The Egyptian Journal of Hospital Medicine (October 2020) Vol. 81 (6), Page 2235-2245

Long Noncoding RNA MALAT-1 and Mirna-9 Expression Profile Levels in Patients with Diabetic Polyneuropathy (DPN) and Their Correlations with The Severity of Painful DPN

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ABSTRACT

Background*:* Diabetic polyneuropathy (DPN) is the major microvascular complication of type 2 diabetes mellitus (T2DM). Painful-DPN is a major cause of mortality as well as morbidity. Long non-coding RNAs (lncRNAs) and microRNAs (miRNA) have emerged as critical regulators of many diseases, however, little is known about their expression patterns and functions in T2DM and its complications.

Objective: To investigate the expression profile levels of lncRNA MALAT-1 and miRNA-9 in Egyptian patients with T2DM and to explore their associations with clinical and electrophysiological tests of both painful and painless DPN. **Patients and Methods:** This cross-sectional controlled study enrolled 55 patients with DPN and 40 controls. All participants were subjected to a complete neurological examination and electrophysiological tests involving nerve conduction studies. The expression levels of lncRNA MALAT-1 and miRNA-9 were measured by quantitative real-time polymerase chain reaction (qRT-PCR).

Results: The relative expression levels of MALAT-1 and miRNA-9 were significantly upregulated in patients with DPN (0.219±0.061, 0.006454±0.0018, respectively) compared to controls (0.111±0.013, 0.0033±0.004, respectively). Interestingly, the relative expression levels of MALAT-1 and miRNA-9 were significantly upregulated in patients with painful DPN (0.206±0.037, 0.0045±0.0008, respectively) compared to patients with painless DPN 0.219±0.083, 0.0058±0.0017, respectively). Patients with DPN had sensory-motor axonal polyneuropathy which was affecting both lower limbs more than upper limbs. P<0.001*.

Conclusions: The relative expression levels of MALAT-1 and miRNA-9 were significantly upregulated in patients with DPN more specifically in patients with painful DPN groups, hence, MALAT-1 and miRNA-9 could be used as useful and reliable diagnostic biomarkers of DPN.

Keywords: DPN, LNC RNA, MALAT-1, miRNA-9, Nerve conduction studies.

INTRODUCTION

The prevalence of diabetes continues to increase and is reaching epidemic proportions, according to an estimation by the International Diabetes Federation (IDF), the global population affected by diabetes will reach 552 million by the year 2030[(1)]. Type 2 diabetes mellitus (T2DM) results from ineffective insulin response. The clinical manifestations of DM restrict its timely diagnosis, which may be delayed by several years. This delayed prediction often results in chronic complications including diabetic polyneuropathies (DPN)[(2)].

DPN is characterized by diffuse damage to the peripheral nerve fibers. Despite the limited knowledge on the molecular mechanisms underlying the pathogenesis of DPN, emerging scientific evidence has disclosed that 30–90% of patients with diabetes have peripheral neuropathy [(3)]. Additionally, diabetic sensory-motor polyneuropathy (DSPN) which is the most common form of neuropathy in T2DM is associated with impaired quality of life, significant morbidity, and increased healthcare costs. Furthermore, 16–34% of patients with diabetes report painful neuropathic symptoms[(4)]. It has been widely described that the symptoms of DPN can be debilitating and can cause sleep disturbances, anxiety, and interference with physical functioning[(5)].

There have been tremendous advances over the last 2 decades in understanding the processes that regulate the epigenetic mechanisms and their potential implication on diabetes and its complications. Epigenetics or epigenomics refer to all the alterations and ensuing phenotypes such as changes in DNA conformation, transcription, or translation, that do not involve changes to the underlying DNA sequence. There are multiple stages of epigenetic regulation including DNA cytosine methylation, histones post-translational modifications in chromatin, and lately noncoding RNAs (ncRNAs) such as long noncoding RNAs (lncRNAs), microRNAs (miRNAs), and circular RNAs (circRNAs) [(6)].

Given the recognized differences in the epigenetic regulators, long noncoding RNAs (lncRNAs) family (>200nucleotides) could participate in various biological processes, such as X-chromosome inactivation, genomic imprinting, quiescence, immune response as well as cellular and metabolic processes [(7)]. MALAT1 (metastasis-associated lung adenocarcinoma transcript 1), programmed by the MALAT1 gene on chromosome 11q13.1 with two exons is in many cells all over the

Received: 24/7/2020
Accepted: 23/9/2020

body [8], primarily associated with lung cancer but later on, emerging evidence supports the concept of wide spreading of MALAT1 in many other diseases[9].

