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Role of cardiac TBX20 in dilated cardiomyopathy

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Abstract Dilated cardiomyopathy (DCM) is an important cause of heart failure and sudden cardiac death worldwide. Transcription factor TBX20 has been shown to play a crucial role in cardiac development and maintenance of adult mouse heart. Recent studies suggest that TBX20 may have a role in pathophysiology of DCM. In the present study, we examined TBX20 expression in idiopathic DCM patients and in an animal model of cardiomyopathy, and studied its correlation with echocardiographic indices of LV function. Endomyocardial biopsies (EMBs) from intraventricular septal from the right ventricle region were obtained from idiopathic DCM patients (IDCM, $n = 30$) and from patients with ventricular septal defect (VSD, $n = 14$) with normal LVEF who served as controls. An animal model of DCM was developed by right renal artery ligation in Wistar rats. Cardiac TBX20 mRNA levels were measured by real-time PCR in IDCM, controls, and in rats. The role of DNA promoter methylation and copy number variation (CNVs) in regulating TBX20 gene expression was also investigated. Cardiac TBX20 mRNA levels were significantly increased (8.9 fold, $p < 0.001$) in IDCM patients and in RAL rats as compared to the control group. Cardiac TBX20 expression showed a negative correlation with LVEF ($r = -0.71$, $p < 0.001$) and a positive correlation with left ventricular end-systolic volume ($r = 0.39$, $p = 0.038$). No significant difference in TBX20 CNVs and promoter methylation was observed between IDCM patients and control group. Our results suggest a potential role of TBX20 in pathophysiology of DCM.

Keywords Dilated cardiomyopathy · Heart failure · TBX20 · LVEF and gene expression

Introduction

Dilated cardiomyopathy (DCM) is one of the most common causes of heart failure [1] and is characterized by enlargement of one or both of the ventricles with associated systolic dysfunction [2]. It has been reported to be responsible for 1/3rd of the total cases of heart failure (HF) and can result from diverse etiologies, ranging from genetic to metabolic factors or infections with varied prognosis [1]. HF in DCM is a consequence of the molecular events of underlying pathology and involves alterations at molecular level including re-deployment of developmental transcription factors and 'fetal' cardiac gene activation [3–5]. These molecular changes have been suggested to be useful in diagnosis and prognostication of DCM [6, 7].

T-box transcription factor 20 (TBX20) is a member of a family of ancient T-box genes [8] that has been shown to play a crucial role in cardiac development and maintenance of adult mouse heart [9]. TBX20 interacts directly with other transcription factors such as Gata4, Gata5, Nkx2-5, and Tbx5 to regulate cardiac-specific gene expression [10, 11]. The knockdown of Tbx20 homolog gene in Drosophila has been found to result in slower heart rate, arrhythmias, and abnormal myofibrillar architecture [12]. Adult mice with heterozygous Tbx20 knockout were found to have left ventricle dilation and contractile abnormalities, whereas homozygous conditional cardiomyocyte Tbx20

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knockout resulted in DCM and contraction-related dysfunctions and death of mice within 2 weeks [12]. Furthermore, mutations in the T-box DNA-binding domain of TBX20 have been shown to be associated with cardiac pathologies including septation defects and cardiomyopathy [13, 14]. R334Q variant of TBX 20 gene has been reported to show apparent clinical DCM at the time of evaluation, thereby suggesting a possible role of TBX20 in development of DCM [12]. However, the status of TBX20 expression in DCM patients remains unexplored.

In the present study, we measured TBX20 gene expression in idiopathic DCM patients and in a rat model of DCM, and studied its correlation with echocardiographic indices of LV function. We also investigated the role of promoter DNA methylation and TBX20 copy number variations (CNVs) in regulating cardiac TBX20 expression.

