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Original Article

Novel candidates for chronic diabetic wound healing

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Abstract

Background Diabetic Foot Ulcer (DFU) is a leading cause of amputation in adult population all over the world. Molecular pathology of delay in wound healing of diabetic foot ulcer patient is not completely understood till today. Numerous works has been required to explore the cause of delay in wound healing which ultimately end in amputation.

Objective To find the role of long non-coding RNAs is a promising strategy which could be beneficial in treating the chronic wound timely and progressively.

Methods We have selected long non-coding RNAs (ANRIL and its target miR-181a, H19 and its target miR-29b) which are involved in the inflammation, proliferation and remodeling phase of wound healing process and do comparison of their expression in DFU patients and normal healthy individuals.

Results Our results revealed that ANRIL down regulation and H19 up regulation in DFU patients and similarly miR-181a up regulation and miR-29b down regulation. These results needed further validation by sequencing work therefore these can be used as diagnostic marker for an early detection in diabetic patients to halt the progression of DFU wound healing delay.

Key words
Diabetic Foot Ulcer; amputation; long non-coding RNAs.

Introduction

Diabetes mellitus (DM) is a multifactorial chronic disease.[1] With the advancement of modern era, life has become very easy and efforts to do everyday life activities has become very simple. With the sedentary lifestyle, a normal person becomes prone to several diseases including DM.[2] DM itself is not the only destructive disease but its associated complications are also harmful. One of the DM complications is diabetic foot ulcer (DFU), which is not only making life of affected individuals difficult but also causing a serious socioeconomic burden to the families because no proper treatment is available to cure the disease.[3] It is one of the complications of diabetic neuropathy as well as peripheral neuropathy, which mainly affects lower distal parts and hands of a diabetic patient. Additional symptoms include pain, loss of sensation, dry feet, fissuring or cracks in the feet. In the case of infections abscess develop (collection of pus cells), which ultimately results in ulcer.[4]

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Among the diabetic, DFU develops in 15% of the patients, 84% of them experience amputation. In Pakistan, regardless of being an emerging economy, prevalence of DFU is similar to several developed nations.[5] Untreated constant hyperglycemic conditions consequently result in the development of DFU from a simple infection in DM patients. Because of amputation life becomes extremely difficult and ultimately leads to long term disability, which sometimes results in tragic death.[6] Currently the molecular aspects and pathophysiology of DFU are very poorly understood. In diabetics determining the disease causative factors and their underlying molecular pathophysiology is challenging.[7]

Studies have shown number of aberrated physiological factors that are involved in failure of wound healing in DM patients. Among them many growth factors are reported to be deregulated, along with angiogenic response towards wound, macrophages, collagen accumulations, and epidermal barrier. In addition, granulation tissue, migration of fibroblast and keratinocyte is abnormal in DFU patients.[8] The wound healing therefore becomes slow or delayed in diabetic patients because of aberrated mentioned mechanisms.

Methods

Skin biopsy samples obtained with the consent taken from all the participants, ethical approval was already taken from the tertiary care hospitals. Demographic features of all the subjects and all the basic biochemical tests were performed in the research laboratory **(Table 1)**.

RNA extraction was done with the help of kit and following the protocol provided with the kit (Norgen Biotek Corp, Thorold, Ontario, Canada). To check the quality of extracted RNA, quantification was performed using the spectrophotometer and counter check on gel electrophoresis system. RNA was converted to cDNA using RevertAid First Strand cDNA synthesis kit (Thermo Fisher Scientific Inc., Waltham, MA) following the protocol provided with the kit. Using the Livak and Schmittgen, 2001 method to calculate the normalization with GAPDH and U6. SPSS used to get statistical analysis.

Table 1 Basic demographic features and biochemical test results.

Parameters, Mean (SD)	*Healthy*	*T2DM*	*DFU*
Age	51.92±9.6	52.13±6.8	51.63±8.2
Diabetes of Duration	N/A	10.81±4.8	14.01±5.0
HbA1c	5.8±2.3	7.7±4.5	9.9±4.2
B.P. (U)	120.54±15.8	125.3±5.2	123.8±7.4
B.P. (L)	79.91±12.5	84.97±2.7	82.48±6.8
Body Fats	26.72±11.3	34.23±4.6	32.67±5.8
Visceral Fats	8.85±4.3	12.88±7.4	10.85±8.3
Cholesterol	182.06±38.9	212.56±18.92	256.24±12.69
Triglyceride	118.09±35.1	182.14±15.41	174.34±11.4
HDL	32.72±10.0	45.12±5.7	55.39±12.5
LDL	120.29±41.3	129.82±21.35	126.23±22.7
VLDL	15.56±2.34	23.67±7.11	28.44±8.43
Sex	Male=27 (54%)	Male=20 (40%)	Male=28 (56%)
	Female=23 (46%)	Female=30 (60%)	Female=22 (44%)
Smoking	No=11 (55.16%)	No=13 (55.16%)	No=19 (55.16%)
	Yes=39 (44.84%)	Yes=37 (44.84%)	Yes=31 (44.84%)
BMI	Normal Weight=3 (6%)	Normal Weight=5 (10%)	Normal Weight=4 (8%)
	Over Weight=24 (48%)	Over Weight=24 (48%)	Over Weight=28 (56%)
	Obese=23 (46%)	Obese=21 (42%)	Obese=18 (36%)