MicroRNAs (miRNAs), are involved in various cellular processes such as cell proliferation, apoptosis, and differentiation. Abnormal expression of miRNAs has been found in a variety of diseases including cancer, cardiovascular diseases, autoimmune diseases, and type 2 diabetes [10]. Growing evidence highlights the direct regulatory link between two important classes of non-coding RNAs, miRNAs, and lncRNAs, on one hand, miRNAs could be sequestered and prevented from acting on the protein-coding mRNAs; on the other hand, miRNA binding to lncRNAs and circRNAs could promote their decay[11]. Despite the limited knowledge on the molecular mechanisms underlying the pathophysiology of microvascular complications of T2DM in particular DPN, emerging scientific evidence has indicated that abnormally expressed miRNAs have pivotal roles in key pathogenic processes of microvascular complications, such as fibrosis, apoptosis, inflammation, and angiogenesis. Mirna-based diagnosis and therapy are highly likely to be the future of treatment and prevention, especially in multifactorial disease processes.

DPN has two forms, a painful and non-painful form. The non-painful variant is the most dangerous because of its insidious nature, and gradual loss of sensation in the feet and lower limbs. Neuropathy might therefore go unnoticed by the patient until irreversible nerve damage has occurred. Therefore, early prediction and diagnosis of DPN are warranted. Thus, this study was designed to investigate the expression profile levels of lncRNA MALAT-1 and miR-9 expression in Egyptian patients with T2DM and to explore their associations with clinical and electrophysiological tests of painful and painless DPN.

PATIENTS AND METHODS

Fifty-five patients with DPN (male/female 17/38), their mean age was 46.23 ± 8.34 years, were enrolled from the internal medicine and neurology outpatient clinics at Zagazig University Hospital, Egypt, and forty healthy subjects (male/female 13/27), their mean age was 45.55 ± 9.61 years with no family history of diabetes mellitus as well as age, sex, and ethnic origin matched to the patients, were served as a control group. The enrolled patients were subjected to thorough history taking, data were collected through a predesigned structured questionnaire to collect information about age, smoking habits, type of diabetes, duration of the disease, previous screening for diabetic complications, history of renal disease (dialysis or transplantation), and history of retinal laser photocoagulation as well as symptoms of diabetic neuropathy. A thorough neurological examination. Assessment of peripheral neuropathy was done by neuropathy disability score (NDS)[12] and the severity of neuropathy was graded according to the Toronto Clinical Scoring System (TCSS) into mild, moderate, and severe DPN [13]. Nerve conduction studies were also done for patients. Patients with DPN were classified into painful and painless DPN patients according to McGill's visual analog scale (VAS).VAS is a common research tool to assess pain, it is formed of 10 cm a straight line which provides a continuous scale for subjective magnitude estimation, and the limits of this line represent the extreme of the symptoms, so in the McGill pain index assessment 0 represents no pain and 10 the worst pain ever [14]. Nerve conduction studies (NCS) were done for all subjects and measured by the Dantec Keypoint Workstation (Suite, CA, USA) [15]. Routine NCS included evaluation of the motor function of the median, ulnar, common peroneal, and tibial nerves, and sensory function of the median, ulnar, and sural nerves. Distal latencies, amplitudes, and conduction velocities of both motor and sensory nerves were recorded.

According to this cross-sectional controlled study, patients with DPN were classified as patients with painless DPN (n=35) and patients with painful DPN (n=20). We excluded patients with any other type of neuropathic pain of non-diabetic origin. Also, we excluded patients with active inflammatory or chronic diseases.

Ethical approval:

The study was approved by the Ethics Committee of the Faculty of Medicine, Zagazig University. Written informed consent was obtained from all study participants.

Blood samples and biochemical Analysis:

The serum and plasma samples were separated and stored at -20^{0}C till the time of use. Fasting plasma glucose (FPG), total cholesterol (TC), and triglycerides (TG) were measured by routine enzymatic methods (Spinreact). HDLc was determined after precipitation of the apo B-containing lipoproteins. LDLc was calculated using the Friedewald formula [16]. Glycated hemoglobin (HbAlc) was estimated colorimetrically by using (Biosystems, Barcelona, Spain).

Gene expression analysis:

The total RNA was extracted from the serum of all participants using the Qiagen RNeasy Kit (Cat. No. 74104, Qiagen, Hilden, Germany) following the protocol supplied by the manufacturer. The potential genomic DNA contamination was eliminated by the application of the DNase step in the provided protocol. The concentration and purity of the extracted total RNA at the absorbance ratio of 260/280 nm were determined by NanoDrop ND-1000 spectrophotometer (NanoDrop Tech., Inc. Wilmington, DE, SA) and the integrity was checked on 2% agarose gel. Then high Capacity cDNA Reverse Transcription (RT) Kit (Applied Biosystems, P/N 4368814) was used for RT reaction housekeeping gene GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) was used for data normalization, and appropriate negative controls; a no template control (NTC) which omits RNA template