Methods

Study population

Thirty consecutive IDCM patients attending cardiology clinic between Jan 2011 and 2014, at the Department of Cardiology, Postgraduate Institute of Medical Education and Research, Chandigarh, India, were enrolled and biopsies were taken from intraventricular septum of the right ventricle region. DCM was diagnosed after echocardiography, defined by left ventricular ejection fraction (LVEF) $\leq$40 % and chronic mild to severe HF (NYHA functional class II–IV). All patients underwent left cardiac catheterization and coronary angiography before their inclusion in the study. Exclusion criteria were as follows: the presence of significant coronary artery disease defined as lumen stenosis in >50 % of any coronary artery, severe primary valve disease, uncontrolled systemic hypertension, hypertrophic or restrictive cardiomyopathy, chronic systemic disease like myocarditis, thyrotoxicosis, HIV disease, and drug abuse. Blood samples ($n = 150$) were also obtained from idiopathic DCM patients after taking written informed consent. All recruited IDCM subjects were on optimal medication, angiotensin-converting enzyme inhibitors, and beta-blockers but had persistently low LVEF despite drug regime at the time of biopsy. Endomyocardial biopsies from intraventricular septum from the right ventricle region ($n = 14$) were obtained from subjects undergoing surgery for ventricular septal defect (VSD), and blood ($n = 114$) from healthy subjects without any history of cardiovascular disease served as controls. The VSD patients recruited in the study have normal LVEF that there is no right or left ventricular dysfunction. The study was approved by the Institutional Ethics Committee (8443-PG-1TRg-10/4497) and written informed consent was obtained

from all patients for participation in the study. Baseline data of patients and control subjects are presented in Tables 1 and 2.

Generation of renal artery ligation (RAL) model for cardiomyopathy

Briefly, RAL model was generated by ligating right renal artery of 20 week-old male rats (250–300 g; $n = 4$) as described earlier [15]. Sham-operated rats that underwent a similar procedure without aortic ligation were termed as control group ($n = 4$). Animals were maintained in optimum condition for 14 days and were sacrificed on the 15th day after surgery. Hearts were taken out and stored in liquid nitrogen for RNA isolation.

Echocardiography

Standard views for M-mode and two-dimensional echocardiography were obtained, and the parameters: (i) LVEF (ii) left ventricular diameter (LVEDD), (iii) left ventricular end-systolic volume (LVESV), (iv) interventricular septal thickness (IVSed), (v) left ventricular end-systolic volume (LVEDV), (vi) E-wave deceleration time (EDT), and (vii) peak early transmitral filling velocity (E-wave velocity) were measured.

RNA isolation and TBX20 gene expression

Endomyocardial biopsies were collected in RNAlater® (Life technologies) and blood samples were collected in EDTA (ethylenediaminetetraacetic acid) collection tubes. Total RNA was isolated from tissues using the RNAqueous® Micro Kit (Ambion), and 200 ng of RNA was reverse-transcribed using the high-capacity RNA to cDNA kit (Life technologies) in 20-µl reactions. Gene expression was measured by real-time polymerase chain reaction (qRT-PCR) using the Applied Biosystems 7500 qPCR system.

PCR assays were performed with 1 µl of cDNA template using the Taqman® Universal Master Mix with UNG (Life technologies). Serial dilution of known quantities of cDNA template was done to generate standard curves for matching the PCR efficiency. All reactions were performed in triplicate. For TBX20 expression, Taqman® gene expression assay (Hs00396596_m1) and, for housekeeping and endogenous reference gene, glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) assay (Hs03929097_g1) (Life technologies, USA) were used.

TBX20 copy number variation analysis

Peripheral blood mononuclear cells (PBMCs) were isolated from the blood of DCM ($n = 150$) and control subjects

Table 1 Demographic and echocardiographic characteristics of patients and control who underwent endomyocardial biopsies

	Patients ($n = 30$)	Controls ($n = 14$)
Gender (male/female)	19/12	9/6
NYHA class (I/II/III/IV)	0/15/11/5	–
LVEF (%)	20.06 ± 1.38	–
LVEDD (mm)	63.3 ± 1.93	–
IVSed (mm)	8.65 ± 0.43	–
Aortic diameter (mm)	27.0 ± 1.23	–
LVEDV (mL)	175.4 ± 12.9	–
LVESV (mL)	135.9 ± 11.09	–
E-wave deceleration time (ms)	116.0 ± 12.55	–
E-wave velocity (cm/s)	63.1 ± 8.1	–

NYHA represents New York Heart Association; *LVEF* left ventricular ejection fraction; *LVEDD* left ventricular end-diastolic diameter; *IVSed* interventricular septum in diastole; *LVEDV* left ventricular end-diastolic volume; *LVESV* left ventricular end-systolic volume; *E-wave velocity* peak early transmitral filling velocity

