Title: The Network Analysis of Peripheral Diabetic Neuropathy Involved Genes

Authors: Vahid Mansouri[1], Mostafa RezaeiTavirani[2,*], Farshad Okhovatian[3]

1. *Proteomics research center, Faculty of rehabilitation sciences, Shahid Beheshti University of Medical Sciences, Tehran, Iran.*
2. *Proteomics research center, Shahid Beheshti University of Medical Sciences, Tehran, Iran.*
3. *Faculty of rehabilitation sciences, Physiotherapy research center, Shahid Beheshti University of Medical Sciences, Tehran, Iran.*

***Corresponding Author**: Email: tavirany@yahoo.com

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Abstract:

Aim: Screening of candidate genes related to sural nerve diabetic neuropathy to find the critical ones is the aim of this study.

Back Ground: Diabetes mellitus is a chronic disease causes by insulin uptake or deficiency. Side effects of diabetes are numerous according to severity of disease. Diabetes could harm the peripheral nerves with chronic pain, lead to nerve damage entitled diabetic neuropathy (DN). Signs and symptoms of DN are sharp pains, numbness, and tangling. Many patterns of nerve injuries could happen during DN but distal symmetric polyneuropathy (DSP) is most common. On the other hand, network analysis is a useful tool to assess incidences and progression of diseases.

Methods: Expression of different genes in diabetic patients with and without progressive neuropathy of surreal nerve (GSE24290) is considered as including data. GEO2R was applied to first step analysis to find the significant differentially expressed genes (DEGs). The queried significant DEGs plus 100 first neighbors were included in a network by Cytoscape software. The network was analyzed by Network analyzed application of Cytoscape and the central nodes were determined.

Results: The total 26 significant DEGs plus 100 first neighbors were interacted to form the network. INS, ALB, AKT1, APP, SNAP25, NEFL, GFAP, IL6, NEFM, TNF, MAPT, GAP43, and MBP were identified as 13 hubs of the network. NEFL and NEFM were highlighted as the queried hub genes. Insulin as the top hub node was determined among all interacted genes (the queried and added genes).

Conclusions: INS**,** NEFL, and NEFM are key genes in DN which are involve in metabolism regulation and intra cellular transportation into axons and denderites respectively.

Key words: Neuropathic diabetes, Network analysis, Metabolism, Intracellular transport.

Introduction

Deregulated metabolic pathways and genetic predispositions could cause diabetes mellitus (1), a disease that lots of people in the world are living with it. Diabetic neuropathy is one of the most common complications of diabetes, seen in 50% of patients (2). This disorder is a long-term complication in type I diabetic patients and, conversely, in type II diabetic individuals (2). Initial classification of diabetic neuropathy includes sensory-motor neuropathy and autonomic neuropathy (3). DN is characterized by progressive loss of peripheral axons, decreasing sensation, and pain feeling (4). The detection of DN is accompany with irreversible damages caused by disease (5). Patients may show only one or all three types of neuropathy. Distal symmetrical neuropathy is the most common kind of neuropathies (6). DN can manifest as motor impairment (7), latent cardiac ischemia (8), situational hypotension (9), vasomotor impairment (10), increased sweating, bladder dysfunction (11), and sexual dysfunction (12). Careful monitoring of blood sugar and daily food care are key to preventing DN (13). The variety of complications from diabetes reflect the wide range of damages.

Lack of knowledge about the cause of post-diabetes complications such as DN has increased our distance from understanding the mechanism of the disease (14).

Recently, researchers try to identify and study the biomarkers which are secreted in the body fluids of diabetic individuals as the other diseases to improve management of the patients. Theyuse proteomic techniques as a powerful tool with high accuracy in this regard (15).Although western blot and immunohistochemistry were used for protein quantification in pathological and normal tissues, however their inherent ability to process some proteins at a time is a part of technical limitations (16). Proteomics techniques could solve this problem by analyzing thousands of proteins quantification simultaneously. In addition proteomics could provide information about protein structure and protein-protein interaction (17). Proteomics with the assistance of system biology could explore the network interaction between proteins to understand pathophysiology of diseases (18). Most of the proteomic studies for protein quantification about diabetic neuropathy were done using 2D-PAGE (19). It is possible to separate thousands of proteins with 2D-PAGE technique but limitation to abundant and soluble proteins is inevitable (20).