from the reaction to test the possibility of extraneous nucleic acid contamination and a no reverse transcriptase control (NRT) which assesses the amount of DNA contamination (if any) present in an RNA preparation, were included in each run. Real-time PCR reactions for lncRNA MALAT1 were carried out using specific Taqmanprobes Hs00273907in a final volume of 20 µl which included 1.33 µl RT products, 10 µl 2× TaqMan Universal PCR Master Mix, 1 µl TaqMan RNA assay. The PCR was performed in StepOne™ Real-Time PCR System (Applied Biosystem, Foster City, California, United States) and incubated as follows: 95°C for 10 min followed by 40 cycles of 92°C for 15 s and 60°C for 1 min. All tests were performed in duplicate and the real-time PCR reactions were carried out following the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines [17,18]. The sequences of primers specific for lncRNA MALAT1forward: A CTTACATGTCTGCC TTGG, reverse TCAAAGCTGGTACAGCCA. The sequences of primers specific for GAPDH forward: AGTAGTCACCTGTTGCTGG reverse: TAATACGGAGACCTGTCTGGT. Regarding miRNA-9, serum was isolated by using the miRNeasy Mini kit (Qiagen, Germany) according to the manufacturer's instructions. Then finally, miRNA was recovered in 30 uL of RNase-free water.

The sequences of primers specific for miR-9forward: TAAAGCTAGATAACCGAAAGT, reverse TTGGGGTGCTCGTGCAGATCGAA. The sequences of primers specific for U6forward: ATGACGTCTGCCTTGGAGAACreverse: TCAGTGTGCTACGGAGTTCAG, U6 was considered as the internal reference of miR-9The $2^{-\Delta\Delta CT}$ method was used to calculate the relative expression of the target genes.

Statistical analysis

Statistical analyses were performed using the Statistical Package for the Social Sciences for Windows (version 21.0; SPSS Inc., Chicago, IL, USA). Sample size and power calculations using G power- 3 software showed that with the specified study design (i.e. gene expression), allowable error rates. Alpha error = 0.05, a medium effect size = 0.5, and sample size 50 for each group can give 80% power of the study (http://www.gpower.hhu.de/).

Data were expressed using descriptive statistics (mean ± standard deviation). Categorical variables were presented as frequency counts and compared using the chi-square ($\chi 2$) test, while student's t-tests, Mann–Whitney U (MW), and Kruskal–Wallis (KW) tests used to compare continuous variables according to a data type as mentioned Pearson correlation coefficient was used to assess the association between the expression profile of lncRNA MALAT1and miRNA-9 with TCSS and nerve amplitude and nerve conduction velocity in patients with DPN. We explored the main independent variables of lncRNA MALAT1 and miRNA-9 among patients with DPN by linear regression analysis. Receiver operating characteristic (ROC) analysis was performed to assess the potential diagnostic accuracy of lncRNA MALAT1 and miRNA-9 levels; area under the curve (AUC), and the cutoff values for diagnosis of DPN among diabetic patients. We considered P to be significant at <0.05 with a 95% confidence interval (CI).

RESULTS

This cross-sectional controlled study conducted on 55 Egyptian patients with DPN,69.6 % were females and 30.4% were males, their mean age was 43.63 ± 12.17 years, and 40 healthy subjects as control, 64.3% were females and 35.7% were males, their mean age was 41.55 ± 10.45 years. The case and control groups were matched for age, sex, and smoking. As expected, patients with DPN had higher values of metabolic risk factors as well as TCSS and NDS compared to the control group as shown in table 1.

Table (1): Clinical and laboratory characteristics of the studied groups

Variables	Control group (*n* =40)	Patients with PDN (*n* =55)	*P*-value
Body mass index (kg/m^2)	22.18± 1.189	37.03± 4.96	<0.001*
Waist/hip ratio	0.864±0.011	1.07±0.21	<0.001*
Systolic blood pressure (mmHg)	117.8± 8.40	159.8±20.44	<0.001*
Diastolic blood pressure (mmHg)	75.6±4.589	91.92± 14.33	<0.001*
Total cholesterol (mg/dl)	184.3± 19.90	221.88±29.1	<0.001*
Triglycerides (mg/dl)	175.26±15.019	271.16±44.6	<0.001*
LDL cholesterol (mg/dl)	100.08±23.067	1255.91±31.4	<0.001*
HDL cholesterol (mg/dl)	58.48±6.87	38.25± 5.63	<0.001*
Fasting plasma glucose (mg/dl)	87.72± 6.304	196.97±30.04	<0.001*
HbA1c (%)	5.63±0.524	9.49±2.36	<0.001*
NDS	6.63±1.524	8.49±1.36	<0.001*
VAS	0	6.49±1.36	<0.001*
TCSS	2.165±0.211	6.54 ± 1.22	<0.001*

DPN, diabetic polyneuropathy; DS, neuropathy disability score; VAS, visual analog scale; TCSS, Toronto Clinical Scoring System.* *P*< 0.05.

General characteristics of diabetic patients:

Among 55 Egyptian patients with DPN, patients with painful DPN had a statistically significant long duration of diabetes as well as higher values of BMI, waist/hip ratio, diastolic blood pressure, TG, FPG, HbA1c, VAS, NDS, and TCSS compared to patients with painless DPN. Regarding diabetic treatment, the prevalence of patients with painful DPN treated with insulin was more common than patients with painless DPN.