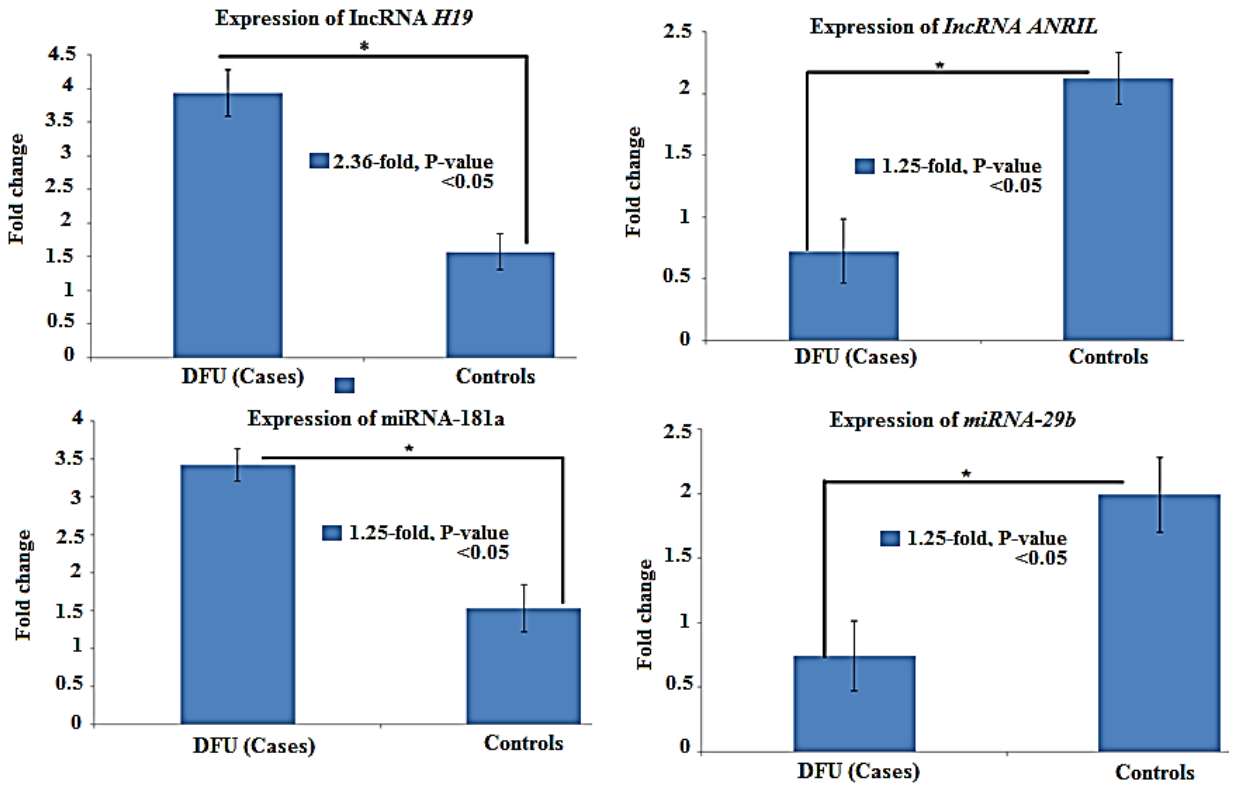

Figure 1 Expression analysis of lncRNAs and their target miRNAs (Fold Change Difference)

Results

The sample size of DFU and controls were 50 in each group (50 tissue samples of DFU, 50 tissue samples of DM and 50 healthy individuals without any DM history). Predicted from miRTarbase database the selected genes and microRNAs. Our findings represent expression of miRNAs were decrease in the DFU samples compared to controls samples. All the three miRNAs were lower in DFU as compared to target genes. P-value<0.05 also proved results were significant. Expression of target genes was higher in control samples as compared to selected miRNAs. Fold change difference in selected miRNAs and their target genes represented separately to illustrate their lower expression in miRNAs in DFU and respective target genes. Biochemical tests (Lipid profile) and basic Diabetes tests (BSR, BSF, and HbA1C) were performed using spectrophotometer GO system with the locally available kits. Fold change difference represented in **Figure 1**. Correlation of lncRNA H19 with demographic features, biochemical tests, and target miRNA 181a represented in the **Table 2** while the correlation of lncRNA

Table 2 Fold Change lncRNA H19 correlation with demographic features, biochemical test results and target miRNA.