It should be noted that most of the proteins in the nervous system are hydrophobic and membrane receptors (21) and mass spectrometry based proteomics could rapidly identify both heavy or low

abundant hydrophilic and hydrophobic proteins. Treatment of DN is still a challenging task. The molecular mechanism for DN is remained unclear and there are many different hypothesis about origin of the disease (22). Genes and protein data banks such as GEO are useful sources to find key genes and proteins related to diabetic neuropathy (23). Powerful network analysis softwares such as Cytoscape and STRING are useful complement in this regard (24, 25). Numerous studies have been performed on neuropathic pain in animal models, and due to its diverse origins, changes in proteins levels, depend on pain model and time of its development. However there are several proteomic overlapping models which are defined in relation to protein expression changes and their role in neuropathic pain (26). Fundamentally categorized proteins for neuropathic pain, arranged based on their physiologic functions, as proteins related to homeostasis, neuronal functions, chaperons and heat shock proteins, proteins related to neurodegeneration and apoptosis, immune system related and signaling proteins, and neurodegenerative and regenerative proteins(26). Inhibition of tumor necrosis factor (TNF) pathway may cause DN progression in animal models (27).

Another study revealed that changes in ion channel functions and energy metabolism that are related to axon-glia interaction may cause DN development (23). Chen et al reported that "Nucleotid binding oligomerization domain like receptor 3" (NLRP3) activation mediated by signaling of ATP-P2X4 may cause inflammation related to DN (28). In vivo researches suggested that knocking down of micro-RNA; miR-29c and miR-27a, could reduce DN progression (29). Scientists introduced miR-21 and miR-29 as biomarkers of DN progression (30). Demyelination is an early pathological aspect of peripheral diabetic neuropathy (PDN) with precedes axon degeneration (31). Molecular biology of demyelination could assist to find biomarkers of PDN. Systematic research has been investigated the relationship between the molecules which are involved in the PDN. In a study genes expressions in different diabetic neuropathy patients compared and concluded that the intervention genes which are involved in the DN were associated with lipid metabolism and inflammatory reactions (32). A study based on network analysis using weighted genes databases related to DN, introduced genetic differences between progressive and non-progressive types of DN (33). Annotation methods as gene ontology or mapping genes and proteins are helpful instruments for understanding and gaining a better view of biological features of the interest sets of proteins in DN (29).

The identification of interventional biomarkers in the development of diabetes and subsequently diabetic neuropathy can help us to identify the contradictory and diverse factors of diabetes-related diseases to assist treating them. In this study, we use existing data to try to find the key genes involved in the development of diabetic neuropathy of the sural nerve via network analysis.

Methods:

The gene expression dataset GSE24290 downloaded from GEO (http://www.ncbi.nlm.nih.gov/geo) as a secondary study which included 35 samples from patients with progressive and none progressive sural nerve diabetic neuropathy (SDN). The GSM597469-85 as non-progressive sural nerve and GSM597451-68 as progressive sural nerve samples were selected to analyze. The samples were statistically matched via GEO2R software. Number of 250 top score genes (based on p-value) selected to calculate difference between progressive and non-progressive SDN groups via GEO2R. The 26 significant DEGs among the 250 DEGs were identified based on P-value <0.05 and FC>1.5 as cutoff criteria. The network was constructed by the 26 DEGs plus 100 first neighbors from STRING database by Cytoscape software 3.7.2. Network analyzer application of Cytoscape was used to analyze the network. Central parameters such as degree, betweenness centrality, closeness, and stress were determined for the elements of network. 10 percent of top nodes based on degree value were selected as hub nodes. The 10 first neighbors of the queried potent hub node were identified from STRING database.

Results:

As it shown in fig 1, 17 control samples (non-progressive sural nerve neuropathy) gene expression profiles matched with 18 progressive sural nerve neuropathy samples. There were aligned midpoints demonstrated that the samples were comparable.