As regard diabetic vascular complications, the prevalence of CHD was more common in patients with painless DPN compared to patients with painful DPN, P <0.001, table 2.

Table (2): Clinical and laboratory characteristics of patients with DPN

Variables	**Painless DPN (n=35)**	**Painful DPN (*n* =20)**	***P*-value**
Duration of diabetes (years)	7.17±0.63	8.58±2.33	<0.001*
Body mass index (kg/m^2)	34.73±2.32	38.03±4.96	<0.001*
Waist/hip ratio	0.96±0.102	1.27±0.234	<0.001*
Systolic blood pressure (mmHg)	157.08±12.457	161.01±16.09	0.042
Diastolic blood pressure (mmHg)	84.52±10.61	103.84±13.12	<0.001*
Total cholesterol (mg/dl)	216.73±31.064	230.61±28.116	0.058
Triglycerides (mg/dl)	232.86±34.98	234.4±6.2	<0.001*
LDL cholesterol (mg/dl)	113.78±29.378	132.95±34.76	0.981
HDL cholesterol (mg/dl)	48.78±5.248	36.79±5.694	0.168
Fasting plasma glucose (mg/dl)	227.78±42.20	246.76±16.93	<0.001*
HbA1c (%)	8.8±1.265	10.79±2.206	<0.001*
NDS	5.8±1.265	8.79±2.206	<0.001*
VAS	0	7.49±1.36	<0.001*
TCSS	5.75±1.96	8.28±2.33	<0.001*
Medication			
Diet	15(42.8%)	10 (50%)	0.800
Oral	25(71.4%)	12(60%)	0.131
Insulin	18(51.4%)	16(80%)	<0.05
Complication of diabetes			
Retinopathy	20(57.1%)	11(55%)	0.610
Nephropathy	19(54.2%)	15(75%)	0.249
Stroke	14(40%)	9(45) 0	0.902
CHD	29(82.8%)	11(55%)	<0.05

HbA1c: hemoglobin A1c, DPN, diabetic polyneuropathy; NDS, neuropathy disability score; VAS, visual analog scale; TCSS, Toronto Clinical Scoring System, CHD: coronary heart disease* *P*<0.05.

Relative expression of lncRNA MALAT1 levels in the studied groups:

Our results show that the patients with painful DPN had statistically significant higher values of the relative expression levels of lncRNA MALAT1(1.87±0.568) compared to patients with painless DPN(1.616±0.288) and the control group (1.014±1.03), P <0.001, figure 1.

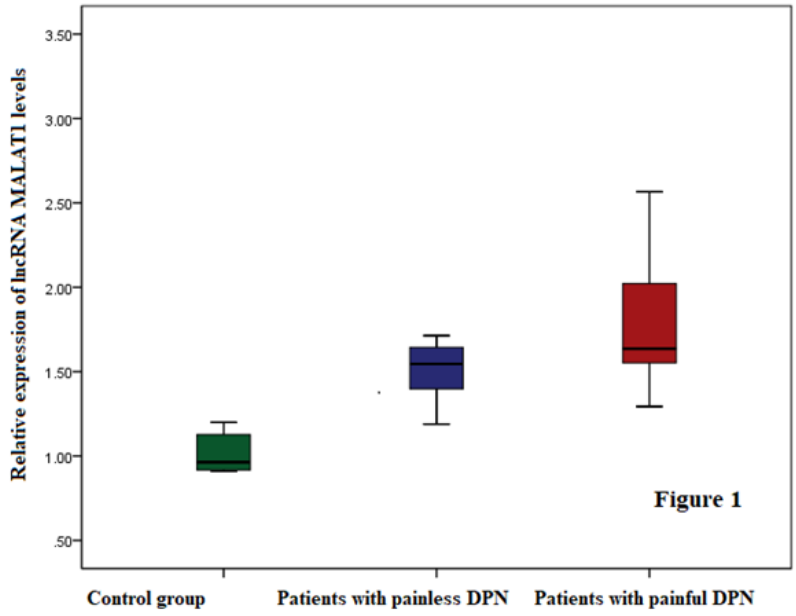

Figure (1): Relative expression of lncRNA MALAT1 levels in the studied groups

Relative expression of miRNA-9levels in the studied groups:

Our results show that the patients with painful DPN (1.463±0.45) had statistically significant higher values of the relative expression level of miRNA-9compared to patients with painless DPN(0.889±0.164) and the control group (0.209±0.0314), P <0.001, figure2.

