

Circulating Antimicrobial Peptide LL-37 Status in Type 1 Diabetes Mellitus and its Relation with Glycemic Control

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ABSTRACT

Antimicrobial-peptides are important molecules of constitutive innate immunity. Though patients with diabetes mellitus are generally prone to infections, there is limited information on their antimicrobial-peptide status. We assessed the circulating LL-37 antimicrobial peptide (also referred as cathelicidin) levels in patients with type 1 diabetes mellitus and its relation with their glycemic status. The LL-37 mRNA expression was assessed in the peripheral blood mononuclear cells (PBMC) by quantitative RT-PCR using β -actin and *cytochrome-C1* as the reference genes in 154 subjects (Type 1 diabetes, n=111 and healthy subjects, n=43). Serum LL-37 was quantified using sandwich-ELISA. Average HbA1c over last 2 years and current HbA1c were used to determine long-term and short-term glycemic status. LL-37 mRNA expression and serum LL-37 levels were correlated with the glycemic status. The LL-37 mRNA copies were comparable between type 1 diabetes and healthy subjects [median (IQR) = 6.7 (1.8–15.28) vs. 7.2 (2.23–21.86), respectively, P = 0.42]. There was no significant difference in serum LL-37 levels between the two groups [median (IQR) = 3.9 (2.88–7.52) vs. 5.0 (3.19–9.05) ng/ml, respectively, P = 0.52]. The LL-37 mRNA and its protein concentration showed no significant correlation with the average or current HbA1c values. The constitutive circulating antimicrobial peptide LL-37 status is not significantly altered in patients with type 1 diabetes mellitus and also not affected by their glycemic status.

Keywords: Innate-immunity, diabetes mellitus, antimicrobial peptide LL-37, cathelicidin, glycosylated hemoglobin.

Introduction

Recurrent bacterial and fungal infections of genitourinary tract, skin, and lungs are common in patients with type 1 diabetes mellitus (T1DM) (1-3). The reasons of increased susceptibility to infections in diabetes are not clear but could involve hyperglycemia mediated abnormalities in innate immunity involving polymorphonuclear cell dysfunction and various antimicrobial peptides (4-5). The LL-37 antimicrobial peptide (cathelicidin) is an essential component of the innate immunity. It is

produced by several cells such as leukocytes, keratinocytes and mucosal epithelial cells to promote phagocytosis in macrophages, dendritic cell differentiation, chemokine production, keratinocyte migration and wound healing. LL-37 also inhibits apoptosis of leukocytes and biofilm formation (6-9).

Though there is increasing interest in the role of LL-37 in bacterial infections, HIV and tuberculosis, the information on LL-37 expression in diabetes is limited. Since patients with uncontrolled diabetes may be particularly

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prone to infections, LL-37 expression might be altered in them (10-13). Earlier studies involving limited number of patients showed variable status of LL-37 in diabetes. Gonzales *et al* and Santiago *et al* reported reduced LL-37 mRNA expression in circulating leukocytes in DM and peri-ulcer skin biopsy of patients with diabetic foot, respectively (11, 12) and Brauner *et al* showed reduced LL-37 levels in T1DM as compared to patients with type 2 diabetes (13). The relationship of glycemic control with circulating LL-37 has not been studied till date. Present study investigated LL-37 mRNA expression in peripheral blood mononuclear cells (PBMC) and serum LL-37 concentration in a cohort of 111 patients with T1DM and assessed its relation with their glycemic status.

Materials and Methods

The study included 111 patients with T1DM attending 'Diabetes of young' clinic at the All India Institute of Medical Sciences, Delhi. Pregnant, lactating women and patients with overt bacterial infections were excluded. Their details including age at onset of diabetes, nephropathy, retinopathy, pulmonary tuberculosis and serial HbA1c values during previous two years were noted from clinical records. Forty three healthy subjects with normal HbA1c who consented for the study were included for comparison.

Quantitative Real Time Reverse Transcription PCR (qRT-PCR) for LL-37 mRNA Expression

Ten ml blood was collected in fasting state in nuclease-free heparinized tube for isolating PBMC by Ficol and RNA extraction using Trizol (Invitrogen, Carlsbad) (14). RNA was quantified using UV spectrophotometer (GeneQuant, Amersham) and its quality was assessed by agarose gel electrophoresis and RNA integrity number (Bio-analyser 2100, Agilent Technologies Inc.). First strand of cDNA was prepared using 2.0µg of total RNA, random hexamer nucleotides and M-MuLv reverse

transcriptase in 20µl reaction incubated at 25°C for 10 min followed by 42°C for 1 hour (14).

