Research Article

Evaluation of DNA damage in Type 2 diabetes mellitus patients with and without peripheral neuropathy: A study in South Indian population

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Abstract

Background: The increasing incidence of Type 2 diabetes mellitus globally has collaterally increased the incidence of diabetes-associated complications such as neuropathy. Oxidative stress induced DNA damage is one of the mechanisms implicated in the pathogenesis of diabetic complications. Here we aimed to evaluate the extent of DNA damage in diabetes patients with and without clinical neuropathy using the Cytokinesis Block Micronucleus Cytome assay, in a group of South Indian population. **Materials and Methods:** The Cytokinesis Block Micronucleus Cytome assay was performed in lymphocyte cultures of 42 type 2 diabetes patients (22 with neuropathy and 20 without neuropathy) and 42 age and sex matched controls. Nuclear aberrations like Nuclear Buds, Nucleoplasmic Bridges and Micronuclei were analyzed. **Results:** The frequency of nuclear aberrations in diabetes patients with neuropathy was higher than compared to diabetes patients without neuropathy. The mean frequencies of nuclear aberrations per cell in diabetes patients with neuropathy and without neuropathy were 0.02 ± 0.02 and 0.01 ± 0.01, respectively. This was significantly higher than in the controls (0.002 ± 0.002) ($P < 0.0001$). An increasing trend of nuclear aberrations in correlation with the duration of diabetes was observed. **Conclusion:** This study highlights the use of the Cytokinesis Block Micronucleus Cytome assay as a potent tool for the identification of DNA damage, which may prove to be useful biomarker to assess the severity diabetes-associated complications such as neuropathy. Implementation of this technique at the clinical level would potentially enhance the quality of management of patients with diabetes and its complications like neuropathy.

Key words: CBMN Cyt assay, DNA damage, diabetic neuropathy, nuclear aberrations, oxidative stress, Type 2 diabetes mellitus

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INTRODUCTION

Epidemic of Diabetes mellitus is growing globally, with 90% of all diabetes patients having type 2 diabetes mellitus (T2DM).[1] T2DM is one of the major public health problems in India. The progressive nature of the disease leads to the development of microvascular complications resulting in increased morbidity and health care costs. About 50% of patients suffering from diabetes eventually

develop various forms of neuropathy of which sensorimotor peripheral neuropathy is the most common.[2] Of concern, 50% of patients with peripheral neuropathy are asymptomatic and the insensate feet pose a risk of injury which may lead to subsequent amputation.[3] In India, one of the common causes, among diabetics for admission to a hospital, is diabetic foot infection.[4] Efficient management of peripheral neuropathy is extremely important so as to minimize the risk of amputation.

Oxidative stress is associated with pathogenic mechanisms leading to diabetic neuropathy.[5,6] In diabetes, the oxidative stress is augmented leading to DNA damage which is potentially linked to the diabetic complications.[7] The Cytokinesis Block Micronucleus Cytome (CBMN Cyt) assay comprehensively measures this kind of DNA damage which manifests in the form of nuclear aberrations, such as Nuclear Buds (NBUDs), Nucleoplasmic Bridges (NPBs) and Micronuclei (MNi).[8] Thus by measuring DNA damage with the use of this assay, we can understand the role of oxidative stress in diabetic neuropathy. Cytogenetic assays have been used in earlier studies to evaluate DNA damage in type 1 diabetes mellitus [9] and pre-diabetes patients.[10] In this study done in a group of South Indian population, we aimed to evaluate the use of CBMN Cyt assay to assess the extent of DNA damage in patients suffering from T2DM, both with and without neuropathy.