Table 2 Demographic profile and clinical characteristics of study population

	DCM ($n = 150$)	Controls ($n = 114$)
Age (years)	41.94 ± 14.37	42. 86 ± 14.16
LVEF (%)	28.31 ± 10.26	–
LVEDD (mm)	58.64 ± 11.12	–
Smokers (%)	28.1	55.7
Non-smokers (%)	71.9	44.3
Alcoholic (%)	22.7	43.4
Non-alcoholic (%)	77.3	56.6
Diabetes (%)		
Diabetic	10.1	0
Non-diabetic	89.9	100
Hypertensive (%)	25.6	0
Non-hypertensive (%)	74.4	100

n number of subjects

Data expressed as mean ± SD and percentage

LVEF left ventricular ejection fraction; *LVEDD* left ventricular end-diastolic diameter

($n = 114$). Genomic DNA was isolated from PBMCs using the commercially available kit (Macherey–Nagel, Germany). RT-PCR-based assays were used for measuring TBX20 copy number (Assay ID: Hs03631198_cn; Assay Location Chr7:35281585) [16]. This assay was based on amplification and quantification of TBX-20 in relation to reference gene, RNase P, in a multiplex PCR using ABI PRISM 7500 sequence detection system (Life technologies). The amplification efficiencies of the target genes and the reference gene were tested to be approximately equal in pre-run optimization.

All assays were conducted in 96-well optical reaction plates (Life technologies, USA). Each well contained FAM-labeled Taqman probe for TBX20 and TAMRA-labeled Taqman probe for the RNase P gene. PCR was performed in a reaction volume of 20 μl. A sample was used as a calibrator with the known copies of gene (TBX20) and reference gene (RNase P) which served as a positive control.

Analysis of promoter methylation of TBX20

The methylation status of the TBX20 promoter region was determined by methylation-specific PCR (MSP) in both the biopsies and blood samples of DCM and control groups. Primers distinguishing unmethylated (U) and methylated (M) alleles for TBX20 were designed using the Methyl Express software from Applied Biosystems. Bisulfite modification of DNA was done by commercially available kit (Zymo Research, USA). Each MSP reaction contained 40 ng of sodium bisulfite-modified DNA, 10 pmol of each primer, 1X PCR buffer, 100 pmol of deoxynucleoside triphosphate, and 2 units of fire pol taq polymerase from Solis Biodyne, in a final reaction volume of 25 μl. Cycling conditions were as follows: initial denaturation at 95 °C for 60 s, 35 cycles at 94 °C for 30 s, 58.3 °C (TBX20 methylation) for 30 s, 52.0 °C (TBX20 unmethylation) for 20 s, and 72 °C for 30 s, and a final extension at 72 °C for 7 min. For each set of MSP reactions, in vitro methylated genomic DNA treated with sodium bisulfite served as a positive control and pooled DNA treated by sodium bisulfite served as a negative control.

Statistical analysis

The statistical analysis was carried out using Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, and

version 21.0 for Windows) or GraphPad Prism 5.0. All quantitative variables were estimated using mean and measures of dispersion (standard deviation and standard error). Normality of data was checked by measures of skewness and Kolmogorov–Smirnov tests of normality. Expression of genes was compared using Mann–Whitney test. All statistical tests were two-sided and performed at a significance level of $*p \leq 0.05$, $**p \leq 0.01$, and $***p \leq 0.001$.

Results

Cardiac TBX20 in DCM patients and renal artery ligation (RAL) model

Cardiac TBX20 gene expression was significantly increased in DCM patients as compared to control subjects ($p < 0.001$). An 8.9-fold increase in TBX20 mRNA levels was observed in DCM samples as compared to control biopsies (Fig. 1a). Cardiac TBX20 expression was also significantly increased in RAL rats as compared to control rats ($p = 0.028$). A 7-fold increase in TBX20 mRNA was observed in RAL group as compared to control group (Fig. 1b).