The qRT-PCR for LL-37 mRNA was carried out using SYBR-mix (Biorad, Hercules, USA) in CFX96 cycler (Bio-Rad) in 20µl reaction using LL-37 gene-specific primers (sense-5'*gcggtggctcactggtgctcctgctgct3'* and antisense-5'*gaagaaatcaccagcagggcaaatc3'*) (14). The RNA expression of housekeeping-genes (*β-actin* and *cytochrome C1*) was used as control. The sense and antisense primers for *β-actin* (5'*catgtacgttgctatccaggc3'* and 5'*ctccttatgtcacgccagat3'*) and *cytochrome C1* (5'*agctgccacaacggagcat3'* and 5'*gactgaccactgtgccgct3'*) were designed using 'Primer-3-software' (<http://www.ncbi.nlm.nih.gov/tools/primer-blast>). The PCR conditions were 94°C x 3 min, 39 cycles of 94°C x 30s, 55°C for *β-actin* and 60°C for LL-37 and *cytochrome C1* each x 30s, 72°C x 30s, 86°C x 20s, final extension 72°C x 10 min. The intra-assay and inter-assay variation were 0.9-1.7% and was 3-5%, respectively. All the reactions were performed in duplicates, and specificity of amplified products was checked by post-PCR melt-curve analysis and agarose gel electrophoresis (Fig.1). The specificity of amplified products of LL-37 was checked by DNA sequencing and that of *β-actin* and *cytochrome C1* by RFLP using Eco0109I and TaqI restriction enzymes, respectively. The mRNA copy number of LL-37 was determined per 10³ copies of geometric mean of *β-actin* and *cytochrome C1*.

ELISA for Serum LL-37 Concentrations

A sandwich ELISA with LL-37 specific antibody precoated on to 96 well microplate was used (MyBiosource Inc., USA). Briefly, blood was allowed to clot at room temperature, centrifuged at 1000xg for 15 minutes at 4°C and serum was stored at -80°C. Assay was carried out using 100 µl of serum along with seven standards ranging from 1.56-100 ng/ml. Avidin-HRP was used in conjunction with biotinylated