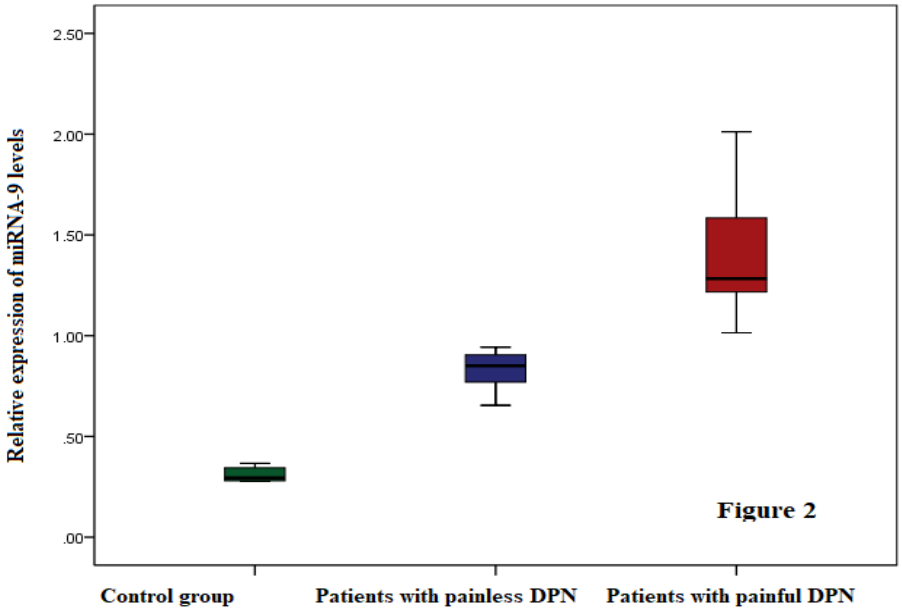

Figure (2): Relative expression of miRNA-9 in the studied groups

Nerve conduction studies of the studied groups of DPN:

Nerve conduction studies in the studied groups showed that MNCV and CMAP of the posterior tibial nerve (PTN) and common peroneal nerve (CPN)were significantly decreased in patients with painful DPN compared to patients with painless DPN. Moreover, SNCV and SNAP of the sural nerve were significantly decreased in patients with painful DPN compared to patients with painless DPN, (p < 0.001), while all other parameters of NCS were not significant. table 3.

Table (3): Nerve conduction studies among patients with DPN

Nerve conduction studies	Painless DPN (n=35)	Painful DPN (*n* =20)	*P*-value
MNCV (m/s)			
PTN	41.8±10.92	38.66±4.11	<0.001*
CPN	40.96±10.54	39.13±6.47	<0.001*
Median	49.96±10.76	45.8±8.93	0.204
Ulnar	50.54±8.12	47.1±11.13	0.352**
SNCV (m/s)			
Sural	40.12±11.04	34.66±4.1	<0.001*
Median	55.4±4.4	54.78±8.4	0.432
Ulnar	54.41±4.317	54.01±5.6	0.512
CMAP amplitude (mV)			
PTN	3.38±1.22	1.36±0.41	<0.001*
CPN	2.36±1.22	1.54±0.411	<0.001*
Median	4.25±1.64	3.23±1.41	0.229
Ulnar	5.18±1.82	4.68±1.38	0.194
SNAP amplitude (*μ*V)			
Sural	4.67±1.27	3.19±1.38	<0.001*
Median	6.77±2.0	6.29±1.59	0.174
Ulnar	4.68±1.25	3.19±1.380	0.121

MNCV, motor nerve conduction velocity; SNCV, sensory nerve conduction velocity; CPN, common peroneal nerve; PTN, posterior tibial nerve; CMAP, compound muscle action potential; SNAP, sensory nerve action potential.* *P*< 0.05

Correlations between relative expression of lncRNA MALAT1 and miRNA-9 with TCSS, nerve amplitude, and nerve conduction velocity in patients with DPN:

Our results demonstrated that the relative expression of lncRNA MALAT1 and miRNA-9 significantly positively correlated with TCSS and significantly negatively correlated with MNCV of PTN and CPN as well as SNCV of the sural nerve. Interestingly, CMAP amplitudes of PTN, CPN, median and ulnar nerves as well as SNAP of sural, median, and ulnar were significantly negatively correlated with the relative expression levels of both lncRNA MALAT1 and miRNA 9, *P*<0.001, table 4.

Table (4): Correlations between the relative expression levels of MALAT-1 and miRNA-9 with TCSS, nerve amplitude and nerve conduction velocity in patients with DPN

