortest paths" pass through them).[28] In system biology, there are some researches that have focused on hubs while others have argued bottlenecks are more crucial proteins.[28] Therefore, we further focused on both features of high degree and high betweenness to obtain a better predictor of protein essentiality in both regulatory and interaction networks. The topologically significant nodes for both networks were extracted as hubs and bottlenecks as the proteins that were in the top 10% in both terms of degree and betweenness centrality. The determined hubs and bottlenecks for the two states are shown in Tables 4 and 7. There are 10 and 3 hub and bottleneck proteins for RD and treated states respectively. As shown in Tables 4 and 7 the hub proteins are also bottlenecks. The introduced hub-bottleneck proteins for disease state are Aldoa, HSPD1, Pgam2, Mapk3, SLC2A4, Ctnnb1, Ywhab, HSPA8, GAPDH and Actb. ALDOA is a key enzyme in glycolysis and acts in regulation of cell proliferation and actin cytoskeleton organization.[33,34] It is reported that the anti-aldolase autoantibody serves as a useful marker for DR diagnosis.[35] *HSPD1* or *HSP60* plays a key role in preventing apoptosis as well as immune response.[36] Expression of *HSPD1* increases in dry eye.[37] Differential gene expression of *HSPD1* in retinoblastoma compared to normal retina is reported.[38] In addition, age-related changes in expression of heat-shock proteins, including *HSPD1* is reported in rats.[39] PGAM is a glycolytic enzyme that catalyzes the reversible conversion of 3-PGA to 2-PGA.[40] This enzyme is up regulated in many human cancers.[41] However, there was no obvious evidence of its relation with retinopathy until now. Mapk3 acts in a signaling cascade that regulates various cellular processes such as proliferation and cell cycle.[42] Alteration

in activation of Mapk3 influences cancer development.[43,44] This enzyme is involved in the release of VEGF in diabetic rat retina; therefore, it may be a potential therapeutic target of DR.[45] Ctnnb1 encodes β-catenin and regulates the coordination of cell–cell adhesion and gene transcription[46] as well as acting as an intracellular signal transducer in the Wnt signaling pathway.[47] Its abnormal expression leads to various diseases including cancer.[48] WNT/β-catenin signaling also is introduced as a new link between diabetes and cancer.[49,50] A role for β-catenin in glucose and energy homeostasis is reported.[51] Inhibition of connective tissue growth factor overexpression in DR via blocking the WNT/beta-catenin pathway confirms the association of beta-catenin with DR.[52] Ywhab encodes a protein that is involved in signal transduction and plays a role in cell cycle.[53] This protein as a member of the 14-3-3 protein family plays a key role in cellular proliferation and development of breast cancer.[54] The report of 14-3-3 proteins relationship with DR is limited to a study that shows expression change in early diabetic rat retinal proteomes versus normal.[25] Chaperones like HSPA8 increase cell survival[55] and play a key role in the maintenance of epithelial cell structure and function and are also responsible for cell repair processes after damage, proliferation, apoptosis and modulate cell signaling.[56]. HSPA8 protective role was further highlighted in a study that identified HSPA8 alongside other HSP70 proteins suppressed aging brains.[57] Actin, cytoplasmic 1 with highest score among the hub-bottleneck proteins has a conserved gene in eukaryotes, which play an essential role in retina development.[58] Glyceraldehyde-3-phosphate dehydrogenase (GADPH) is a critical enzyme in glucose-induced apoptosis of retinal Muller cells.[59] Moreover, the role of GADPH in the development and progression of DR was investigated by Kanwar and Kowluru.[60] Regulation of actin-based motility by Rho was introduced as an important pathway according to analysis of the DR saliva.[61] ENO1 is a hub-bottleneck in treated network. Its overexpression has been associated with multiple tumors.[62-64] In many of these tumors, ENO1 promoted tumorgenesis via activating and regulating the PI3K/AKT signaling pathway.[63] It is shown that the knockdown of ENO1 expression led to suppressed cell growth and migration by inactivating the PI3K/Akt pathway in glioma cells.[63] Tear proteomic analysis of patients with type 2 diabetes with dry eye syndrome shows alteration in expression of ENO1[65] as well as results on DR in rat.[25] Another hub-bottleneck after laser therapy is ALDOA, which can be suggested as a common hub-bottleneck for DR progression and laser therapy. It is introduced as a potential metastasis-associated marker of lung squamous cell carcinoma.[66] Aldolases A and C are mainly involved in glycolysis[67] and glycolysis itself has been reported to be increased in DR.[68] Some data demonstrate that the anti-aldolase autoantibody can serve as a useful marker for DR diagnosis because of high level in serum[35]; interestingly, following laser treatment the ALDOA expression decreased in DR.[26] The alteration of glycolysis in laser therapy of diabetic retina may reflect metabolic improvement caused by laser treatment. GAPDH is another common hub-bottleneck protein for the 2 networks, which has highest centrality score. GAPDH is the key glycolytic enzyme. Increase in glycolysis, specially overexpression of GAPDH is considered as an essence of many types of cancer.[69] Non-glycolytic role of GAPDH in cancer by interacting with telomerase RNA component is reported.[70] Diacylglycerol (DAG) is a key activator of protein kinase C, it increases glycolysis and endothelial cell proliferation as well as endothelial permeability.[71,72] Down regulation of aldolase A and GAPDH after laser treatment[26] can control the hyper activity of glycolysis which occurs in DR patients.[68] Agents that regulate GAPDH can be potential candidates in inhibiting the progression of DR.[73] In this study, we introduced 3 hub-bottleneck proteins that are involved in laser treated DR rats. Two of these key proteins (66%) are in common with the central proteins that differentiate DR rats from the healthy groups; so, it can be concluded that the treated are approximately normalized towards the healthy rats.

The GO pathway clusters enriched by crucial proteins in DR rats versus healthy controls (see Table 5) provide a series of candidates for mechanism research, such as response to lithium ion, response to cadmium ion, protein stabilization, and response to heat and pyridine-containing compound. Their role is plausible as most of the enriched pathways including protein stabilization, and response to heat pathways were previously shown to be associated with DR.[74-76] The related GO pathway clusters after laser therapy are determined as columnar/cuboidal epithelial cell development and pyruvate metabolic process (see Table 8). DR involves an abnormal pathology of major retinal cells, including retinal pigment epithelium, inter-retinal edema, microaneurysms.[77] So involving the pathway in epithelial development such as columnar/cuboidal epithelial cell development is not unexpected. This process is involved in most of the mentioned characteristic features of RD and the introduced crucial proteins are attributed in regulation of this process. Pyruvate metabolic process is another detected pathway. Pyruvate is the end-product of glycolysis[78] and its dysmetabolism contributes to failure of the pancreatic islet β-cells during late type 2 diabetes.[79] Its up regulation can lead to excessive fatty acid oxidation and ROS formation in the mitochondria.[80]

Conclusion

Aldoa, HSPD1, Pgam2, Mapk3, SLC2A4, Ctnnb1, Ywhab, HSPA8, GAPDH and Actb are the 10 key proteins in PPI network of DR rats. However ENO1, ALDOA and GAPDH are the 3 central proteins related to treatment of DR rats by laser. This provides molecular evidence in support of therapeutic effect of laser.

Conflict of Interests

None.

Ethical Considerations

Not applicable.

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