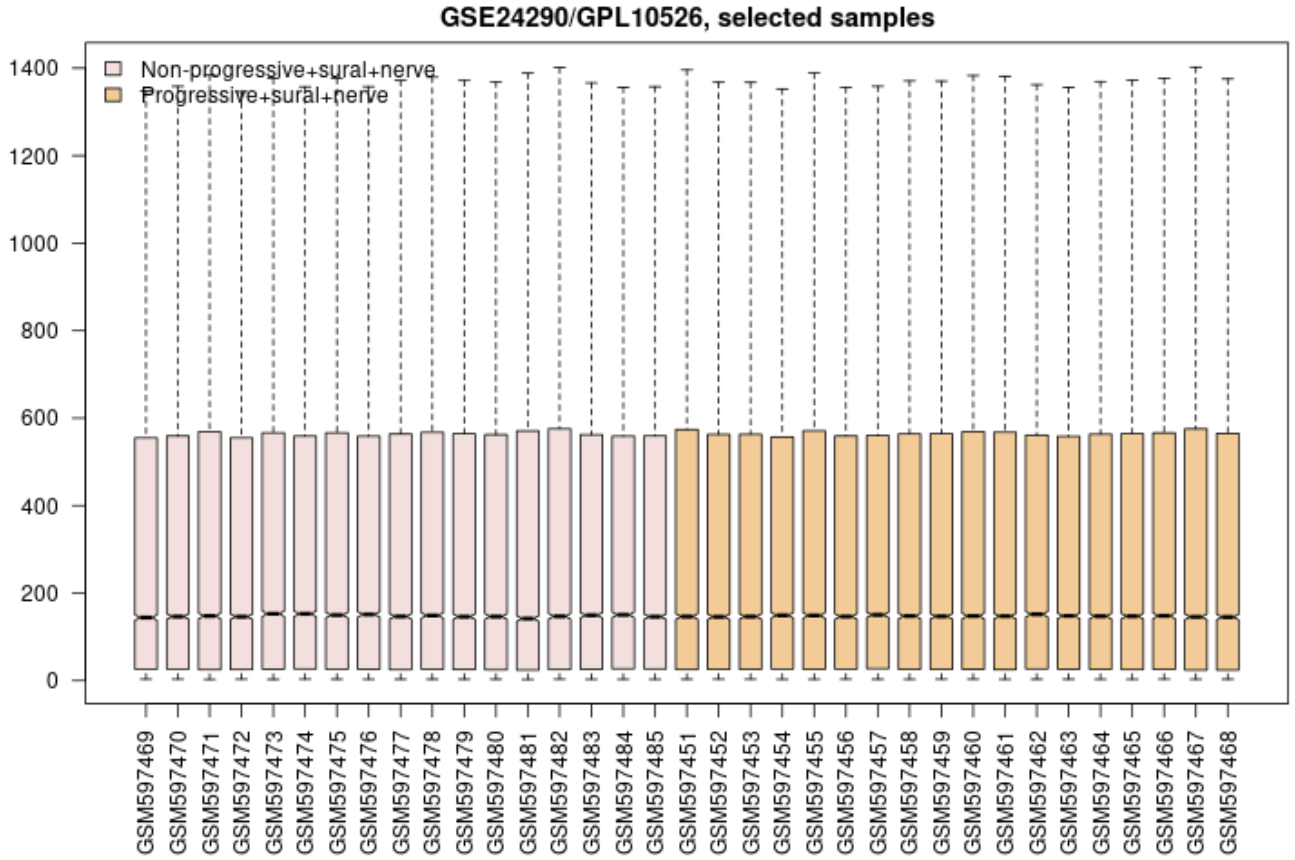

Fig 1: The number of 17 RNA profiles of non-progressive sural nerve diabetic neuropathy (pink color)and 18 progressive sural nerve diabetic neuropathy(orange color) were matched via boxplot illustrations. Vertical axis revealed normalized gene expression amounts.

Among 250 top score genes, 26 genes (FC≥1.5 and p-value <0.05)were identified as significant DEGs (see Table 1).

Table 1: 26 significant DEGs including 16 up-regulated and 10 down-regulated ones are presented.