Correlation of cardiac TBX20 expression with echocardiographic indices

In order to correlate TBX20 expression with echocardiographic indices, the median values of mRNA expression were used as cut-off to stratify the TBX20 expression into low and high expression levels. We observed a significant negative correlation between LVEF and the TBX20 expression ($r = -0.71$, $p < 0.001$) (Fig. 2a). LVESV was found to be positively correlated with TBX20 expression ($r = 0.39$, $p = 0.038$) (Fig. 2b). No significant difference in LVEDD, IVSed, and LVEDV were observed between DCM patients with high TBX20 expression and those with low TBX20 expression (Table 3). TBX20 expression showed a negative correlation with LVEF in RAL rats too ($r = -0.99$; $p < 0.001$) (Fig. 2c; Table 4).
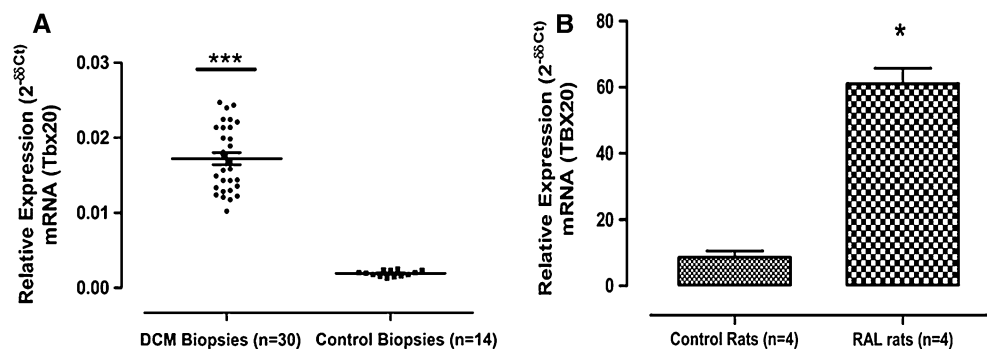
Promoter methylation of TBX20 gene in DCM

Promoter methylation status of TBX20 gene was assessed by methylation-specific PCR (MSP) in biopsies and PBMCs of DCM and control subjects. TBX20 promoter was found to be hemi-methylated in both the patients and control samples (Fig. 3; Table 5). The promoter methylation pattern was similar in all the subjects screened for TBX20 gene. For each set of MSP reactions, in vitro methylated genomic DNA treated with sodium bisulfite served as a positive control and pooled DNA treated by sodium bisulfite served as a negative control.

Copy number variations (CNVs) and TBX20 expression in DCM patients

There was no significant difference in the distribution of TBX20 copies (1/1) between patients and controls as clear from the amplification plot (Fig. 4a). Mean ΔC_t value for TBX20 gene for carrying two copies of TBX20 was 0.57 (SD = 0.02) and the RQ values thus obtained ranged from 1 to 1.105 (Fig. 4b). Our results showed that all the subjects in both DCM and control groups harbored two copies of TBX20 gene indicating that the copy number of TBX20 remained conserved in DCM.

Discussion

In this study, we provide evidence that cardiac TBX20 expression is increased in DCM and shows a negative correlation with LVEF, suggesting that an increased myocardial TBX20 gene expression may be an indicator of deteriorating cardiac function.

The increased TBX20 expression seen in our study appears to reflect an adaptive response of a stressed heart. Increased TBX20 expression has been reported in tetralogy of fallot (TOF), and no significant change in cardiac TBX20 levels was observed in VSD patients [13]. TBX20 overexpression has been shown to induce activation of multiple cardiac proliferative pathways via activation of
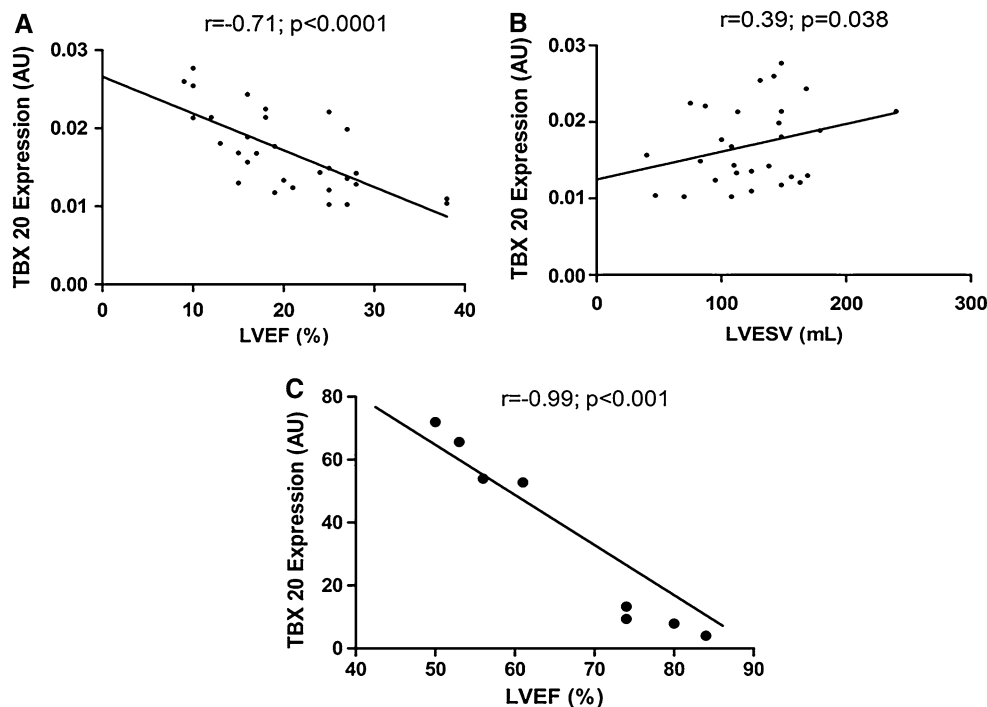