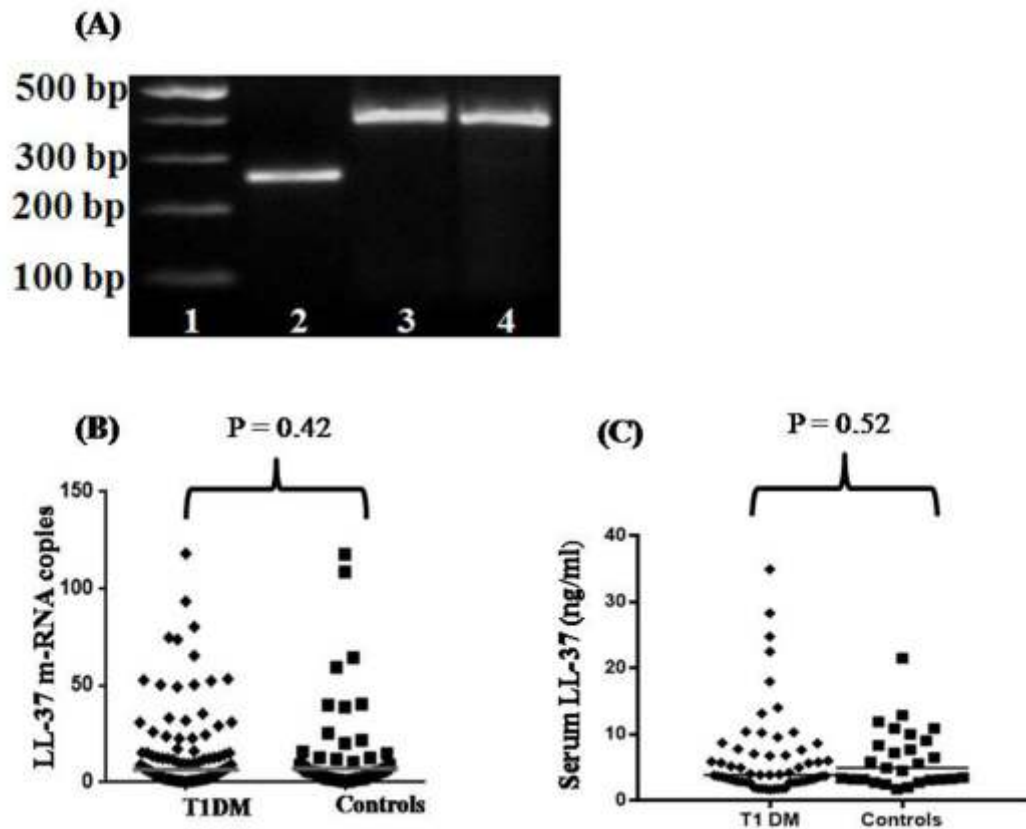


Fig.1: Agarose gel (1.5%) electrophoresis showing the PCR products of β -actin, CYC1 and LL-37 (lanes 2,3,4, respectively), Lane 1: 100 bp DNA ladder (A); LL-37 expression in T1DM and controls, mRNA copies/ 10^3 reference genes (B) and serum LL-37 concentration (C)

detection antibodies as second step reagent for indirect enzymatic labeling. Serum samples from patients and healthy subjects were put in the ELISA plates in 2:1 ratio to minimize assay-related variation. The ELISA was carried out as per the kit protocol with optical density measured at a wavelength of 450 nm using i-Mark reader (Biorad). The specific absorbance was plotted by four-parameter logistic curve fitting. The assay had minimum detection limit of 0.94 ng/ml of natural and recombinant LL-37, coefficient of variation < 10% and reportable range between 1.56-100.0 ng/ml.

The study was approved by the Institutional Ethics Committee of All India Institute of Medical Sciences, New Delhi. Written informed consent was obtained from all subjects.

Statistical Analysis

Data are shown as mean \pm SD, frequencies and median with interquartile range. The differences in the study parameter between patients with diabetes and healthy subjects were compared using Student's 't' test or by Mann' Whitney 'U' test as appropriate. Differences in the frequency of subject with serum LL-37 value below the detection range of the assay (<1.56 ng/ml) between diabetes and controls were analyzed using chi Square test. Pearson's correlation coefficient was used to assess relationship between LL-37 mRNA expression with average HbA1c over last two years and current HbA1c. Similar analysis was also carried out for serum LL-37. All the statistical analyses were implemented on SPSS 11.5 (SPSS Inc., USA). A two-tailed P value of < 0.05 was considered significant.

Results

Table 1 shows the clinical and biochemical characteristics of the study subjects. The mean age and male: female ratio of patients (28.6 ± 11.68 years and 56:55) and healthy subjects (29.6 ± 5.86 years and 23:20) were comparable. The mean BMI was significantly less in the diabetes group than healthy subjects (20.6 ± 4.0 kg/m² and 25.1 ± 2.79 kg/m², respectively, $P < 0.001$). The mean HbA1c was higher in diabetes than healthy subjects [$9.2 \pm 1.98\%$ (77.1 ± 21.78 mmol/mol) and $5.4 \pm 0.33\%$ (35.1 ± 3.62 mmol/mol), $P < 0.001$]. The mean duration of diabetes was 15.4 ± 9.12 years and history of diabetic ketoacidosis was present in 76 (68%) patients. GAD₆₅ auto-antibodies were positive in 58.6% of the 87 patients tested. Retinopathy and proteinuria >150 mg/24 hours were present in 22.5% and 5.4%, respectively. The mean hemoglobin and leukocyte counts were in normal range in both patients and healthy subjects.

LL-37 mRNA Expression and its Serum Concentration in Diabetes

The mean LL-37 mRNA expression was comparable between diabetes and healthy groups. In view of the skewed distribution of LL-37 mRNA expression, the two groups were compared using non-parametric test. There was no difference in the median copy number of LL-37 mRNA between the two groups (Table 1). The median serum LL-37 protein concentration were comparable between diabetes and healthy subjects [median (IQR) = 3.9 (2.88–7.52) vs. 5.0 (3.19–9.05) ng/ml, respectively, $P = 0.52$]. Similarly, the frequency of subjects with serum LL-37 below the detection range were comparable between diabetes and control groups (45.9% vs. 37.2%, respectively, $P = 0.42$).