Row	**Gene Symbol**	**Gene.title**	**logFC**	P.Value
1	GJB6	gap junction protein beta 6	1.40	0.005
2	CCL26	C-C motif chemokine ligand 26	1.24	0.002
3	HAMP	hepcidin antimicrobial peptide	1.01	0.009
4	SCGB1D2	secretoglobin family 1D member 2	0.84	0.008
5	NEFM	neurofilament, medium polypeptide	0.82	0.010
6	RNF128	ring finger protein 128, E3 ubiquitin protein ligase	0.81	0.001
7	GSTM5	glutathione S-transferase mu 5	0.81	0.001
8	VSNL1	visinin like 1	0.77	0.000
9	UPK1B	uroplakin 1B	0.75	0.001
10	CRYBA2	crystallin beta A2	0.72	0.014
11	NEFL	neurofilament, light polypeptide	0.71	0.009
12	OLIG1	oligodendrocyte transcription factor 1	0.70	0.004
13	RARRES1	retinoic acid receptor responder 1	0.66	0.004
14	OLFM4	olfactomedin 4	0.66	0.006
15	OLR1	oxidized low density lipoprotein receptor 1	0.64	0.001
16	KERA	keratocan	0.61	0.002
17	MTTP	microsomal triglyceride transfer protein	-0.61	0.012
18	GABRE	gamma-aminobutyric acid type A receptor epsilon subunit	-0.64	0.007
19	PROK1	prokineticin 1	-0.65	0.011
20	NAT8L	N-acetyltransferase 8 like	-0.77	0.013
21	GPAM	glycerol-3-phosphate acyltransferase, mitochondrial	-0.82	0.015
22	LRP1B	LDL receptor related protein 1B	-0.85	0.013
23	ABCD2	ATP binding cassette subfamily D member 2	-0.90	0.016
24	ANO3	anoctamin 3	-0.93	0.013
25	PFKFB1	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 1	-1.03	0.010
26	GYS2	glycogen synthase 2	-1.05	0.014

The network including26 DEGs plus 100 first neighbors was constructed. The analyzed network based on degree value is shown in the figure 2.