Variables	lncRNAMALAT-1		miRNA-9	
	r	p	r	p
TCSS	0.576	<0.001*	0.629	<0.001*
MNCV (m/s)				
PTN	-0.401	<0.001*	-0.435	<0.001*
CPN	-0.438	<0.001*	-0.806	<0.001*
Median	-0.162	0.107	-0.066	0.452
ulnar	-0.134	0.103	-0.180	0.058
SNCV				
Sural	-0.385	<0.001*	--0.4533	<0.001*
Median	-0.159	0.051	-0.022	0.286
Ulnar	-0.178	0.077	-0.128	0.201
CMAP amplitude (mV)				
PTN	-0.505	<0.001*	-0.493	<0.001*
CPN	-0.614	<0.001*	-0.840	<0.001*
Median	-0.479	<0.001*	-0.674	<0.001*
Ulnar	-0.687	<0.001*	-0.824	<0.001*
SNAP amplitude (μV)	.			
Sural	-0.568	<0.001*	-0.812	<0.001*
Median	-0.517	<0.001*	-0.763	<0.001*
Ulnar	-0.600	<0.001*	-0.419	<0.001*

lncMALAT1, long non-coding, metastasis-associated lung adenocarcinoma transcript 1; miRNA-9, microRNAs -9; MNCV, motor nerve conduction velocity; SNCV, sensory nerve conduction velocity; CPN, common peroneal nerve; PTN, posterior tibial nerve; CMAP, compound muscle action potential; SNAP, sensory nerve action potential. * *P*< 0.05**.**

Linear regression analyses in patients with DPN:

Parameters associated with the relative expression of lncRNA MALAT1 and miRNA-9 levels. Our results showed that TCSS, BMI, HbA1c, and TG were independently correlated with both epigenetic biomarkers, P<0.001, table 5.

Table (5): linear regression analyses to test the influence of the main independent variables against the relative expression levels of MALAT-1 and miRNA-9 (dependent variable) in patients with DPN

Model		Unstandardized Coefficients		Standardized Coefficients	t	p	95% C.I.	
		B	SE	Beta			Lower Bound	Upper Bound
lncRNA MALAT-1	(Constant)	-0.874	0.058		-15.14	0.000	-0.990	-0.758
	TCSS	0.617	0.010	0.584	63.915	0.000	0.597	0.636
	BMI	3.489	00.088	0.408	39.727	0.000	3.312	3.665
	HbA1c	0.586	.008	0.555	76.037	0.000	0.570	0.601
	TG	3.775	0.069	0.441	54.722	0.000	3.637	3.914
miRNA-9	(Constant)	0.962	0.790		1.218	0.229	-0.626	.962
	TCSS	8.996	0.081	1.110	110.40	0.000	8.832	9.160
	BMI	0.074	0.015	0.028	4.872	0.000	0.043	0.104
	HbA1c	0.491	0.020	0.197	24.953	0.000	0.452	0.531
	TG	0.074	0.015	0.028	4.872	0.000	0.043	0.104

lncMALAT1,long non-coding, metastasis-associated lung adenocarcinoma transcript 1; miRNA-9, microRNAs -9

The accuracy of relative expression of lncRNA MALAT1 and miRNA-9 levels for discriminating patients with DPN from the control group by ROC analysis:

We explored the potential diagnostic value of the relative expression of lncRNA MALAT1 and miRNA-9 levels in discriminating patients with DPN from the control group, the cutoff values of the relative expression of lncRNA MALAT1 and miRNA-9 levels were 1.1629 and 0.971, respectively and the AUC was 0.770 (95% CI =0.647-0.893) and 0.799(95% CI =0.669-0.928), additionally, the sensitivities and the specificities were 90% and 65.7% for lncRNA MALAT1 and 95% and 74.3% for the mRNA expression level of miRNA-9 respectively<0.001, figure3.

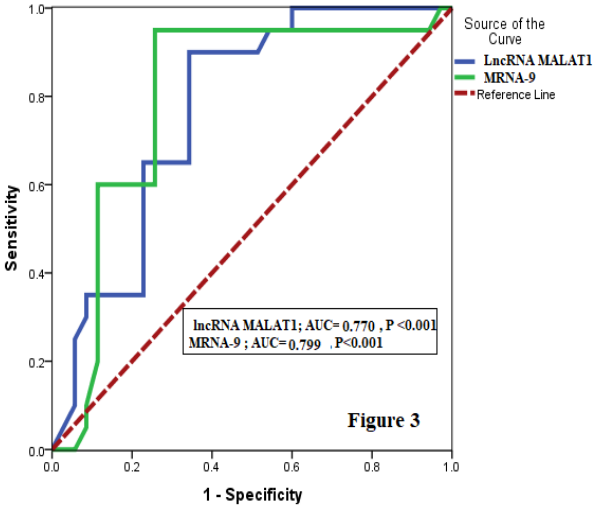

Figure (3): The accuracy of the relative expression levels of lncRNA MALAT1 and miRNA-9 for discriminating patients with DPN from the control group by ROC analysis.

The accuracy of relative expression of lncRNA MALAT1 and miRNA-9 levels for discriminating patients with DPN from patients without DPN by ROC analysis.

We applied the ROC test to discriminate patients with painful DPN from patients with painless DPN, the cutoff values of the relative expression of lncRNA MALAT1 and miRNA-9 levels were 1.109 and 0.338, respectively the AUC was 0.812 (95% CI =0.690-0.934) and 0.809 (95% CI =0.690-0.929), additionally, the sensitivities and the specificities were 90% and 58%for lncRNA MALAT1 and 83.3% and 87.8% for the mRNA expression level of miRNA-9 respectively, P<0.001, figure 4.

