Expression of LncRNA-MALAT1 in Type 2 Diabetic Patients with or without Coronary Artery Disease

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Abstract: The development of type-2 diabetes mellitus (T2DM) has been linked to environmental, genetic, and epigenetic risk factors. Long non-coding RNAs (lncRNAs) play a role in the pathophysiology of complicated disorders and can also be used as diagnostic indicators. The literature strongly supports the implication of these lncRNAs in certain metabolic disorders. In this regard, one of the lncRNAs Metastasis Associated Lung Adenocarcinoma Transcript 1 (MALAT1) expressions is also under investigation concerning diabetes and its related complications. There are few researches on the relationship between T2DM progression and MALAT1 dysregulation, and this study was undertaken to investigate this link further and gain a better knowledge of its clinical significance in the diagnosis and prognosis of T2DM. Therefore, the purpose of this study was to assess the gene expression of lncRNA MALAT1 in healthy controls, type 2 diabetics, and T2DM patients with the presence of coronary artery disease complications. This study was the case-control one performed on blood samples followed by centrifugation to separate the buffy coat. Then total RNA was extracted from buffy coat using triazole method followed by quantifying its expression levels by quantitative polymerase chain reaction (qPCR). The results of our study showed the significant up-regulation of lncRNAs-MALAT1 expression in patients with T2DM 1.22 (3.7) and T2DM with related coronary artery disease (CAD) complications 1.95 (2.10) (p-value <0.001). These findings suggest that modulating the expression of lncRNA MALAT1 may be a future strategy for diagnosing and treating diabetes-related complications like CAD.

Keywords: long non-coding RNAs, metastasis associated lung adenocarcinoma transcript 1, quantitative polymerase chain reaction.

長鏈非編碼核糖核酸-轉移相關肺腺癌轉錄本一在伴或不伴冠狀動脈疾病的二型糖尿病患者中的表達

摘要：二 型糖尿病的發展與環境、遺傳和表觀遺傳風險因素有關。長鏈非編碼核糖核酸在復雜疾病的病理生理學中發揮作用，也可用作診斷指標。文獻強烈支持這些長鏈非編碼核糖核酸對某些代謝紊亂的影響。在這方面，長鏈非編碼核糖核酸轉移相關肺腺癌轉錄本 一表達之一也在研究糖尿病及其相關並發症。關於二 型糖尿病進展與轉移相關肺腺癌轉錄本 一失調之間關係的研究很少，本研究旨在進一步探討這一聯繫，並更好地了解其在二 型糖尿病診斷和預後中的臨床意義。因此，本研究的目的是評估長鏈非編碼核糖核酸轉移相關肺腺癌轉錄本 一在健康對照、二 型糖尿病患者和存在冠狀動脈疾病並發症的二型糖尿病患者中的基因

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表達。這項研究是對血樣進行的病例對照研究，然後離心以分離血沉棕黃層。然後使用三唑法從血沉棕黃層提取總核糖核酸，然後通過定量聚合酶鏈反應量化其表達水平。我們的研究結果顯示，在二 型糖尿病 1.22 (3.7) 和具有相關冠狀動脈疾病並發症 1.95 (2.10) 的二 型糖尿病患者中，長鏈非編碼核糖核酸-轉移相關肺腺癌轉錄本 一 表達顯著上調（p 值 <0.001）。這些發現表明，調節長鏈非編碼核糖核酸轉移相關肺腺癌轉錄本 一的表達可能是診斷和治療糖尿病相關並發症（如冠狀動脈疾病）的未來策略。

关键词：長鏈非編碼核糖核酸，轉移相關肺腺癌轉錄本 一，定量聚合酶鏈反應。

1. Introduction

Diabetes is one of the most common diseases globally, with significant rates of morbidity and mortality among adults. According to the 2019 Atlas of the International Diabetes Federation (IDF), the estimated number of cases suffering from diabetes is figured as 463 million [1, 2]. It has been assessed that around 700 million individuals will be affected by this disorder by 2045. The middle-east and North African regions are the most affected, carrying the highest prevalence of diabetes (10.9%). As far as Pakistan is concerned, the overall prevalence is 17.1. The International Diabetes Federation (IDF) considers diabetes to be one of the most serious health problems, affecting 8.8% of the world's population, with 91% of those suffering from adult-onset type 2 diabetes (T2DM) which is noninsulin-dependent diabetes that develops in adults [1]. It occurs secondary to progressive dysregulation and dysfunction of β-cells of the pancreas resulting in insulin resistance. It is also evident from previous literature that ecological and underlying genetic factors amplify its pathogenesis like any other multifactorial conditions [3]. Compared to non-diabetic patients, T2DM patients have an increased chance of getting CAD (Coronary artery disease) [4]. It has been estimated that 52% of the deaths of T2DM patients occur as a result of subsequent development of cardiovascular complications, including CAD [5].

