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Miniature Non-Coding RNA- A Key Player in the Chronic Wound Healing

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Abstract

MicroRNAs (miRNAs) are associated with several diabetes complications especially the diabetic foot ulcers development. miRNAs regulate the expression of genes which can lead to delay in wound healing in diabetic foot ulcer and ultimately amputation, a life threatening risk. To trace the role of miRNAs which are associated in the development of diabetic foot ulcer and ultimately amputation, we performed a RT-PCR approach. miRNA-146a, miRNA-21, and miRNA-210 were analyzed with RT-PCR expression analysis difference between diabetic foot ulcer and controls. miRNA-210 over-expressed in diabetic foot ulcers while miRNA-146a and miRNA-21, down regulated as compare to diabetes patients. Screened miRNAs can be used for further investigation therefore their role will be clear to save foot ulcer individuals from amputation.

Keywords

Diabetic foot ulcers, microRNA, Amputation

Introduction

Diabetes Mellitus (DM) delineates the entire population of the world with a devastating force, not only in the developing world but also in the developed world affected with this life time threatening disease [1]. Prevalence stands 9% among all over the world and the Asian population has prevalence rate is much higher as about 10% and almost 60% diabetic individuals falls in Asia and China [2]. Pakistan is among the top ten countries in which DM is upsetting the population with a greater earnestly. If it is affecting with the same rate, then in coming future Pakistan will fall in top five countries with DM burden [3]. DM is connected with a myriad of all life threatening diseases but before it transformed into untreatable condition where reversal is quite impossible it alert the individual be careful that no more mistake now, therefore after DM encounter either genetic factors or environmental circumstances contribution, needs extra care with life activities [4].

Person having prediabetic signs and symptoms or having positive family history has an opportunity to maintain a balance life plan with consultation of endocrinologist to figure out how proper diet plan and regular exercise can save from future complications as follow the footsteps to a healthy life with a million thank to DM that abstained from its complications [5]. Life becomes horrible with uncontrolled DM, sooner or later medications have no effect on the improvement, if proper and regular medicine schedule not followed. After the

complication started having uncontrolled DM, not only for the patient but also for the physician are in trouble to cure its complicatedness [6]. With the passage of time risks of harm to any organ of the body is quite obvious, one of the major organ which affected with it is foot, and foot not only lift the whole weight of the body for movement but also stable the body in its proper form. Life stops if the problem in the foot and how critical life becomes if the complete foot is lacking [7]. Structure and size of the foot looking very simple and short as compared to other body parts but it is very important and complicated organ, there is a solution to fracture but no solution to infection if it is not treated in time and with proper diagnosis as well under the keen observation of diabetologist [8].

One of the greatest upset confront of uncontrolled DM is the Diabetic Foot Ulcer (DFU), very serious and very costly human problem which starts with a simple infection and ends with an amputation [9]. With every thirty second an amputation results in the entire world which is an alarming condition but the world is not taking is as seriously as it need to be. It is the most common complication in DM patients who have uncontrolled DM in the form of substandard glycemic level, peripheral vascular and neuropathy disease, most common cause of osteomyelitis and amputation [10]. It is considered a disease of the old people but young are also affected now days with a same ratio, one of the examples is excess use of the choice of junk foods which shoot the sugar level to extreme end and basic nutrients are far away from these ready to use foods as life becomes fast and not having time for healthy life plan and routines, plus involved in smoking and drugs as a fashion, putting an extra harmful effect [11]. A person already having DM and involved in mistake welcome the terrible face of diabetic complication, therefore young or adult both are affected with this foot ulcer disease [12].

The prevalence of diabetic foot ulcer in diabetic patients is about 15% worldwide with a little plus minus variation. Almost every diabetic patient encounter with the foot ulcer problem, foot ulcers are with the advancement of medical treatment heals 60 - 80%, with proper treatment and care. Only 15 - 25% takes extra time for healing and remaining 5 - 10% are not able to recover as healing is not occurring with the passage of time and before they cause affecting remaining part of the body, therefore needs amputation, to save the remaining part of the body from damage [13]. Distal part of the body which is usually ignored by a person if never feel anything by touch or if the case is in which sensations are disturbed with the neuropathy [14]. Following cleaning the body parts practice not only saves us from dirt but also germs action to our body [15]. Movement is the key to healthy life therefore move every muscle of the body so that it will be remaining active [16, 17].

Some studies shows men are mostly affected with DFC as compared to women but cases are quite different with region and according to customs of different cast systems; women are also high in number more than 54% females in Pakistan as they are more susceptible, as they subsidized their responsibilities in adult age which also affect their overall health [18]. Person who is smoking is also on the risk of many or most of the diseases. Here again data is an eye opener for a smoker as major diseases represent strong connection with smoking, and now the smoking is transformed to increase expeditiously its effect on health as exist in very dangerous form that is using a pipe process and equal to harm the body in a single encounter similar to smoke a complete pack at one time [19]. Dealing with ulcer at home such as called bathroom surgery are very dangerous and lack of education encounters it largely.