MATERIALS AND METHODS

Forty-two patients with T2DM were recruited from the Institute of Diabetology, Madras Medical College and Rajiv Gandhi Government General Hospital in Chennai, Tamil Nadu, India. Of the 42 patients, 22 patients were with clinical peripheral neuropathy and 20 patients were without neuropathy. Forty-two age and sex-matched controls - with normoglycemia as demonstrated by the Oral Glucose Tolerance Test - were included in this study. The screening of patients for neuropathy included a thorough diabetic history including history for symptoms of peripheral neuropathy, clinical examination for peripheral neuropathy and biothesiometry (also known as Vibration Perception Threshold [VPT] test) by a diabetologist. Those T2DM patients who had VPT readings greater than 20 on at least three points on their feet were grouped as "type 2 *diabetes* with clinical *neuropathy* (DN). Patients with VPT readings less than 10 and duration of T2DM for at least 10 years were classified into the "type 2 *diabetes* with *no* clinical *neuropathy* (DNN)" group. Patients with history of chronic alcohol consumption, renal failure, hypothyroidism and those who were on medications for conditions such as tuberculosis, leprosy, human immune deficiency virus infection and malignancy were not considered. Pregnant women were not included. Close relatives of the study subjects were not taken as controls.

General and lifestyle information about the patients along with family pedigrees were recorded. Informed written consent was obtained from all the patients and the study was carried out with the approval of the institutional ethical committee.

Cell culturing and harvesting

Blood samples were drawn by venipuncture into sodium-heparin vacutainers. Lymphocyte cell culturing was carried out for the collected blood samples on the same day.[11] 0.5 mL of the whole blood sample was added to 5.0 mL of RPMI 1640 culture medium [Hi Media]. Subsequently, 1.2 mL of Foetal Bovine Serum (FBS) [Hi Media] and 0.3 mL of Phytohaemagglutinin [GIBCO] were also added to the cultures. Incubation of the cultures was carried out at 37°C for a period of 72 h.

Cytokinesis block micronucleus cytome assay

This assay was performed by adding Cytochalasin B [GIBCO, 6 μg/mL] at the 44th hour of incubation at 37°C. The cells were harvested at the end of 72 h of incubation. The cells were treated with cold (8°C) hypotonic solution (0.075 M Potassium chloride) and followed by fixation with methanol: Glacial acetic acid (3:1). The slides were prepared and stained using Giemsa solution (4%) for 3 min.

Scoring and data evaluation

The scoring criteria established by Fenech[8] were used for CBMN Cyt assay analysis. Bi-nucleated cells were analyzed and the number of nuclear anomalies such as NPBs, NBUDs and MNi [Figure 1] were scored. The scoring of this assay was carried out in a blinded manner, with the group, *i.e.* DN, DNN or control being concealed from the person carrying out the scoring.

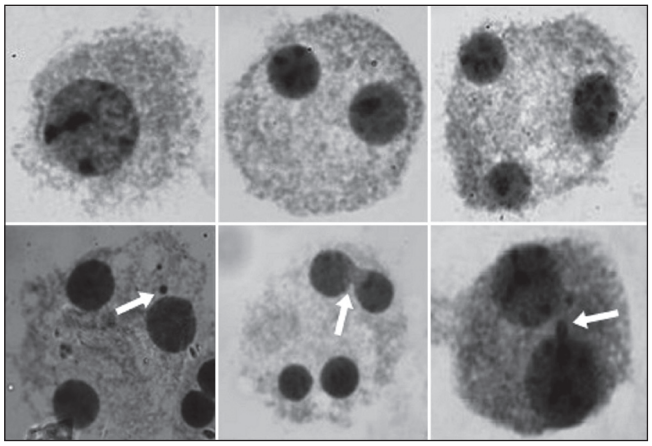

Figure 1: Observations in the Cytokinesis Block Micronucleus Cytome (CBMN Cyt) Assay. Top row-left to right-mononuleate cell, binucleate cell and trinucleate cell. Bottom row-left to right-micronucleus, nucleoplasmic bridge and nuclear bud

Statistical analysis

The comparison of frequencies of nuclear aberrations between the two patient groups and the control group was carried out by using an Analysis of Variance (ANOVA) test. For analysing variance in nuclear aberrations in different age groups of the subjects and ratio of binucleates to mononucleates, ANOVA was used. For correlation analysis of age of the subjects and duration of diabetes with frequency of aberrations, Pearson correlation coefficient test was used. The comparison of nuclear aberrations between the patient groups, with or without family history of T2DM was done using the *t*-test. A "*P*-value" of <0.05 was considered to be statistically significant. Numerical data are presented as mean ± standard deviation (S.D.). Microsoft Excel software was used for the statistical analysis.