Fig. 1 Quantitative results of mRNA expression of cardiac TBX20 in **a** DCM patients and control biopsies **b** cardiac TBX20 expression in renal artery ligation (RAL) and control rats. Normalization of total RNA was done by GAPDH. Data given are mean $\pm$ SEM; $*p < 0.05$; $***p < 0.001$

Fig. 2 Scatter plots showing the correlation of cardiac TBX20 gene expression with **a** left ventricular ejection fraction (LVEF), **b** left ventricular end-systolic volume (LVESV) in DCM patients, and **c** LVEF in RAL rats. Data given are mean ± SEM; 8$p < 0.05$; ***$p < 0.001$

Table 3 Association of TBX20 expression with disease severity

Echocardiographic indices	Low expression	High expression	p value
LVEDD	63.47 ± 2.6	63.13 ± 2.9	0.931
LVEF	25.1 ± 1.7	15.4 ± 1.4	**0.000*****
LVESV	138.5 ± 17.0	133.2 ± 14.45	**0.038***
LVEDV	174.8 ± 21.0	176.1 ± 15.3	0.232
IVSed	8.6 ± 0.64	8.7 ± 0.6	0.921
Aortic diameter	27.07 ± 2.35	27.0 ± 1.0	0.98
EDT	137.3 ± 20.7	96.42 ± 13.2	0.11
E-wave velocity	49.3 ± 10.8	76.1 ± 11.3	0.09

Data are presented as mean ± SEM

LVEDD left ventricular end-diastolic diameter; *LVEF* left ventricular ejection fraction; *LVESV* left ventricular end-systolic volume; *LVEDV* left ventricular end-diastolic volume; *IVSed* interventricular septum in diastole; *E-wave velocity* peak early transmitral filling velocity

* $p < 0.05$, *** $p < 0.001$

BMP2/pSmad1/5/8 and PI3 K/AKT/GSK3β/β-catenin signaling pathways [17]. Tbx2 and N-myc1, which are direct downstream target genes of Tbx20, regulate cardiomyocyte proliferation [18]. TBX20 overexpression decreased the cell proliferation, ERK phosphorylation, and N-myc1 levels [19]. TBX20 target genes decreased with TBX20 knockdown in mice, encoding proteins with critical functions in cardiomyocytes. These included genes encoding the gap junction protein Gja1 (connexin 43); potassium channel genes Kcnd2, Kcnd3, Kcnj2, Kcnj3, Kcnip2, and Kcnh2; calcium channel genes Cacna1c, Cacna1 g, Cacna2d1, Cacnb2, and Cachd1; genes encoding calcium cycling or regulatory proteins Serca2 (Atp2a2), phospholamban (Pln), ryanodine receptor 2 (Ryr2), and calmodulin-dependent protein kinase type II delta chain (Camk2d); and those encoding cytoskeletal proteins cypher (Ldb3) and desmin (Des) [9]. Thus, increased TBX20 in DCM hearts may result in activation of cardiac genes/signaling pathways leading to cardiac remodeling and ventricular dysfunction. Increased cardiac TBX20 expression in RAL rats which showed cardiac hypertrophy and fibrosis and ventricular dysfunction further support the

