

Assessment of DNA damage using cytokinesis-block micronucleus cytome assay in lymphocytes of dilated cardiomyopathy patients

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Summary

Studies on the extent of DNA damage are undertaken to elucidate the nature and causes of genomic instability in any syndrome or disease progression in human. In this study, cytokinesis-block micronucleus cytome (CBMN Cyt) assay was employed to evaluate the extent of chromosomal instability or DNA damage in lymphocytes of patients suffering from dilated cardiomyopathy (DCM), a serious cardiac muscle disorder. Effect of DNA damage on the disease was also assessed by analysis of mutations in cardiac Troponin C type I (TNNC1) gene. Blood samples were collected from 48 DCM patients and 48 age- and sex-matched controls from Vellore region of South India. Significantly high frequencies of micronuclei (MNI) and genomic damage such as nuclear buds (NBUDs) and nucleoplasmic bridges (NPBs) were observed in the patient group as compared with the control group ($P < 0.001$). Molecular analysis revealed that no mutations were found in the TNNC1 gene. It was observed that although there was a high frequency of DNA damage in the lymphocytes of the patients, no correlation between severity of the phenotype and the frequencies of MNI, NPBs and NBUDS could be established. Our study appears to be the first one in which chromosomal instability was estimated using CBMN Cyt assay for DCM patients. Studies with a larger population size may help in validating the use of genetic markers for establishing frequencies and type of DNA damage in DCM. It will also help in understanding the effect of DNA damage on this disease.

1. Introduction

Dilated cardiomyopathy (DCM) is a cardiac muscle disorder that leads to cardiovascular morbidity and mortality. It is associated with heart failure and sudden cardiac death. This disorder is known to develop at any age, in either sex and in people of any ethnic origin (Taylor *et al.*, 2006). The prevalence of DCM in the general population of India is not well defined (Ushasree *et al.*, 2009).

Over the past few years, studies on the application of genetic and genomic techniques to understand the aetiology of DCM have increased. With a focus on

molecular diagnostics and genetic intervention, it is aiding in the improvement of clinical practice and routine management of the patients. Of the many approaches in genetics, studies on DNA damage remain an important aspect to understand the complete molecular nature of disorders. Very few studies have reported DNA damage in cardiovascular patients using DNA adducts and cytogenetic damage in peripheral blood lymphocytes as endpoints (Botto *et al.*, 2001; Murgia *et al.*, 2007). However, no data exist on DNA damage in lymphocytes of DCM patients. The cytokinesis-block micronucleus cytome (CBMN Cyt) assay is an efficient technique to assess cellular and nuclear malfunctions and dysfunctions by measuring the frequencies of MN and other genomic aberrations in cultured human cells (Fenech, 1997).

In this study, the possible effects of DNA damage on the development and progression of the disease were also investigated by mutation analysis of the

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cardiac Troponin C type I (TNNC1) gene. TNNC1 was identified as a novel gene for DCM in the study conducted by Mogensen *et al.* (2004). A few studies have reported the presence of mutations in the gene TNNC1 coding for cTnC in DCM patients. The cTnC mutations E59D and D75Y were detected in only one patient with DCM. This raised the question of whether this mutation co-segregated with the disease. However, from functional studies it was identified that these mutations have a synergistic effect on Ca^{2+} sensitivity and ATPase activation.

The aim of the present study was to assess the DNA damage by employing CBMN Cyt assay in cultured peripheral blood lymphocytes of DCM patients. An effort was also made to understand if there was any association between DNA damage and disease progression.

2. Materials and methods

(i) Populations studied

Informed written consent was obtained from 48 patients suffering from DCM and undergoing treatment at Heartline Medical and Research Centre, Vellore, India and 48 age- and sex-matched controls ($n = 48$). Blood samples were collected by venipuncture in heparinized and EDTA-coated vacutainers.

(ii) Ethical standards

Ethical clearance for this study was obtained from the institutional review board of VIT University. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008.