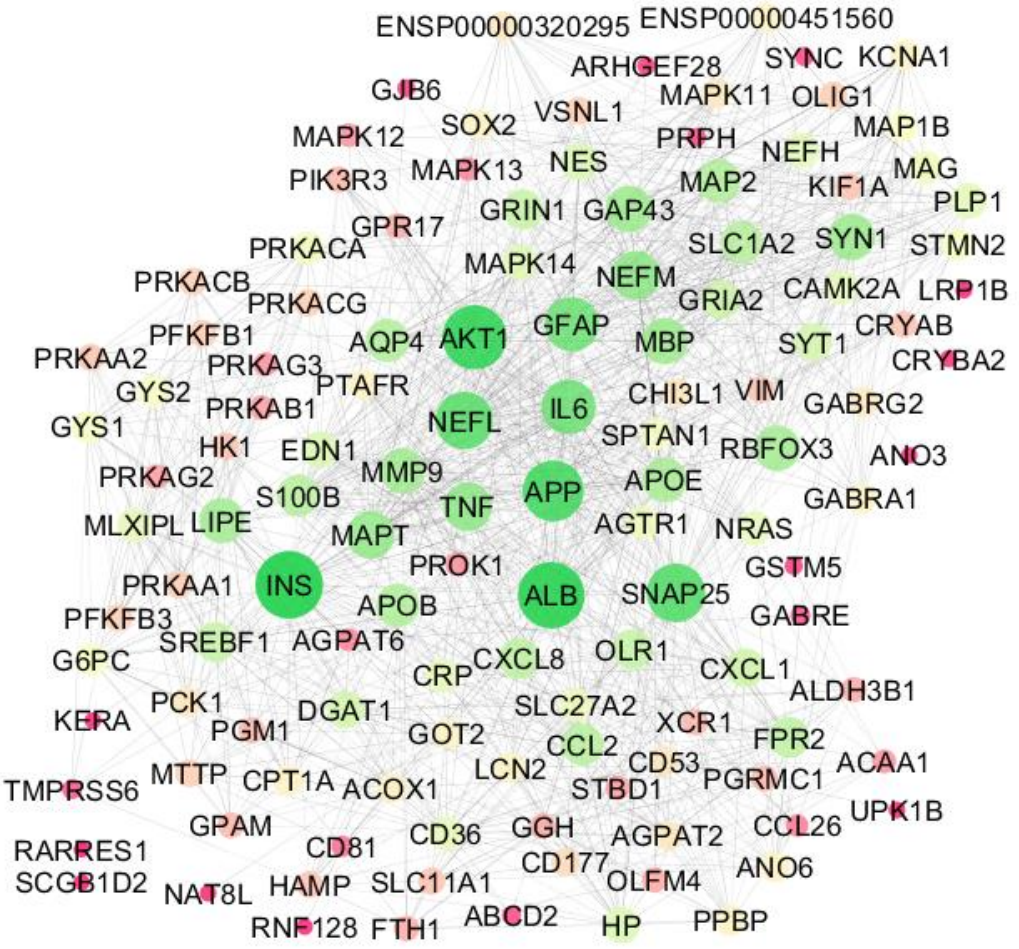

Fig 2: PPI network of sural nerve diabetic neuropathy. The nodes degree value makes the network layout. The node size corresponds to higher value of degree and the color change from red to green indicates increment of degree value.

The13 hub nodes were determined and tabulated in table 2 .Among the 13 hubs only 2 nodes are queried genes named NEFL and NEFM. The 10 first neighbors of NEFL (as the potent queried hub nodes) as an interactome is shown in the Fig 3.

Table 2: Selected 13 hub nodes for sural nerve diabetic neuropathy. The genes are sorted by degree value. The values of degree (K), betweenness centrality (BC), closeness centrality (CC), and Stress are represented in columns 4-7 respectively.

R	Gene	description	K	BC	CC	Stress
1	INS	Insulin; Insulin decreases blood glucose concentration. It increases cell permeability to monosaccharides, amino acids and fatty acids. It accelerates glycolysis, the pentose phosphate cycle, and glycogen synthesis in liver.	52	0.093	0.62	13640
2	ALB	Serum albumin; Serum albumin, the main protein of plasma, has a good binding capacity for water, Ca(2+), Na(+), K(+), fatty acids, hormones, bilirubin and drugs. Its main function is the regulation of the colloidal osmotic pressure of blood.	51	0.065	0.62	11604
3	AKT1	V-akt murine thymoma viral oncogene homolog 1; AKT1 regulates many processes including metabolism, proliferation, cell survival, growth and angiogenesis. AKT is responsible of the regulation of glucose uptake. AKT is also thought to be one mechanism by which cell proliferation is driven.	49	0.079	0.61	12110
4	APP	Amyloid beta (A4) precursor protein; N-APP binds TNFRSF21 triggering caspaseactivation and degeneration of both neuronal cell bodies (via caspase-3) and axons (via caspase-6); Endogenous ligands	46	0.055	0.59	7884
5	SNAP25	Synaptosomal-associated 25 kDa protein; t-SNARE involved in the molecular regulation of neurotransmitter release. May play an important role in the synaptic function of specific neuronal systems.	44	0.070	0.59	7754
6	**NEFL**	Neurofilament, light polypeptide; Neurofilaments usually contain three intermediate filament proteins: L, M, and H which are involved in the maintenance of neuronal caliber.	43	0.033	0.55	4664
7	GFAP	Glial fibrillary acidic protein; GFAP, a class-III intermediate filament, is a cell- specific marker that, during the development of the central nervous system, distinguishes astrocytes from other glial cells.	40	0.024	0.57	5314

8	IL6	B-cell stimulatory factor 2; Cytokine with a wide variety of biological functions. It is a potent inducer of the acute phase response. Plays an essential role in the final differentiation of B-cells into Ig- secreting cells Involved in lymphocyte and monocyte differentiation.	40	0.028	0.58	5734
9	**NEFM**	See description about NEFL	35	0.015	0.49	2136
10	TNF	Tumor necrosis factor ligand superfamily member 2; Cytokine that binds to TNFRSF1A/TNFR1 and TNFRSF1B/TNFBR. It is mainly secreted by macrophages and can induce cell death of certain tumor cell lines.	35	0.017	0.56	3640
11	MAPT	Microtubule-associated protein tau; Promotes microtubule assembly and stability, and might be involved in the establishment and maintenance of neuronal polarity.	34	0.017	0.55	3636
12	GAP43	Axonal membrane protein GAP-43; This protein is associated with nerve growth. It is a major component of the motile "growth cones" that form the tips of elongating axons. Plays a role in axonal and dendritic filopodia induction.	33	0.008	0.54	2040
13	MBP	Myelin membrane encephalitogenic protein; The classic group of MBP isoforms (isoform 4-isoform 14) are with PLP the most abundant protein components of the myelin membrane in the CNS. They have a role in both its formation and stabilization.	33	0.013	0.54	3102