Being a unique and novel class of non-coding RNAs, Long non-coding RNAs (lncRNAs) are transcripts that are nearly 200 nucleotides long [6] that can control and regulate the expression of certain genes by several roles in cell cycle control, epigenetic regulation, chromatin modification, transcription, cellular differentiation, and other essential natural processes [7]. LncRNAs (long non-coding RNAs) regulate transcription in various functional and pathological processes. In addition, the unusual aberrant expression of these lncRNAs has been discovered in patients who have type 2 diabetes [8]. Some recent studies have linked lncRNA dysregulation to several ailments, so this is one of the widely studied lncRNA in this regard. The expression levels of MALAT1 have been linked with the processes associated with diabetes-related inflammation and hypoxia, along with diabetic retinopathy, nephropathy, and glucose metabolism dysregulation. [9]. Recent studies proved the active role of MALAT1 in the pathogenesis of various metabolic diseases such as diabetes mellitus and its related cardiovascular complications such as CAD [10]. MALAT1 stands for Metastasis Associated Lung Adenocarcinoma Transcript 1. NEAT2, NCRN00047, HCN, PRO2853.20, and LINC00047 are other names for this gene. It was discovered in NSCLC (Non-Small Cell Lung Cancer) patients for the first time [8]. It has over and above 800 nucleotides and is positioned on chromosome 11 (11q13.1) and localized in the nucleus. It is expressed in almost every tissue of human beings, but the lungs and pancreas have shown the maximum levels of its expression [11].

Jingshu Chen et al. suggested that MALAT1 generates reactive oxygen species (ROS), which activates JNK Pathway inhibiting insulin receptor substrate 1 (IRS-1) and insulin-induced phosphorylation of serine/threonine kinase Akt, eventually leading to insulin resistance that in turn leads to insufficient glycolysis, inappropriate fatty acid oxidation and augmented gluconeogenesis causing hyperglycemia [12]. Long-standing hyperglycemia-associated complications are classified as macrovascular and microvascular. Hyperglycemia-induced inflammation damages the endothelium of the vessel wall, accelerating deposits of lipid, cholesterol, and plaque buildup in the arteries leading to atherosclerosis, ultimately resulting in one of the macrovascular complications, i.e., CAD [13]. As there are limited studies on the correlation of T2DM progression with MALAT1 dysregulation, this study was conducted to explore this relationship further and better understand its clinical relevance in the diagnosis and prognosis of T2DM. This study aims to evaluate and compare the expression levels of lncRNAs MALAT1 in healthy controls, diabetics, and diabetes with CAD.

2. Materials and Methods

2.1. Study Population

It was a case-control study comprising 200 participants, including 100 control individuals and 100 cases, which comprised 50 people with diabetes (T2DM) and 50 diabetic patients with CAD. One hundred healthy volunteers who were matched based on sex, age, and without diabetes participated in this study as controls, whereas cases were recruited from Ziauddin Hospital Clifton campus Karachi from Nov 2020 to Sept 2021. Sampling was done using the purposive sampling technique. The Ethics Review Committee has approved the current research of Ziauddin University (ERC No 2520820SAPAT). Informed consent in writing has been obtained from all participants of the study. Demographic details and the comprehensive history of all the participants were collected and documented through a questionnaire. Controls and Diabetic patients were selected based on history and lab reports of HbA1c and lipid profile. Diabetic with CAD patients were selected based on patients' HbA1c, lipid profile, and angiography reports. CAD patients were selected amongst known diabetic patients who got a referral to the hospital due to symptoms depicting CAD necessitating coronary angiography. Adamson et al. defined CAD in angiography as the evidence of more than 50% stenosis in at least one major epicardial coronary artery [14] were recruited. Patients with Type 1 diabetes, any congenital heart disease, infectious heart disease, or any other macrovascular or microvascular complication of T2DM, dilated CMP, conduction defects, or any complications of T2DM like neuropathy or nephropathy and Pancreatic retinopathy disorder (Pancreatitis or pancreatic cancer) were excluded.