Correlation with	*Correlation Coefficient (r)*	*p-value*
Age (Years)	0.127	Non-significant
BMI (kg/m2)	-0.41	Significant
Systolic	0.059	Non-significant
Diastolic	0.084	Non-significant
HbA1c (%)	-0.57	Significant
BSF (mg/dL)	0.36	Non-significant
BSR (mg/dL)	0.44	Non-significant
TC (mg/dl)	-0.52	Non-significant
LDL-C (mg/dl)	-0.167	Non-significant
HDL-C (mg/dl)	0.26	Significant
TG (mg/dl)	-0.398	Non-significant
miR-181a	-0.612	Significant

Table 3 Fold Change lncRNA ANRIL correlation with demographic features, biochemical test results and target miRNA.

Correlation with	*Correlation Coefficient (r)*	*p-value*
Age (Years)	0.131	Non-significant
BMI (kg/m2)	-0.39	Significant
Systolic	0.043	Non-significant
Diastolic	0.096	Non-significant
HbA1c (%)	-0.66	Significant
BSF (mg/dL)	0.41	Non-significant
BSR (mg/dL)	0.48	Non-significant
TC (mg/dl)	-0.44	Significant
LDL-C (mg/dl)	-0.187	Non-significant
HDL-C (mg/dl)	0.21	Significant
TG (mg/dl)	-0.33	Non-significant
miR-29b	-0.56	Significant

ANRIL with demographic features, biochemical tests, and target miRNA 29b represented in **Table 3**.

Significant correlation of lncRNA H19 with BMI, HbA1c, HDL and miR-181a and significant correlation of lncRNA ANRIL with BMI, HbA1c, HDL and miR-29b.

Discussion

Understanding the molecular aspects of the development of DFU is important to counter the DFU associated amputation.[9] LncRNAs are involved in many chronic diseases and their regulatory roles in DFU development is of great importance in its treatment and early detection of its worse effect.[10] Pathways by which miRNAs modulate their bad outcomes and their target genes which modulate these pathways are of great significance.[11] Molecular pathways hidden in the development of DFU needs to be explore with advanced bioinformatics analysis to cope up the failure in DFU treatment. Interestingly miRNAs are also recognized in previous studies that these can also play a vital role in controlling these biological pathways associated with the healing of DFU linked wounds. To understand the functional role of lncRNAs and their target miRNAs provide an insight to treat DFU accurately and efficiently.

Research work not only evaluated the expression of lncRNAs and their target genes but also represent that miRNAs play a major role in the occurrence of DFU and wound healing process.[13] Real-time PCR analysis of expression of tissue samples, prove to be a reliable and effective tool to trace role of miRNAs and their respective genes.[14]

One of the main consequences of the DFU patients the impairement of self repairing abilities result in impair wound healing either angiogensis disorders, prolonged inflammation phase, destruction of extracellular matrix, or increasing oxiditive stress therefore prevent the ulcer from healin process.[15,16] LncRNAs have both RNA and protein like function, ANRIL is an example of lncRNA which is famous for the regulation of extracellular matrix, and lack of it promote poor healing process which also confirmed by our results low expression in diabetic patients as compared to healthy individuals.[17,18]

ANRIL is also involve in the regulation of growth factors which enhance the regeneration of the rupture skin, enhance the activeness of microvescels, ANRIL is a key player in several other complications of the diabetes as well, such as in renal injury and retinopathy, confirmed it as a promising candidate in foot ulcer treatment as well.[19,20] H19 promotes insulin sensitivity of the skeltal muscles and act as sponge to miR-29b, enhanced the wound healing process in DFU.

Silencing of H19 represent poor wound healing as well as facilitate the healing process in DFU.[21,22] miR-29b delivery through collagen enhance the wound healing by extracellular remodeling and mesenchymal transition in the epithelial matrix formation.[23,24] It also accelerate

the wound healing process involved in extracellular formation. MiR-181a regulate the toll like receptors to promote and enhance wound healing, regulate wound healing process.[25,26]

Conclusion

Selected lncRNAs and their target miRNAs play a vital role in chronic wound healing process, difference among DFU cases and control validate their importance. Further advance and cutting edge technologies are required to unveil the role of these lnvRNA which act as dual player an RNA and a protein and control the function of miRNAs as well.

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