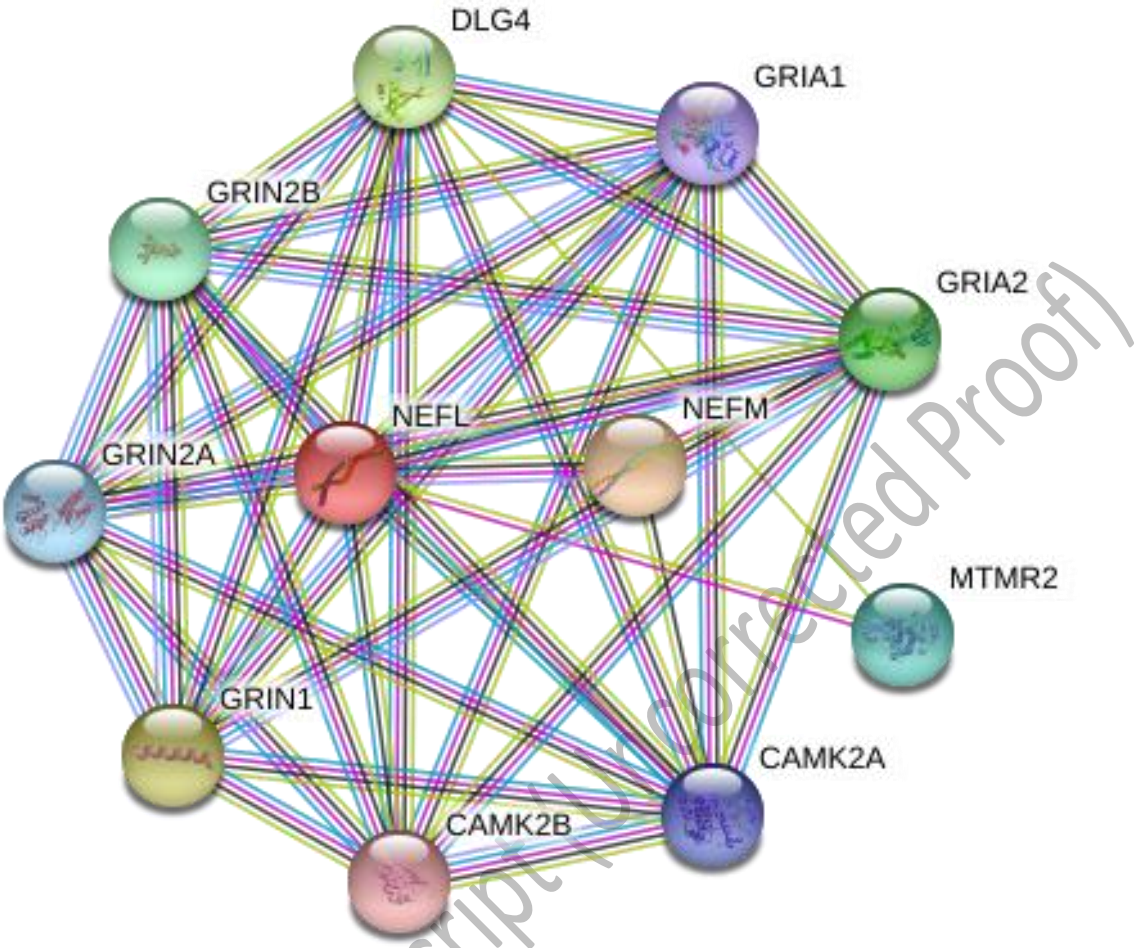

Fig 3: NEFL (the potent queried hub node) is related to the 10 first neighbors.