Table 4 TBX20 mRNA expression and echocardiography parameters in RAL rats and control rats

Groups	LVEDD (mm)	% FS	LVEF (%)	TBX20 mRNA expression
Control rats (Sham)	4.34 ± 0.20	62.08 ± 1.18	78 ± 8.8	8.6 ± 1.9
Ligated (RAL)	5.83* ± 0.38	34.6* ± 3.8	55* ± 6.7	61.1* ± 4.6

LVEDD left ventricular end-diastolic diameter; *LVEF* left ventricular ejection fraction; *FS* fraction shortening

Data are presented as mean ± SEM

* $p < 0.05$

involvement of TBX20 in pathophysiology of cardiomyopathy. Our results are further supported by a study by Zhang et al. (2011), which showed that forced expression of TBX20 has been shown to cause ventricular hypertrabeculation and septal defects in transgenic mice, which are known factors in DCM development [20].

Reduction in LVEF is a commonly observed phenomenon in severe forms of HF, and decreased LVEF alone and in combination with ventricular arrhythmias has been shown to increase the risk of HF [21, 22]. We observed a negative correlation of cardiac TBX20 expression with LVEF and a positive correlation with LVESV, respectively, in DCM patients and in RAL rats, suggesting that increased cardiac TBX20 was associated with decreased cardiac function. Thus, a decline in LVEF and an increase in LVESV function with elevation in TBX20 gene expression reflect a detrimental effect of TBX20

dysregulation in DCM. Thus, cardiac TBX20 expression needs to be investigated as a potential predictor of disease severity and prognosis in HF.

Our study suffered from a limitation that cardiac biopsy data from DCM patients could not be compared with those from subjects without any disease as endomyocardial biopsy from healthy subjects was unethical. Further, autopsy samples from individuals dying of non-cardiac causes showed extensive mRNA degradation, resulting in inconsistent and non-reproducible data. However, an earlier study by Hammer et al. (2008) had reported unaltered cardiac TBX20 expression in VSD samples; thus, VSD biopsies were used as control in our study [13].

Sheng et al. (2012) reported promoter hypomethylation of TBX20 in TOF and suggested that decreased promoter methylation was responsible for increased TBX20 expression in TOF [23]. However, we did not find any significant
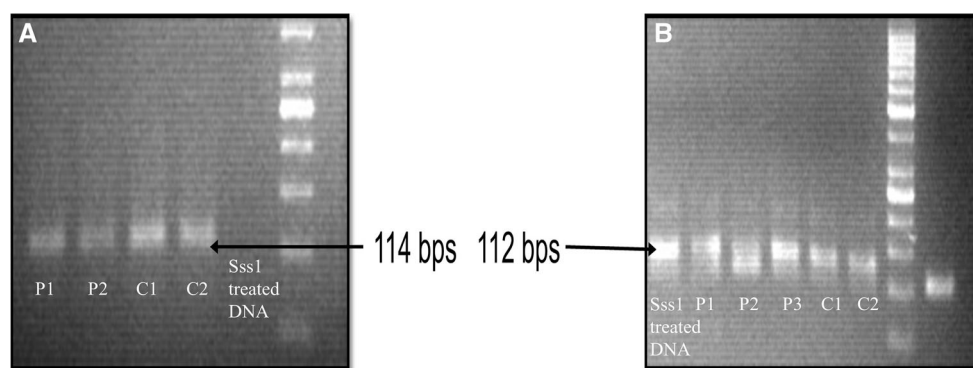
Fig. 3 Promoter methylation pattern determined by methylation-specific PCR in DCM and control subjects. Representative agarose gel from **a** unmethylation (114 bps) and **b** methylation (112 bps) PCR showing similar methylation patterns in patients and controls. P1–P3 represent patient biopsy 1 to 3; C1–C2 represent control biopsy 1 to 2, respectively. In vitro methylated (sss1-treated) genomic DNA served as a positive control and pooled DNA treated by sodium bisulfite served as a negative control