RESULTS

The general characteristics of patients studied are listed in Table 1. The mean age of the DN patients was 60.13 ± 11.53 years, with a mean duration of diabetes of 8.83 ± 5.70 years (range: One month-20 years). The mean age of the DNN patients was 58.3 ± 10.90 years, with a mean duration of diabetes of 13.35 ± 4.0 years (range: 10-20 years). 8 DN patients and 7 DNN patients had a family history of T2DM. Table 2 shows the mean frequencies of nuclear aberrations observed in the three groups. DN and DNN groups showed higher frequency of nuclear aberrations (0.02 ± 0.02 and 0.01 ± 0.01, respectively) than controls (0.002 ± 0.002) ($P < 0.0001$). The frequency of aberrations was higher in the DN group than in the DNN group. In DN and DNN groups the ratios of binucleates to mononucleates were observed to be significantly higher (1.30 ± 0.29 and 1.35 ± 0.64, respectively) than in the controls (0.98 ± 0.02) [Table 2]. The mean frequencies of nuclear aberrations in the different age groups of patients and controls are presented in Table 3. They were found to be significantly higher in the DN and DNN groups than controls. An increasing trend of nuclear aberrations in correlation with the duration of diabetes was observed ($r = 0.964$, $P = 0.017$) [Figure 2]. We did not find any significant correlation between age of subjects and frequency of aberrations ($r = 0.073$, $P = 0.34$). There was no significant variation in the frequency of aberrations between the DN patients who had a family history of diabetes and those without family history ($P = 0.79$). Similar pattern was also observed in DNN patients ($P = 0.28$).

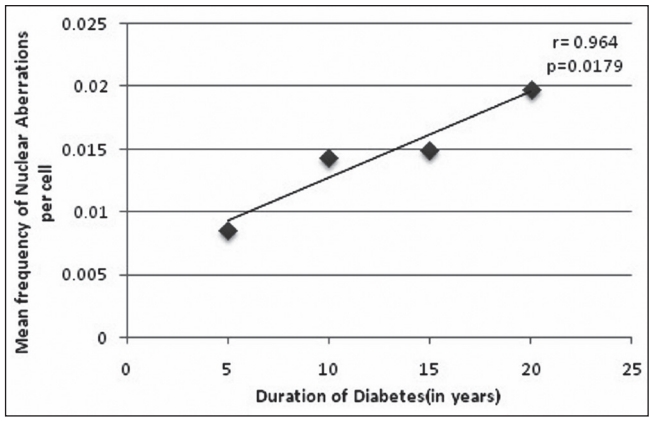

Figure 2: Correlation between the mean frequencies of nuclear aberrations and the duration of diabetes. Scatter plot analysis of aberrations with respect to duration of diabetes. Significant Correlation was observed ($r = 0.964$) ($P = 0.0179$)

DISCUSSION

Oxidative stress can cause DNA damage in patients with diabetes mellitus.[9,10] The scoring and statistical comparison of the CBMN Cyt assay data are previously used to analyze the DNA damage or genotoxicity due to environmental and lifestyle conditions.[12,13] The investigation of DNA damage was considered in our study as oxidative stress plays a prominent role in the pathogenesis of diabetic neuropathy. Early diagnosis and treatment of diabetic neuropathy is essential so as to minimize further complications.[14] DNA damage in patients with type 1 diabetes mellitus is previously demonstrated by the frequencies of sister chromatid exchanges,[9] and in patients with pre-diabetes, significant correlation of DNA damage and cytotoxicity with A1C is reported.[10] The present study provides data on DNA damage using CBMN Cyt assay in patients with T2DM with (DN) and without (DNN) clinical peripheral neuropathy.