Discussions:

The current study takes an essential step toward the goal of identifying set of special genes whose expressions accurately related to surreal nerve diabetic neuropathy onset and progression. By analyzing genes interaction within known cellular pathways we could identify common elements in this complicated network to yield novel insights into disease pathogenesis and therapeutic targets to identify potential DN biomarkers. Proteomics is a suitable screening tool for extracting large amount of data to identify biomarkers related peripheral DN. A proteomic research for

example suggested serum apolipoprotein C-1 precursor for detecting and classification of DN (34). Our initial analysis of data sets classified as progression of diabetic neuropathy. The reported data related to DN were screened by PPI network analysis to trace key related elements. The samples including progressive and non-progressive diabetic neuropathy were matched as equal boxplots demonstrated in Fig1 and statistically comparable. Twenty six significant genes were selected considering restricted conditions for more investigations (Table 1). As shown in table 1, two over expressed genes were GJB6, CCL26, respectively. The GJB6 gene or gap junction protein beta 6 as the highest over expressed gene (Log FC =1.4) provides instruction for making protein named gap junction beta 6 or connexin 30. Its related pathways are gap junction trafficking and vesicle mediated transport (35). GJB6 up regulation in progressive diabetic neuropathy is associated with fatty acid homeostasis and glucose homeostasis which confirmed by our results (36). GJB3 is an important Para loge of this gene and expressed in peripheral and auditory nerves , could cause peripheral nerve neuropathy and auditory impairment (37). Second over expressed one was CCL26 or C-C motif chemokine ligand 26, which is a protein coding gene involve in PEDF induced signaling and AKD signaling. Gene ontology related to this gene has chemokine activity and an important para loge of this gene is CCL2 enhanced microglial activation lead to increase expression of receptor CCR2, suggests important role of chemokine CCL2 and its receptor CCR2 in development of neuropathic pain (38).On the other hand two lower expressed genes were GYS2 and PFKFB1respectively.GYS2 involved in glucose metabolism and its expression is decreased in diabetic patients, conform to our findings (39). GYS2 gene provides instruction for making liver glycogen synthtase, an enzyme produced in liver to form glycogen from glucose monomers. GYS2 plays role in AMPK signaling pathway in peripheral diabetic neuropathy (36). PFKFB1 (6-Phosphofructo-2-Kinase/Fructose-2,6-Biphosphatase 1) is a protein coding gene and one of its related pathways is glucose metabolism. Signaling pathway of adenosine monophosphate (AMP)-activated protein kinase (AMPK) in progressive and non progressive diabetic neuropathy is significantly enriched with PFKFB1 and other genes as PPARG and SDC. Network analysis suggested AMPK and PPAR pathways may implicated in diabetic neuropathy confirmed by our results (23). Table 2 demonstrated the identified hubs that are ranked and tabulated based on degree value. Myelin formation is result of circumferential wrapping of Schwann cells cytoplasm and it is particularly enriched with cholesterol and saturated long chain fatty acids (40). Some researchers believed that diabetic hyperglycemia leads to malfunction in

differentiation and re-differentiation of Schwann cells in accompany with myelin damages (41). New myelin formation after Schwann cells malfunction could be difficult and may cause DN progression (42). As it is depicted in the table 2, 13 nodes of the constructed network were highlighted as hub genes. Among the hub nodes (INS, ALB, AKT1, APP, Snap25, NEFL, GFAP,IL6, NEFM, TNF,MAPT , GAP43 , and MBP) there are 11 first neighbors. According to the assumptions in Table 2, there are two queried hubs among the introduced hubs including NEFL and NEFM.

According to Table 2, INS gene centrality is accompanied with degree 52 which is more than other 12 genes. It seems that INS gene expression dysregulation following diabetes and hyperglycemia plays critical role in disease progression. As it is shown in the table 2 INS is the potent bottleneck and also is characterized by the highest values of closeness centrality and stress. Further findings were obtained from roles of NEFM and NEFL as the only queried hub genes in progression of disease Base on published documents, NEFL and NEFM encode the neurofilament proteins and mutation of them leads to Charcot Mariye neuropathy and peripheral nerve neuropathy (43). Neurofilaments are intermediate filament type IV heterodimers composed of three different heavy, intermediate and light chains and they functionally maintain the neural caliber because neurofilaments comprise axoskeleton (44). They may play role in intra cellular transportation into axons and denderites. NEFL encodes light chain neurofilament protein. Mutation of this gene could cause disorders of peripheral nerves characterized by neuropathy (45). As it is demonstrated in Fig 3, NEFL and NEFM are linked with edges together and other 9 neighbors genes that can be considered as the other related genes which have significant impact on progressive diabetic neuropathy incidence.

Conclusions:

More in vitro and in vivo investigations suggested for role of NEFL and NEFM genes in progressive diabetic neuropathy incidence.

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Conflicts of interests:

Authors declare that they have no conflict of interests.

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