2.2. Procedure

Buffy coat was extracted from blood samples for RNA isolation. RNA extraction was done using Trizol™ reagent (Thermo Fisher Scientific, Waltham, MA, USA). 1ml Trizol was added in 400 ul of buffy coat and incubation for 10 mins at room temperature. Then chloroform (200 ul) was added, followed by incubation for 15 mins, then centrifugated at 12,000 rpm for 15 mins at 4ºC. For RNA isolation, the upper aqueous phase was used. 2mL isopropanol was added in order to precipitate the RNA. Then centrifugation was performed for 10 mins at 12000 pm followed by careful aspiration of the supernatant, and 1 ml of 75% ethanol was added to the tubes. The supernatant was discarded after centrifugation; the pellet was air-dried and resuspended in 20ul Nuclease-free water. The concentration and purity of the isolated RNA were analyzed by Multi Scan Sky spectrophotometer. 1 ug of RNA was reverse transcribed by using Revert Aid First Strand cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA) using manufacturer's protocol. The reaction mixture of RNA, primer and DEPC treated sterile were incubated at 65 ºC for 5 mins, with the subsequent addition of 5X reaction buffer, dNTPs, Ribolock, and RNAase out (20u/µL). The tubes were incubated at 42°C for one hour and then at 70ºC for 10 mins. *Primer3* design program was employed for designing the primers for both the genes (http://frodo.wi.mit.edu/primer3/) and acquired from Penicon. Reconstitution of primers was carried out in 10 mMTris-HCl/EDTA (TE) buffer (pH 8). The sequences of the primers used are as follows (Table 1).

Table 1 Sequences of the primers used

S.no	Gene of Interest	Primer
1	MALAT1 (F)	GACGGAGGTTGAGATGAAGC
	MALAT1 (R)	ATTCGGGGCTCTGTAGTCCT
2	GAPDH (F)	CCAGAACATCATCCCTGCCT
	GAPDH (R)	CCTGCTTCACCACCTTCTTG

Expression of MALAT1 was analyzed by Real time-PCR. As an internal control, the GAPDH gene was used. 0.4 µl mixture of cDNA and 5µl primer were added to 4.6 µl of SYBR green master mix to make a total volume of 10µl. The reaction was run for 40 cycles of denaturation (92^{o}C), annealing, and extension (72^{o}C). Finally, the acquired CT values and the relative fold change were calculated for further analyzing the expression of the study gene as compared to control by using the following formula:

$\Delta\Delta CT = \Delta CT$ (target gene) - ΔCT (internal reference gene)

where ΔCT is the number of cycles, the fluorescent signal in each reaction tube reaches the set field value, and $\Delta\Delta CT$ is a convenient and simplified form of the formula for calculating relative fluorescence quantification when the samples are being compared for differences or ratios.

2.3. Statistical Analysis

SPSS program (version 20) was used for analyzing the data. The data were statistically analyzed in three comparison groups. For the evaluation of normality of the distribution, the Shapiro-Wilk test was applied. As the data was not normally distributed Kruskal-Wallis test was used, and the variables were represented as median and IQR. The Mann-Whitney U test was applied for inter-group comparison of the not normally distributed variables. Categorical variables were characterized as frequencies and percentages. The ROC curve was used to estimate the discrimination potential of MALAT1 in the groups. A P-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Demographic and Clinical Characteristics of Subjects

In our study total of 200 participants were enrolled, out of which 100 were healthy controls, and 100 were

cases further divided as 50 diabetic patients and 50 diabetic patients with CAD complications. In this study, 81 (40.5%) were women, and 119 (59.5%) were men with a mean age of 49 ± 12 years. In the control group, 58 (29%) were male, and 42 (21%) were females, whereas in other cases, 61 (30.5%) were male, and 39 (19.5%) were females. The demographic data of study participants are presented in Table 2.