Materials and Methods

Collection of Samples

Tissue samples of both controls and patients from human source approval were taken from Ethics Review Board of the Department of Biosciences, COMSATS University Islamabad and International Diabetic Foot Center Islamabad. Skin biopsy samples from patients after informed consent and following strict inclusion/exclusion criteria of adult's age more than 49 and below 71 years of age, non-smokers, not having any other diabetic complication. Samples were collected during routine wound debridement or surgery and stored in pre-filled manually 1 ml RNA Later solution (Applied Biosystems, Carlsbad, CA) in 2 ml cryovials (Thermo Scientific, Darmstadt, Germany) tubes on ice throughout the shipping to Translational Genomics Laboratory, where processed immediately on the particular days.

RNA extraction from Tissue

Total RNA extraction was done using the RNA purification kit (Norgen Biotek Corp, Thorold, Ontario, Canada) as following instructions provided with the kit.

Homogenization

Excised a small part of the tissue and chopped into smaller pieces on petri dish. Added 400 µl of buffer SK containing guanidinium salts, into the screw cap vials along the chopped tissue into the vials and homogenized completely using hand held Tissue Master 125 homogenizer (Omni International, Kennesaw, GA). Spin the lysate for 2 minutes at 14,000 RPM to pellet any cell debris and at the end transfer the supernatant to another RNAse-free micro centrifuge tube. Equal volume of 70% ethanol added and tubes were vortexed for few seconds. Spin columns provided with the kit assembled with the collection tubes 600 µl of lysate added and centrifuged for 2 minutes at 10,000 RPM and then washing with wash solution A followed by repetition with 2 minutes at 13,000 RPM, the flow through was discarded. Blank spin to dry the column completely centrifuged for 2 minutes at 13,000 RPM, the columns were assembled to elution tubes. Elution solution about 50 µl added and centrifuged for 1 minutes at 14,000 RPM. The RNA were stored at -40 °C.

RNA Quantification

RNA extracted was quantified nanodrop spectrophotometer. An amount of 1 µl DEPC water was loaded as a standard reference on RNA/DNA quartz cuvette. Added 1 µl of the extracted RNA on the cuvette, absorbance at 260 nM to 280 nM (A260/A280) was used to get the concentration of

RNA. Thresholds of 250 ng/µl set as standard below that concentration were not used for cDNA synthesis.

RNA Denaturing Gel Electrophoresis

Denaturing gel 1.2% was used to evaluate the extracted RNA, made by 0.84 g of agarose (Invitrogen, Germany) added in 70 ml of 1X TAE buffer by warming in the microwave oven under low power P80 heat used for 1-2 minutes. Bleach (Clorox®) 140 µl poured into the heated solution, after cooling of the solution for few minutes added 7 µl of ethidium bromide (Sigma Aldrich, USA) for visualization and poured the solution to gel caster to solidify. A cocktail of 8 µl was made: 2 µl bromophenol blue (Scharlau, Germany) as loading dye and 6 µl of formamide per sample then 5 µl of the RNA sample. Cocktail denatured at 70 °C for 2 minutes followed by cold shock at -20 °C for 1 minute. Samples were then loaded in the wells and gel apparatus connected with power supply to run at 500 mA and 80 Volts for 40minutes.

cDNA synthesis

The extracted RNA samples were converted to cDNA using RevertAid First Strand cDNA synthesis kit (Thermo Fisher Scientific Inc., Waltham, MA) provided the manufacturer's protocol. For each sample total RNA 5 µl, Random hexamers 1 µl and PCR water 6 µl made total volume of 12 µl. PCR at 65 °C for 5 minutes and hold at 16 °C. For heat shock put the tubes at -20 °C for 1 minute. Second reaction 5X First Strand Buffer 4 µl, RNAse inhibitor 1 µl, dNTPs 2 µl and M-MLV 1 µl and final volume of 20 µl and PCR (25 °C for 5 minutes, 42 °C for 60 minutes, and 70 °C for 5 minutes, hold at 4 °C). Run the PCR product on gel for confirmation.

The relative level of GAPDH and the miRNAs were calculated using Livak and Schmittgen, 2001 $2^{-\Delta\Delta Ct}$ analysis technique. For expression analysis the Ct values of miRNAs were normalized with housekeeping gene (GAPDH).