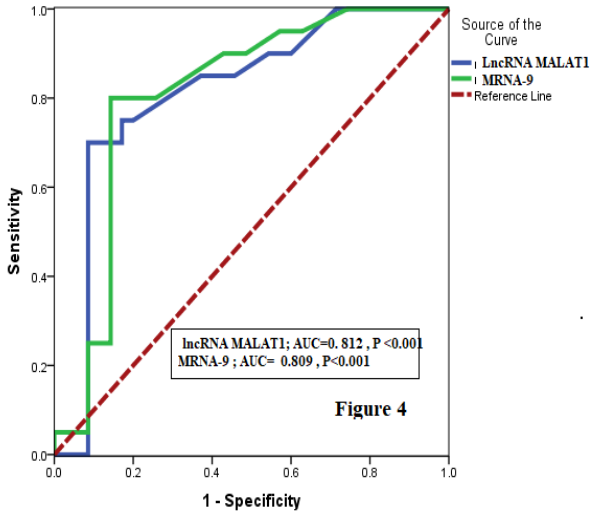

Figure (4): The accuracy of the relative expression levels of lncRNA MALAT1 and miRNA-9 for discriminating patients painful DPN from patients with painless DPN by ROC analysis.

DISCUSSION

Previous numerous studies have demonstrated that the neurovascular complications affect more than 60% of patients with T2DM among which is DPN, growing evidence highlights the complex disease etiologies and the most important of which is chronic hyperglycemia, which may contribute to its pathogenesis by causing changes in gene expression, inflammation, and oxidative stress [19].

Noteworthy, DPN is the major contributor to lower limb amputation and has also a significant negative effect on the patient's quality of life. Given the lack of disease-modifying treatments for DN, the identification of risk factors for DN is key in optimizing treatment and delaying the development and progression. To address these needs, we investigated the expression profile levels of lncRNA MALAT-1 and miRNA-9 expression in Egyptian patients with T2DM and to explore their associations with clinical and electrophysiological tests of painful and painless DPN.

In our study, we first performed a cross-sectional study of our case group as we classified our patients with DPN according to clinical tests, for example, TCSS, VAS, NDS, and NCS to patients with painless DPN(n=35,63.6%) and patients with painful DPN(n=20,36.4%) and the current study revealed clear evidence that patients with painful DPN had a statistically significant long duration of diabetes as well as higher values of BMI, waist/hip ratio, diastolic blood pressure, TG, FPG, HbA1c, VAS, NDS, and TCSS compared to patients with painless DPN.

Similar results confirmed by **Morkrid *et al.*** [20] observed that a lower level of fasting blood glucose and oral hypoglycemic use was associated with lower odds of DPN, which emphasizes the role of intensive glycemic control in DPN prevention and treatment

In accordance with those observations, **Spallone and Greco** [21] suggested that poor diabetic control has been repeatedly associated with the development of DPN. Also, the study conducted by Sorensen et al to evaluate the role of height, gender, ethnicity, and glycemic control in the pathogenesis of DPN observed the importance of good glycemic control in the prevention of DPN [22]. Although a causal relationship between the presence of a long duration of diabetes in addition to poor glycemic control and PN remains to be determined, the possible role of the long duration of diabetes in the pathogenesis of DPN is a key issue [23].

Against our results, **Raputova *et al*** [24] study was not able to confirm an association of painful neuropathy with any of the previously mentioned risk factors. The controversial results between our study and this study are contributed to the limitations of this study which was that the pain scoring was not based on a diary, but on a one-time assessment, and analgesic therapy was not stopped before assessment of pain severity, it might have influenced the results of this study.

According to our results, regarding diabetic treatment, the prevalence of patients with painful DPN treated with insulin was more common than patients with painless DPN. As regard diabetic vascular complications, the prevalence of CHD was more common in patients with painless DPN compared to patients with painful DPN.

In agreement with our results, **Sone *et al.*** [25] found that insulin use is associated with DPN. In this study context, insulin use tends to be an indicator for longer duration and greater severity of diabetes. Similarly, reports of **Adler *et al***. [26] detected a positive association between insulin use and DPN.

In contrast, a study conducted by **Khawaja *et al.*** [27] detected that diabetic retinopathy was significantly higher in patients with DPN [27]. Also, against our results, **Maser and colleagues** [28] demonstrated that nephropathy which is the most common diabetic complication is often concomitant with DPN.