(iii) Clinical study

The clinical evaluation was based on the guidelines prescribed by Dickstein *et al.* (2008). Clinical evaluation consisted of recording a careful patient and family history, electrocardiogram (ECG), echocardiogram, chest X-ray and routine blood chemistry. Based on the clinical presentation, the exclusion criteria were decided. Patients with known history of rheumatic heart disease, joint pain, chronic hypertension, anaemia and those having abnormal values for creatinine, urea and albumin were not included in the study.

DCM was confirmed using an M-Mode, 2D echocardiogram with colour Doppler, by the presence of left ventricular dilatation and dysfunction with ejection fraction (EF) $<50\%$ and fractional shortening (FS) <30 , no region of wall abnormalities in the

echo and normal ECG. The left ventricle dimensions were obtained using the Teichholz method. The frequencies of the clinical characteristics such as EF, FS, mitral regurgitation and tricuspid regurgitation were recorded.

(iv) Detailed history and physical examination

Alcohol use, cocaine use, dietary pattern, past illnesses (including infections involving the heart, history of arrhythmias, etc.), medications taken, pregnancies, occupations of the patients and family history were recorded. The occupational characteristics of the patients and the controls were recorded, to account for exposures to toxins and mutagens, if any.

(v) Cytogenetic studies

Peripheral blood samples were processed immediately on sampling and cultured. Chromosome preparations were made according to the standard procedure (Hungerford, 1965). Cultures were set up by mixing 0.5 ml of whole blood with 6 ml of Ham's F10 media (PAN Biotech GmbH), supplemented with 1.2 ml of fetal bovine serum (Hi Media) and 0.3 ml of phytohaemagglutinin (GIBCO).

(a) CBMN Cyt assay

For this assay, lymphocyte cultures were set up as above. Cells were blocked in cytokinesis at the 44th hour by the addition of Cytochalasin B ($6 \mu\text{g/ml}$ final concentration, Sigma). Harvesting was carried out at the end of 72 h. The cells were treated hypotonically with 0.075 M KCl for 8 min, followed by fixation with methanol-acetic acid (3:1). Subsequently, slides were prepared and stained with Giemsa solution (4%). For each sample, 1000 binucleated cells were scored for abnormalities following the criteria specified by Fenech *et al.* (2003).

(vi) Molecular studies

Blood samples collected in EDTA vacutainers were processed for DNA extraction. Total genomic DNA was extracted from $100 \mu\text{l}$ peripheral blood samples from patients using DNeasy blood & tissue kit (Qiagen, Germany). Primers were designed and the standard PCR reaction for a $20 \mu\text{l}$ reaction mixture was carried out. PCR products were analysed by agarose gel electrophoresis. PCR products were purified using MinElute PCR Purification Kit (Qiagen, Germany). Purified PCR products were quantified in synergy H4 multimode micro-plate reader using Take3 micro-volume plate. The purified exon 4 amplified PCR product was cycle sequenced using BigDye terminator (Life Technologies, USA), and

Table 1. Results of the CBMN Cyt assay

	Patients (<i>n</i> = 48)	Controls (<i>n</i> = 48)
Total number of binucleates (BN) scored (1000 BN/individual)	48 000	48 000
Total number of MNi observed	264	18
Mean frequency of MNi per cell	0.005±0.003	0.000375±0.0006
Total number of NPBs	189	38
Mean frequency of NPBs per cell	0.004±0.002	0.0008±0.00085
Total number of NBUDs	184	28
Mean frequency of NBUDs per cell	0.004±0.002	0.0006±0.0008
Total number of MNi+NPBs+NBUDs	637	84
Mean frequency of MNi+NPBs+NBUDs per cell	0.0133±0.0038	0.00175±0.00134
% of aberrant cells scored (MNi+NPBs+NBUDs)	1.327%	0.175%

the labelling reaction was carried out from both ends using the forward and reverse primers individually. The PCR products were purified by Ethanol/EDTA precipitation method. The purified product was denatured using 10 µl of Hi-Di Formamide and it was run on a AB3730 DNA analyser. The raw data files were analysed using Chromas lite Software (Technelysium, USA) to detect for any mutation in the patient sample compared with the normal sequence of TNNC1 gene.