Results and Discussion

One hundred individuals participated in the current study among them 50 controls and 50 DFU patients. The mean age of controls and participants was 55±10, as adult population affected with the disease. We have divided 25 males and 25 females in both control and diseased individuals to further eliminate the biasness of the research work. Strict inclusion/exclusion criteria followed as described in methodology section. Gene expression of the three selected miRNAs (miR-21, miR-146a, and miR-210) were included in the study. The relative gene expression has been represented in the form of bar graph, and respective fold changes between control and disease groups. These graphs represents the relative gene expression significantly up regulated in DFU in case of miRNA-210 while in the case of miRNA-21 and miRNA-146a significantly down regulated as compared to control groups against DFU patients. The relative fold change expressions were represented in the form of bar graphs.

miRNA-210 over-expressed in DFUs while miRNA-146a and miRNA-21, down regulated as compared to diabetes patients. Hsa-miRNA-210 showed a threefold increased expression in DFU as compare to DM controls (Figure 1).

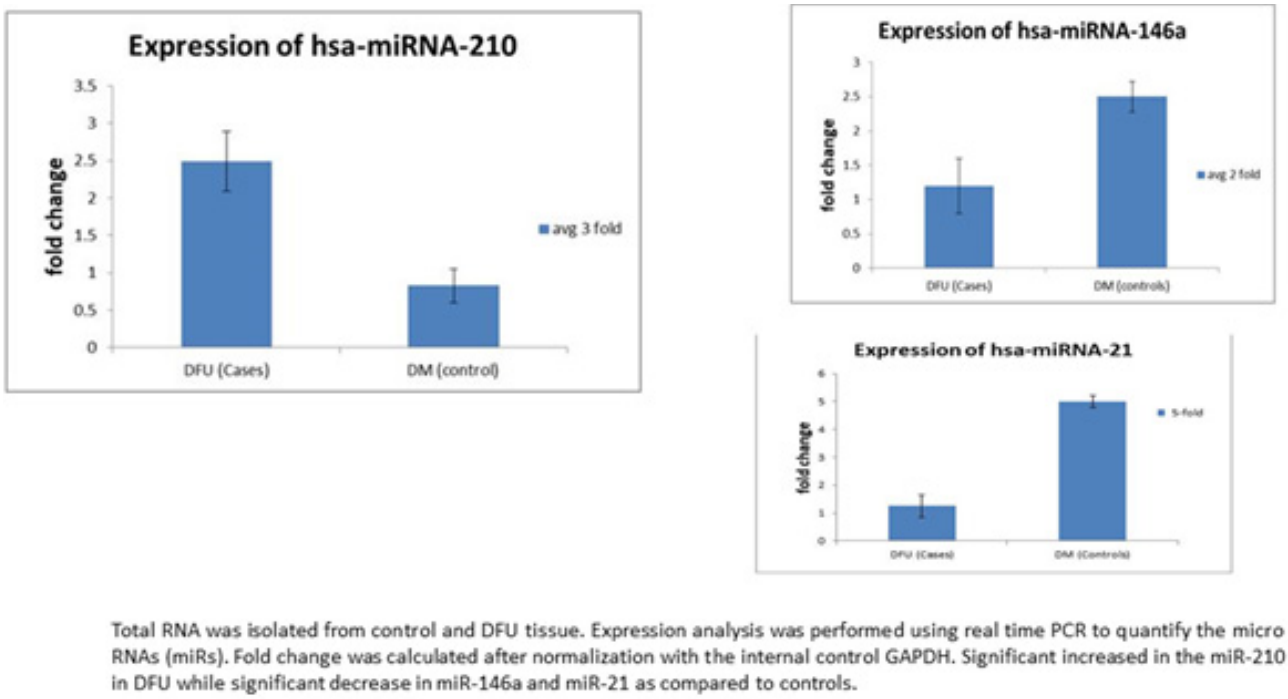

Figure 1: Over expression of hsa-miRNA-210 and decreased expression of hsa-miRNA-146a and hsa-miRNA-21 expression decreased to twofold and fivefold respectively in the DFU patients against the controls. Relative fold change difference represented.

Hyperglycemia is a root cause of diabetic related complication and foot ulcer healing required a strict glycaemic control. Proper and timely diet is also an important factor to get tight glycaemic control. Insulin is more effective as compared to oral medicine. Hypertension is also associated with poor wound healing therefore it should be treated well while treating the foot ulcer. Smoking is another negative impact on wound healing therefore abstained from smoking has a good impact on wound healing [20]. Neuroischaemic ulcers patients should take statins and antiplatelet. Education is the foremost important as it will help them how to get protection of the feet from mechanical, thermal, and chemical trauma [21]. Patients should be educated how to take care of their foot by adopting precautionary measures such as emphasis on the importance of taking the rest, proper and comfortable footwear, regular dressing of the ulcer wound area and have a close look to prevent from infection and focus on early signs of infections [22]. Few things regarding ulcer keep in mind very noticeably, first and foremost thing to observe any swelling, pain on any part, alteration in the color and any breakage in the skin, consult the podiatrist as soon as noticed [23].