Table 5 TBX20 promoter methylation status in DCM patients and control

	TBX20 methylation	TBX20 unmethylation	Odds ratio
Control group	**61** (54 %)	**52** (46 %)	–
DCM group	**81** (54 %)	**67** (46 %)	1.02 (0.62–1.66); $p = 0.5223$

$p > 0.05$ is non-significant

Numbers in bold represent the number of subjects found having methylated or unmethylated TBX20 promoter
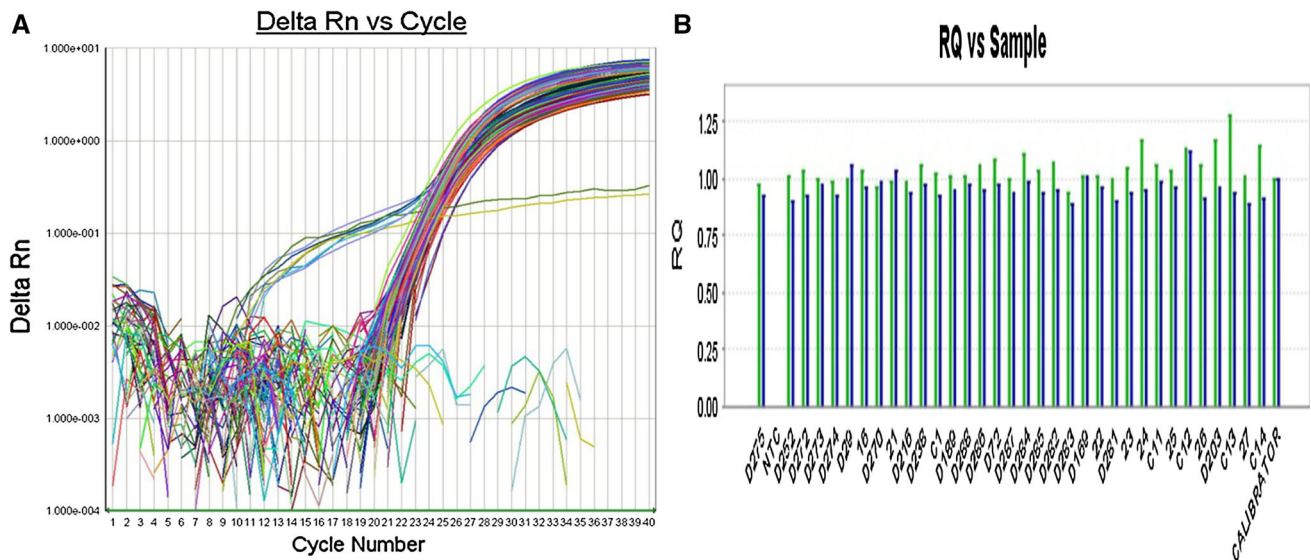
A

B

Fig. 4 Copy number variation determination in DCM and control samples. **a** Amplification plot of TBX20 gene for both the DCM patients and controls showing 2 copies of the gene. **b** Relative difference in TBX20 promoter methylation status between DCM and control samples, indicating that promoter methylation of TBX20 gene was not involved in regulating TBX20 expression in DCM. This is the first report showing the promoter methylation status of TBX20 promoter in DCM patients.

quotient plot showing the distribution of TBX20 copies among DCM and control samples. Sample with known copies of TBX20 and RNase P was used as both the calibrator and positive control

Since CNVs have been shown to contribute to altered expression of various genes [24–26], we compared TBX20 CNVs between DCM and control subjects. However, no significant difference in TBX20 gene CNVs was seen between DCM patients and controls indicating that CNVs were not contributing to altered TBX20 gene expression in DCM. We also explored if microRNAs could be involved in regulating TBX20 expression in DCM. MicroRNAs are important regulators of post transcriptional gene expression in various cardiac diseases [27–30]. We used bioinformatics tools to identify putative microRNAs (miRNAs) targeting TBX20 gene; however, we found that TBX20 does not have a 3′-UTR sequence long enough to support the binding of any miRNA, indicating that microRNA may not be involved in regulating its expression. Thus, other regulatory mechanisms such as histone acetylation need to be examined in TBX20 regulation in DCM.

In summary, our results show an increase in TBX20 cardiac gene expression which showed a negative correlation with LV function in DCM patients and RAL rats. These findings suggest that TBX20 may be playing an important role in the pathophysiology of DCM.

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