Table 2 General characteristics of study participants

Variables	Control Median(IQR)	Diabetic Median(IQR)	Diabetic with CAD Median(IQR)
Age	42 (19)	50 (15)	60 (15)
weight	59 (10.8)	65 (12)	80 (20.5)
Height	5.4 (0)	5.2(1)	5.7 (1)
BMI	21.6 (4.5)	24.9 (3.4)	28.2 (2)
Duration of Diabetes	0	4 (2)	6 (4)

3.2. Fold-Change Expression of LncRNA MALAT1 in Three Comparison Groups

We calculated the fold change of MALAT1, which came out to be 1.22 (p-value = 0.004) for diabetic and 2.1 (p-value = 0.000) for patients having diabetes with CAD (Table 3). The fold change of cases was then compared with the fold change of control, i.e., 1. Fig. 1 shows comparative relative fold change expression of MALAT1 in control, people with diabetes, and people with diabetes with CAD. As compared to control, people with diabetes showed highly significant up-regulation of the MALAT1 gene (p-value <0.01) while people with diabetes with CAD showed very highly significant up-regulation of MALAT1 (p-value < 0.001).

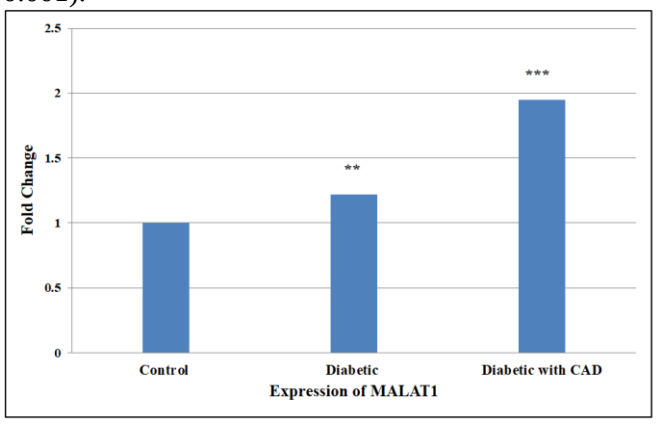


Fig. 1 Gene expression analysis of MALAT1 showing significant up regulation in patients and patients with CAD when compared to control

Notes: * p-value <0.05, ** p-value <0.01, *** p-value <0.001) (* significant, ** highly significant, *** very highly significant

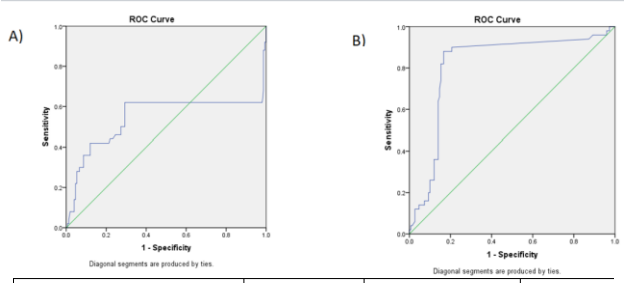
Table 3 Comparison of MALAT1 expression among control, diabetic, and diabetic with CAD groups

Comparison	Fold change Expression	P value
Control	1	<0.001[a]
Diabetic	1.22 (3.7)	
Diabetic with CAD	1.95 (2.10)	
Control	1	0.004[b]
Diabetic	1.22 (3.7)	
Control	1	0.000[b]
Diabetic with CAD	1.95 (2.10)	

Notes: The data is represented as median and IQR
CAD - coronary artery disease
[a] Kruskal-Wallis test, [b] Mann-Whitney U-test

3.3. ROC Curve Analysis

According to the ROC results, AUC values of MALAT1 showed increased diagnostic value and significance in a person with diabetes with CAD than diabetic group alone (AUC = 0.815, p-value < 0.001) (Fig. 2).



Gene(MALAT1)	AUC	CI	P value
A) Diabetic	0.545	0.424±0.665	0.346
B) Diabetic with CAD	0.815	0.742±0.889	<0.001

Fig. 2 Receiver operating characteristic (ROC) curve of lncRNA MALAT1 using fold change expression levels: A) Diabetic patients; B) Diabetic with CAD patients. AUC: Area under the curve; CI: Confidence interval