Expression of molecular factors including growth factors are of prime importance such as platelet derived growth factor (*PDGF*), keratinocyte growth factor (*KGF*), vascular endothelial growth factor (*VEGF*), insulin-like growth factor (*Igf-1, Igf-2*), miRNA-146 (*mir-146*), stromal cell derived factor-1 alpha (*SDF1-α*), hypoxia induced factor-1 alpha (Hifi-α), are reported to be downregulated while the expression of interleukins such as interleukin-6 (*IL-6*), interleukin-8 (*IL-8*), tumor necrosis factor alpha (*TNFα*), regulator gene that control transcription c-Myc, receptor activator of kappa ligand (*RANKL*), and matrix metalloproteinases-9 (*MMP-9*) are reported to be unregulated [24].

In other diabetic complications number of miRNAs are found to be associated with down/upregulation of number of genes e.g. expression of miR-27, miR-146b, miR-185, and miR-146a and miR-143 decreased result increased into down regulation of target genes *Foxo1, SREBP, SIRT1* and *LDLR* while upregulate NF-kappaB. Some of the miRNAs which are found to be associated with DFU includes: has-miR-31-3p, has-miR-31-5p, has-miR-10-5p, has-miR-136-5p, and has-miR-338-3p which are found to be significantly down-regulated in diabetic foot skin. Although a number of studies have been done to elucidate the genetic bases of DFU but comprehensive data is still lacking to unveil the underlying genetic mechanism of the disease [25]. To find a proper remedy for DFU a good population specific genetic data is required which may provide insights to devise new strategies for diagnosis and treatment. Comparative transcriptional profiling in cellular responses is helpful in identification of molecular factors which are involved in intracellular mechanisms [26]. Previously it has been proposed that expression analysis of the particular set of genes which show elevated level of expression in diabetic foot ulcer patients and are not expressed in normal population and also the vice versa that particular set of genes which are present in normal individuals but absent in diabetic foot ulcer snuffer's might be of help in understanding the pathophysiology of DFU and such data might be used to identify specific molecular markers that can be used for disease diagnosis or treatment. As the molecular data regarding pathophysiology is the purpose of this study is to genetically analyze DFUs specific miRNAs and their differentially expressed target genes in Pakistani DFU subjects. Identification of miRNAs and expression analysis of their target genes that are involved in DM and development of DFUs will be conducted [27]. There are many pathways and signaling factors are involved in DFU as result from deregulation miRNAs involved in pathogenesis. They play a vital role in wound healing such as inflammatory response and particularly in the tissue growth [28].

Regulation and signaling governs by miRNAs develop better understanding the phenomena of wound healing. miRNAs are synthesized by polymerase II as mRNAs pattern, but then go processing by multiple enzymes in nucleus and cytoplasm that shape them into mature form called mature miRNAs, process of assembly which is in the form of RISC formation. For functioning of these miRNAs they base pair with mRNAs in a Watson and Crick base pairing with their seed sequence usually 6-8 base pairs long. There is a simple phenomenon of action of these miRNAs that a single miRNAs can target and involved in the regulation of hundreds of genes and similarly single gene is under the control of multiple miRNAs, make them an important part of the regulation mechanism [29]. Specific miRNAs which are present in the skin has important involvement on wound healing process, for example there is an important miRNA-21 has an important role in skin morphogenesis and keratinocyte differentiation by targeting p63. In normal skin the expression of the miRNA-21 is unregulated while in psoriasis its expression is down regulated, it is of great interest that psoriasis and chronic wound healing has the same molecular pathology as same pattern founded in both of them. miRNA-21 and miR-146a are also involved in targeting the wound healing process genes such as early growth factor of wound healing and leptin receptor [30]. Based on the literature search these miRNAs are very promising molecular targets for the early detection and treatment enhancement for the chronic wound healing process. miRNA-210 was previously associated with target gene HIF1α in diabetic mice and in the tumor developing towards cancer. Recently miR-210 over expression was discovered in case control study [31].

Conclusion

The differential expression of miRNAs in case control study was observed on DFU tissues compared to that of control subjects of diabetic without any further complication. These observed findings the miR-210 over expression and miRNA-21 and miRNA-146 down regulation involved in the molecular response on DFU. Most importantly this study demonstrated that the elevated expression of miRNA was positively associated with inflammation signifying its possible diagnostic value. Down regulated miRNAs have their importance as well in therapeutics. Further analyses are required to eradicate the root cause of DFU not healing mechanism and save DFU patients from amputation.

Compliance with Ethical Standards

The work has complied with ethical standards.

Conflicts of Interest

The authors have no competing interests to declare.

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