The analysis of the CBMN Cyt assay data demonstrates that frequency of DNA damage is significantly higher in the patient groups than in the controls. The higher frequency of nuclear aberrations in patients of the DN group as

Table 1: General characteristics of the studied groups

General characteristics	DN	DNN	Controls
Number of subjects	22	20	42
Gender			
Males	13	7	30
Females	9	13	12
Age in years (mean ± S.D.)	60.13±11.53	58.3±10.90	57.78±10.92
Duration of diabetes (range)	One month-20 years	10-20 years	—
Lifestyle:			
Sedentary activity	11	10	12
Moderate activity	5	9	13
Heavy activity	6	1	17

DN: Type 2 *diabetes* with clinical *neuropathy*, DNN: Type 2 *diabetes* with *no* clinical *neuropathy*, S.D: Standard deviation

Table 2: Frequencies of nuclear aberrations observed in the lymphocytes of the patients and controls

Nuclear aberrations	DN (*n* = 22)	DNN (*n* = 20)	Controls (*n* = 42)
Total number of BN scored	22100	20064	42401
Total number of MNi	130	102	14
Mean frequency of MNi per cell	0.006±0.007	0.005±0.005	0.0003±0.0005
Total number of NPBs	141	107	33
Mean frequency of NPBs per cell	0.006±0.005	0.005±0.004	0.0007±0.0008
Total number of NBUDs	102	73	26
Mean frequency of NBUDs per cell	0.005±0.005	0.004±0.003	0.0006±0.0008
Total number of MNi, NPBs and NBUDs	383	282	73
Mean frequency of MNi, NPBs and NBUDs per cell	0.02±0.02	0.01±0.01	0.002±0.002
Ratio of binucleates to mononucleates	1.30±0.29	1.35±0.64	0.98±0.02

The values are depicted as mean ± S.D, The mean frequencies of nuclear aberrations per cell were found to be significantly higher in DN and DNN than controls ($P < 0.0001$), DN: Type 2 *diabetes* with clinical *neuropathy*, DNN: Type 2 *diabetes* with *no* clinical *neuropathy;* BN- Binucleates, MNi: Micronuclei, NPBs: Nucleoplasmic Bridges, NBUDs: Nuclear Buds, S.D: Standard deviation

Table 3: Mean frequencies of nuclear aberrations in different age groups of the subjects

Age groups	DN (*n*)	DNN (*n*)	Controls (*n*)	*P*
41-50 years	0.02±0.01 (7)	0.01±0.007 (5)	0.001±0.001 (10)	0.001
51-60 years	0.01±0.01 (8)	0.01±0.008 (8)	0.002±0.001 (11)	0.0023
>61 years	0.02±0.02 (7)	0.02±0.01 (7)	0.002±0.001 (21)	0.0004

The values are presented as mean ± S.D, The mean frequencies of nuclear aberrations per cell in different age groups of the subjects were found to be significantly higher in DN and DNN than controls, "*n*" indicates number of patients, DN: Type 2 *diabetes* with clinical *neuropathy*, DNN- type 2 *diabetes* with *no* clinical *neuropathy,* S.D: Standard deviation

compared to the DNN group may be due to higher level of oxidative stress in patients of diabetic neuropathy as compared to the patients of the DNN group. The significantly higher ratio of binucleates to mononucleates in patient groups than in the control group could be due to faster cell cycle progression, stress or the effect of drugs taken by the patients.

The frequency of nuclear aberrations was also seen to increase significantly with the duration of diabetes in the patient groups. The reason for this can be attributed to the increasing susceptibility of the patients to DNA damage with the increase in the duration of diabetes. However, the frequency of nuclear aberrations was independent of the age of the patients. As there was no significant variation in the frequency of aberrations between those with a family history of T2DM and those without, it could be inferred that the aberrations are due to the damage caused by diabetes progression. In this study, we have not taken into account the influence of the drugs taken by the diabetic patients for the observed effect and this aspect needs further studies.

CONCLUSION

There is a need to evaluate DNA damage in T2DM patients for early identification of patients developing diabetes-associated complication such as peripheral neuropathy. We suggest the possibility of using the CBMN Cyt assay as a potent tool for the identification of DNA damage in diabetic patients. The implementation of such techniques at the clinical level would greatly enhance the diagnosis, care and management of diabetes patients developing complications such as neuropathy.

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