Growing number of reports suggest a role for epigenetics in the pathogenesis of diabetes [29-31] and a significant utility as diagnostic and predictive biomarkers. Currently, several pieces of evidence exist, demonstrating that miRNAs are involved in the regulation of vascular inflammation and microRNAs are known to exert their functions mainly, if not exclusively, in the cytoplasm [32-35]. Noteworthy, intriguing reports are DN painful manifestation and inflammatory response are also affected by miRNA-23a via chemokine receptor 4-related signaling[36]. This hypothesis was further supported by other studies as **Feng and his colleagues**[37] observed higher levels of miRNA-146 in circulating mononuclear cells and they suggested that miRNA suggesting that modulates the inflammatory response in DPN.

MALAT1 is a nuclear lncRNA that localizes to nuclear speckles in association with splicing regulators raising the possibility that miR-9 targets MALAT1 in the nucleus [38]. The results presented here are innovative as this study was the first Egyptian study that investigated the possible association of lncRNA MALAT1 and miRNA-9relative expression levels with neuropathic pain in patients with DPN in particular patients with painful DPN. Interestingly, we detected that the expression of lncRNA MALAT1 and miRNA-9 were significantly upregulated in patients with DPN compared to healthy control. More specifically, lncRNA MALAT1 and miRNA-9 relative expression were significantly overexpressed in patients with painful PDN compared to patients with painless PDN, with a positive correlation between the levels of both genes and TCSS. However, the expression levels of lncRNA MALAT1 and miRNA-9 were significantly negatively correlated with CMAP, MNCV, and SNCV of both PTN and CPN.

In agreement with our results, the **Zhou *et al***. [39] study found significant upregulation of lncRNA MALAT1 in T2DM compared to healthy controls.

Similar to our result, a study conducted by **Zhang *et al.*** [40] observed that the relative lncRNA MALAT1 expression was upregulated in patients with gestational diabetes mellites(GDM). Moreover, an experimental study conducted by **Liu *et al***.[41] on human retina microvascular endothelial cells treated with high glucose detected the upregulation of relative lncRNA MALAT1 expression level.

There is compelling evidence suggesting the role of epigenetics for example miRNA as possible biomarkers or therapeutic targets in diabetes-induced neuropathy [35]. Consistent with this notion and for additional assessment of our results, we applied a linear regression analysis test to explore the independent markers correlated with relative expression of lncRNA MALAT1 and miRNA-9 levels and we detected that among significantly correlated markers, TCSS, BMI, HbA1c, and TG were the independent markers correlated with those epigenetic biomarkers. Regarding miRNA-9 relative expression levels, In agreement with the present study, **Kong *et al***.[42] confirmed that the expression level of miRNA-9 was upregulated in patients with T2DM. MiRN-9 has a negative control on insulin release through inhibition of the expression of the transcription factor One cut-2, and then by increasing the level of Granuphilin/Slp4, a Rab GTPase effector associated with β-cell secretory granules [43].

In agreement with our findings, **Liu *et al*** [44]conducted an experimental study on rats to evaluate the role of miR-9 in the pathogenesis of

DPN and they detected that the expression of Calcium homeostasis modulator 1 was increased in PDN rat compared with controls. Also, the miR-9 expression level was upregulated in the spinal dorsal horn neurons of PDN rats and they concluded their study with this finding; CALHM1 is involved in the miR-9-mediated ATP-P2X7 pathway between neurons and glias in PDN rat.

On the contrary, a study by **Zhang and Zhu**[45] found that miR-9-5p is downregulated in GDM and participates in the progression of GDM through regulating glycolytic pathways and mitochondrial complex expression by targeting HK-2.

Our findings are in concordance with **Zhou *et al***. [39] results as they detected a positive correlation between lncRNA MALAT1 expression and HbA1c.

To better elucidate the diagnostic power of lncRNA MALAT1 and miRNA-9 relative expression for discriminating patients with DPN from the control group by using ROC analysis, our results revealed that the sensitivities and the specificities were 90% and 65.7% for lncRNA MALAT1 and 95% and 74.3% for the mRNA expression level of miRNA-9 respectively. As regards, the differentiation of patients with painful DPN from patients with painless DPN, the sensitivities and the specificities were 90% and 58% for lncRNA MALAT1 and 83.3% and 87.8% for the mRNA expression level of miRNA-9 respectively.

CONCLUSION

The relative expression levels of lncRNA MALAT1 and miRNA-9 were significantly upregulated in patients with DPN more specifically in patients with painful DPN. The identification of the optimum cut-off point of lncRNA MALAT1 and miRNA-9 could help in evaluating diabetes and DPN in an attempt to decrease health hazards related to neuropathy. Further future multicenter studies with a bigger sample size are needed to validate our findings.

DECLARATIONS

Ethics approval and consent to participate:

Written informed consent was taken from all of the participants after explaining details and benefits as well as risks to them. The ethical committee of Faculties of Medicine, Zagazig University approved the current study.

Consent for publication:

The authors declare that they received consent for publication from all the participants.

Availability of data and material:

The data that support the findings of this study are available from the corresponding author (nrashad78@yahoo.com) upon reasonable request

Competing interests: The authors declare that they have no competing interests

Funding: No fund.

Acknowledgments: Not applicable.

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