(vii) Statistical analysis

A comparison between the data obtained from the patient group (*n* = 48) and the control group (*n* = 48) was analysed using the *t* test. A *P* value of <0.001 with confidence limit of 95% was defined as statistically significant. Numerical data are presented as mean ± SD. Microsoft Excel and WinStat were used for the statistical analysis.

3. Results

No significant difference was observed in the demographic profile and the lifestyle of the patient group and the control group. The individuals studied had varied occupation. The basic diet of both the patient group and control group was a typical South Indian diet.

(i) CBMN Cyt assay

A significantly higher frequency of micronuclei (MNi) was observed in the patient group than that of the control group (*P*<0.001) (Table 1). The mean frequency of MNi per cell observed for the patient group was 0.005±0.003 and for the control group was 0.000375±0.0006 (*P*<0.001). A significant number of nuclear buds (NBUDs) were also observed in the patient group (0.004±0.002 per cell), as compared with the control group (0.0006±0.0008 per cell) (*P*<0.001). A higher frequency of nucleoplasmic

bridges (NPBs) in the patients (0.004±0.002 per cell) was observed as compared with the controls (0.0008±0.00085 per cell) (*P*<0.001). No significant difference was observed in the mean frequencies of aberrations detected by the CBMN Cyt assay in the patient group due to age, gender, smoking, alcohol drinking, dietary patterns or their occupation (*P*>0.005). The frequency of anomalies grouped according to disease severity, established by EF measurement showed no differences. The frequency distributions of various anomalies detected by the CBMN Cyt assay, in the control group and patient group are presented in Fig. 1. All patients had at least three to five aberrations. Seventeen patients had 12–14 aberrations. In one patient, 26 aberrations were scored. Whereas in the control group, 12 individuals had three to five aberrations and 36 individuals had only one aberration. The ratio of binucleated cells to mononucleated cells was calculated as 1.04 for patients and 1.01 for controls. No significant difference was found in the ratio of binucleated cells to mononucleated cells in the patient and control groups. The percentage of aberrant cells containing MNi with NBUDs and NPBs in the patient group is 1.327%, whereas in the control group it is 0.175%.

(ii) Mutation analysis

The primers were designed approximately 100 base pairs up-stream and down-stream of exon 4 with the forward primer sequence of 5'-CTCCCCAG-GCTCCAGAAG-3' and reverse primer sequence 5'-GCATCCCTCTCCCCTATCAG-3'. Sequence data analysis revealed no variations in the gene sequence of the patients and the normal TNNC1 gene sequence.

4. Discussion

Two main causes of death in the global population are cardiovascular diseases and cancer. It is hypothesized that these chronic degenerative diseases share several