4. Discussion

In the current work, the expression level of the lncRNAs, namely MALAT1, was assessed in three comparison groups. LncRNAs have a role in multiple functional and pathological processes. LncRNAs are better recognized as regulatory RNAs, which significantly impact certain genes involved in pathophysiological pathways in some diseases, so the evaluation of these expressions is found to be helpful in diseased conditions. As presented in Fig. 1, the current study results showed that the MALAT1 expression level in diabetics and patients with diabetes with CAD was significantly higher with a p-value of 0.004 and 0.000, respectively, compared with healthy control. So the existence of either of these two states, diabetes alone or diabetes with CAD, was related to the significantly higher expression level of the study gene. Previous studies also report the upregulation of MALAT1 in the endothelium of both small and large arteries and their enhanced expression in the conditions of hypoxia, hyperglycemia, and conditions of oxidative stress [15]. In a study conducted by Vausort and his colleagues, it was found that the individuals having myocardial infarction exhibited increased MALAT1 expression [16]. Another study in this regard has been done by Zhang et al. [17] on diabetic rats, which came up with a similar finding of raised MALAT1 expression in heart tissue of diabetic rats. Other studies reported the association of reduced MALAT1 expression in diabetes-induced animal models with the diminution of the microvascular dysfunction in 2016. Yan et al. tested MALAT1 expression in liver tissue of animal model, which demonstrated correlation of

increased MALAT1 expression with insulin resistance [18].

Sohrabifar et al. has recently carried out a case-control study in the Iranian population, including cases comprising of CAD patients with or without T2DM compared with controls having diabetic and non-diabetic individuals with a purpose to assess the relationship of special lncRNAs including MALAT1 with CAD, T2DM, and both of them concurrently via analyzing their expression. This study concluded that the elevated expression level of MALAT1 may be seen in CAD or diabetic patients or patients suffering from both CAD and DM, thus signifying the adequate diagnostic power of MALAT1 on ROC results in specific results conditions such as patients presenting with both CAD and T2DM. However, the study also indicated that the raised expression of MALAT1 is not definite to these conditions [19]. Another study conducted on patients diagnosed with fewer than five years of type 2 diabetes and compared it with non-diabetic subjects came up with contrary findings with a significant decrease in MALAT-lncRNA levels derived from serum or serum exosomes in association with type-2 diabetes. Additionally, they also reported a significant downregulation in MALAT1 expression levels of serum exosomes in patients having metabolic syndrome. The predictable reason underlying the contrary results of this study compared to most other studies claiming association of its higher expression levels of MALAT1 with progressive T2DM could be due to the analysis of MALAT1 levels in serum and exosomes rather than buffy coat [9]. Alfaifi et al. further elaborated the clinical significance of the elevated expression of circulating MALAT1 in patients with T2DM with poor patient outcomes. A somewhat similar study reported that MALAT1 had been connected to alcoholism, and smoking has been shown to influence the severity and extent of disease in T2DM patients [20]. Eman et al. conducted a case-control study on 110 stable CAD patients and 117 age-and sex-matched controls. The presence of MALAT1 relative expression levels in the circulatory system of stable CAD patients suggests the possibility of a link between MALAT1 with coronary artery disease and reinforces that the association was more apparent in CAD patients with known cardiac ischemia [21].

The studies above have revealed that the level of MALAT1 expression is elevated in CAD and T2DM, and the current work has reached comparable results compared to other studies in our population.

Therefore, there is a significant association between elevated expression of MALAT1 lncRNA and progressive T2DM. However, wide-scale longitudinal studies in this regard are prerequisites to investigate the involvement of hyperglycemic conditions in the overexpression or decreased expression of MALAT1.

5. Conclusion

Some studies highlighted the pathogenic significance of the lncRNAs in the progression of diabetics and their chronic complications and their role as biomarkers for various diseases. There is scarce literature on the relationship between T2DM progression and lncRNA MALAT1 dysregulation. This study was undertaken to investigate this link further. Our study reports the clinical relevance of upregulation of MALAT1 as the disease progresses to severity. This finding suggests that modulating the lncRNA MALAT1 expression may be a future method for diagnosing and treating diabetes-related complications like CAD. The study's limitations included a relatively small sample size, the use of only one biomarker, and the MALAT1 expression level was only investigated in diabetic CAD complications. We suggest that this marker should be investigated in other macrovascular and microvascular complications of diabetes and CAD patients on a larger cohort.

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Ethics Approval

Ethical approval was taken from the institutional approval from the Ethical review committee (Reference code: No 2520820SAPAT).

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