Table 1: Clinical and biochemical characteristics of study subjects

Parameter	Diabetes (n=111)	Healthy subjects (n=43)	t and U-values	P
Age (years)	28.6 ± 11.68	29.6 ± 5.86	U= 2152.0	0.35
Male: Female (n)	56:55	23:20	-	0.86
Body mass index (kg/m ²)	20.6 ± 4.00	25.1 ± 2.79	U = 803	<0.001
HbA1c (mmol/mol)	77.1 ± 21.78	35.1 ± 3.62	U = 1.0	<0.001
Total leukocytes (count/mm ³)	7462 ± 1920	7039 ± 2041	t = 1.2	0.24
Polymorphs (%)	59.1 ± 9.71	58.7 ± 6.05	U = 1051	0.98
Lymphocyte (%)	30.4 ± 8.47	29.9 ± 4.14	U = 1014	0.79
Erythrocyte sedimentation rate (mm/hr)	17.6 ± 13.36	15.7 ± 9.3	U = 2333	0.90
LL-37 mRNA/10 ³ housekeeping copies Median (IQR)	6.7 (1.8 – 15.28)	7.2 (2.23 – 21.86)	U = 2186	0.42
Serum LL-37 (ng/ml) Median (IQR)	3.9 (2.88–7.52) (n = 60)	5.0 (3.19–9.05) (n= 27)	U = 740.5	0.52

Correlation of LL-37 mRNA Expression and Serum LL-37 Protein with Glycemic Status

The LL-37 mRNA expression showed no significant correlation with the current HbA1c ($r = -0.014$, $P = 0.88$) and the average HbA1c during previous two years ($r = 0.105$, $P = 0.27$). Similarly, there was lack of significant correlation between the serum LL-37 concentration and current HbA1c ($r = 0.05$, $P = 0.72$) and average HbA1c ($r = 0.03$, $P = 0.83$).

Eleven of 111 T1DM patients had *M.tb* positive sputum culture. The LL-37 mRNA expression and its serum concentration showed no significant differences between patients with and without sputum culture positive for *M.tb*.

Discussion

The reasons of susceptibility to infections in patients with diabetes and their relation to hyperglycemia are under investigation (7-9, 15-17). Antimicrobial peptides are a key component of the first and second layer of defense of the body. Its presence in the skin and mucosal epithelial barrier supplement the first layer of defense. Polymorphonuclear cells secreting LL-37 pass through various compartments of the body to provide defense against various pathogenic organisms (9, 18). Recently, there has been interest in the altered expression of circulating LL-37 in diabetes (11, 13). However, results have been variable, which could be due to the limited number of subjects in these studies (11, 13). The present study has been carried out to assess possible alteration in the LL-37 expression among patients with diabetes. The strength of the study are a large cohort of patients with T1DM, age matched healthy subjects, measurement of both mRNA expression and serum LL-37 concentration and the availability of HbA1c data of patients during previous two years.

The present study showed no significant alteration in the LL-37 mRNA expression and circulating LL-37 protein concentration in

patients with diabetes. Interestingly, both LL-37 mRNA and LL-37 protein concentration also showed no significant relationship with current or average HbA1c indicating that circulating LL-37 expression is not altered with severity of hyperglycemia. There have been only two previous studies reporting mRNA expression in the PBMC and serum concentration of LL-37 in patients with diabetes. Gonzalez *et al* reported significantly lower mRNA expression of LL-37 in 30 patients with T2DM compared to healthy subjects ($P < 0.001$) (11). Brauner *et al* assessed the serum concentration of LL-37 in 58 patients with diabetes (27 T1DM and 31 T2DM) and 19 healthy subjects (13). The study revealed significantly lower serum LL-37 concentration T1DM compared to those with T2DM. However, the LL-37 levels among patients with T1DM were comparable to the control group. Interestingly, in the current study we did not find any significant difference in LL-37 expression between patients with and without sputum *M.tb* positivity. These preliminary findings need to be further assessed in future studies targeting larger cohort of diabetes patients with and without active tuberculosis.

Thus, the lack of significant difference in the serum LL-37 concentration between patients and healthy subjects in current study coupled with no significant relationship of LL-37 with glycemic control suggest that constitutive LL-37 expression is not altered in patients with diabetes at least in the circulation. Further, the expression of LL-37 peptide is not affected by adverse glycemic status in patients with T1DM.

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Declaration of Interest

The authors have nothing to disclose and there is no conflict of interest.

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Ethics

The study was carried out after approval from the Ethics Committee of the All India Institute of Medical Sciences, New Delhi (Reference IESC/T-296/08.08.2014 vide letter 23-8-2014). Written informed consent was obtained from all the patients and controls.

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