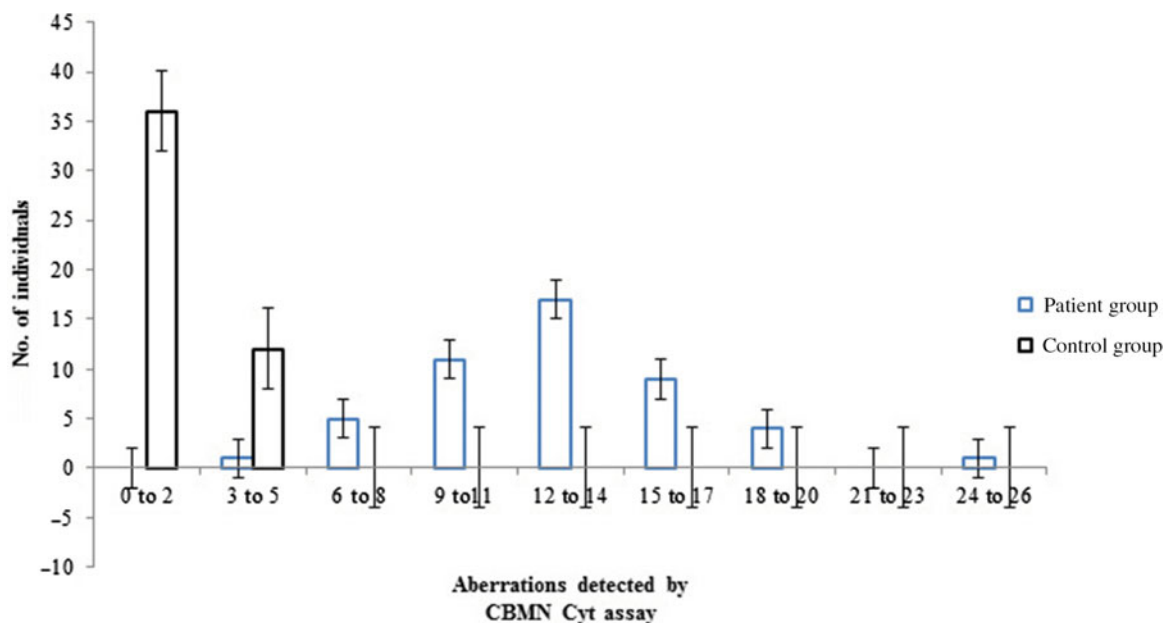


Fig. 1. Frequency distribution of aberrations detected by CBMN Cyt assay in patients.

risk factors, such as oxidative stress and DNA damage (Trosko & Chan, 1980). Although the role of DNA damage and mutation in carcinogenesis is well established, their involvement in vascular diseases is less understood.

In general, in any biological system for the detection and estimation of naturally occurring and induced genotoxic effects, either chromosomal aberration (CA) frequencies or MNi frequencies are used. In an earlier study, we reported high frequency of DNA damage as expressed in various types of CA such as chromosomal breaks, chromatid breaks and dicentric chromosomes (Sitaraman *et al.*, 2012). It was observed in that study that the mean value of all CAs was 0.092 ± 0.04 . In the present study, through the application of CBMN Cyt assay, the NPBs between the binucleate cells (0.004 ± 0.002) and nuclear budding (0.004 ± 0.002) gave added informational parameters along with the frequency of MNi. It is noted that although the frequencies of CAs, as reported previously and the various anomalies detected by the CBMN Cyt assay are comparable, the frequency of CA per cell (0.092 ± 0.04) is higher than the frequency of MNi per cell (0.0133 ± 0.0038).

There was no significant difference between the ratio of binucleated cells to mononucleated cells in the control and patient groups, produced by the CBMN Cyt assay. This indicates that the cell cycle progression in the patient group is similar to that of the control group and is not affected by any extrinsic or intrinsic factor.

The other genomic aberrations as seen in this study may help to have a clearer picture of the complete DNA damage in the DCM syndrome. NBUDS are

known to form due to the elimination of amplified DNA or sequence to distinct region of the nucleus, usually the nuclear periphery. NBUDS have also been shown to be formed when an NPB between two nuclei breaks and the remnants shrink back towards the nuclei (Fenech, 2007; Fenech *et al.*, 2011).

No mutations were identified in the TNNC1 gene. Hence, it is not clear from this study whether mutations may arise as a consequence of DNA damage. Although a few studies have reported functional studies on mutations in cTnC and have shown the effect of cTnC mutations in muscle contractility (Mogensen *et al.*, 2004; Hershberger *et al.*, 2010; Pinto *et al.*, 2011), it is highly likely that cTnC being the Ca^{2+} sensor of cardiac muscle, any mutations in this protein might be lethal and therefore possibly be non-existent in the adult population. It is also possible that the mutations exist but have no overall effect on cardiac function.

It was also observed that no correlation could be established between the severity of the phenotype, as expressed as lower EF and DNA damage measured. Hence, the high frequency of aberrations observed in the patient group may be attributed to co-existing factors of the disease rather than the disease itself. It is hypothesized that psychological stress combined with the clinical presentation of the disease may have led to a higher frequency of DNA damage. Several studies have reported that stress can be responsible for induction of DNA damage (Fischman & Kelly, 1999; Dimitroglou *et al.*, 2003).

Although in this study significant DNA damage in the lymphocytes of the patients has been observed, it is important to note that given the small size of the

cohort, the results may not be representative over a generalized patient population. Studies on larger cohorts may help in establishing the effect that DNA damage has on the functional unit of the muscle, which is seen to be affected in DCM.

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Statement of Interest

None.

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