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DUSP-1 gene expression is not regulated by promoter methylation in diabetes-associated cardiac hypertrophy

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Abstract

Background: The exact mechanism causing decreased expression of the dual specific phosphatase-1 (*DUSP-1*) gene in diabetes-associated cardiac hypertrophy is not known. DNA promoter methylation is often associated with decreased gene expression in many diseases including cardiovascular diseases. So, we investigated whether epigenetic silencing *via* promoter methylation is involved in the decreased expression of *DUSP-1* in diabetes-associated cardiac hypertrophy.

Methods: Real-time polymerase chain reaction (PCR) and Western blotting confirmed the down regulation of the *DUSP-1* gene at transcriptional and translational levels. Bisulfite-converted DNA samples from myocardium of rat model of diabetic cardiomyopathy (DCM), high glucose (HG)-treated neonatal rat cardiomyocytes (NRCMs) and cardiac tissues from archived human myocardial DCM autopsies along with their respective controls were analyzed for methylation in the promoter region of the *DUSP-1* gene.

Results: We observed no methylation in the promoter regions of the *DUSP-1* gene in DCM rat hearts, in HG-treated NRCMs (between –355 bp and –174 bp) and in cardiac tissues from archived human myocardial DCM autopsies (between –274 bp and –73 bp).

Conclusion: Methylation-mediated silencing of the *DUSP-1* promoter does not appear to be associated with reduced expression, indicating the involvement of other factors in specific suppression of *DUSP-1* in diabetes-associated cardiac hypertrophy.

Keywords: cardiac hypertrophy, diabetic cardiomyopathy, methylation

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Introduction

Diabetic cardiomyopathy (DCM), a distinct primary disease process leading to heart failure in diabetic patients, is independent of coronary artery disease. Cardiac hypertrophy and cardiac fibrosis are distinct features of DCM.[1] Mitogen-activated protein kinases (MAPKs) are one of the dysregulated signaling pathways in DCM.[2] MAPKs are highly conserved protein kinases which comprise sequentially activated protein kinases, p38, c-Jun N-terminal kinases (JNKs) and extracellular signal-regulated kinases (ERKs).[3] It has been reported that cardiovascular diseases including DCM are associated with increased activity of all of the three MAPKs.[4]

Dual specific phosphatase-1 (*DUSP-1*) (or MKP-1) dephosphorylates the MAPKs p38, JNK and ERK after localizing to the nucleus *in vitro*.[5,6] Bueno and colleagues have shown that decreased expression of *DUSP-1* induces cardiac hypertrophy in transgenic mice model. Constitutive expression of *DUSP-1* in cultured primary cardiomyocytes blocks the activation of p38, JNK1/2, and ERK1/2 and prevents agonist-induced hypertrophy.[5] Also, Messier and colleagues reported that p38 expression/activity and cardiac hypertrophy increased in *DUSP-1* knockout mice, indicating the involvement of upregulation of MAPKs activities mediated by *DUSP-1* in the pathophysiology of cardiac hypertrophy.[7] Decreased

expression of *DUSP-1* in the myocardium of streptozotocin (STZ)-induced diabetic rats suggests a role of DUSPs and MAPKs in the pathophysiology of DCM.[8]

Recent studies suggest that hyperglycemia is an important epigenetic modifier and results in long-lasting epigenetic modifications that lead to differential expression of genes involved in diabetes-induced pathological changes.[9] Methylation is the most widely studied type of epigenetic modification of DNA.[10] Regulation of gene expression by promoter methylation is shown to be involved in many diseases including cardiovascular diseases.[11] but still the role of methylation in pathophysiology of DCM is not well studied.

The role of epigenetic changes in modulating the cardiac *DUSP-1* expression in DCM/hyperglycemia is not known. Thus, we hypothesized that hyperglycemia-induced epigenetic changes in *DUSP-1* may regulate *DUSP-1* expression in DCM. Thus, we investigated mRNA expression and methylation status of the *DUSP-1* gene in a rat model of DCM, in DCM patients and in high glucose (HG)-treated neonatal rat cardiomyocytes (NRCMs).

Materials and methods

Animal model of DCM

The animal experiments were performed in accordance with Institute Animal Ethics Committee (IAEC), PGIMER, Chandigarh, India (Approval number: IAEC/242). According to the earlier described protocol, the DCM rat model was developed using low doses of STZ along with a high-fat diet (HFD).[12] Briefly, beginning at 180 g of weight, the male Wistar rats were divided in two groups. The first group was made diabetic ($n = 6$) by giving HFD (22% fat, 48% carbohydrate, and 20% protein) for 4 weeks, followed by intraperitoneal injection of STZ (2×30 mg/kg consecutive injections, a week apart in citrate buffer, pH = 4.4). In the second group, sex-matched control rats ($n = 6$) were given an injection of equal volume of citrate buffer along with regular chow (5% fat, 53% carbohydrate, and 23% protein). DCM was confirmed after 3 months by echocardiography, morphological, histopathological examination, and cardiac expression of hypertrophic markers (ANP & β-MHC). Data not shown.[13]

Human patients

Formalin fixed paraffin embedded (FFPE) human myocardial archived tissues from type 2 diabetic patients, showing features of DCM ($n = 6$) and healthy control patients ($n = 6$) were included in the present study. Tissues were screened for DCM features, cardiac fibrosis, myocyte hypertrophy and absence of ischemic lesions, by tissue histology using Hematoxylin and Eosin (H&E) and Masson's trichrome (MT) staining (Supplementary Figure 1). In the control group, archived tissues of patients who died of accident or noncardiac diseases were included. From these FFPE tissues, total RNA was extracted using the recover all total nucleic acid isolation kit following the manufacturer's instructions (Ambion, USA). The study was approved by the Institutional Ethical Review Committee and was conducted in accordance with guidelines of the Declaration of Helsinki.

HG treatment of cardiomyocytes

As described earlier, NRCMs were cultured from new born Wistar rat pups.[14] In brief, 10–15 isolated hearts were washed, minced in M199 medium supplemented with 10% fetal bovine serum and incubated in the presence of collagenase (0.2 mg/ml; Worthington, USA). An hour of pre-plating was done for removal of noncardiomyocytes and on the pre-coated culture plates (1% laminin, Sigma, USA) cardiomyocytes (1×10^6 cells/35 mm) were plated. Fresh medium supplemented with 10% (w/v) fetal bovine serum was added after 48 h of incubation and incubated for another 24 h. Prior to all the experiments the cells were serum-starved overnight followed by 48 h of incubation with 5.5 mmol/l D-glucose (control; NG) or 30 mmol/l D-glucose (HG). A 30 mmol/l mannitol solution was used as an osmotic control.

RNA isolation and qRT-PCR

Total RNA (small and large) from rat cardiac tissue and NRCMs was extracted using mirVana™ RNA isolation kit (Ambion, USA). cDNA was synthesized using manufacturer's instructions (Invitrogen, USA). qRT-polymerase chain reaction (PCR) was performed on cDNA samples using an ABI cycler using following primers: 18s forward = CGCGGTTCTATTTTGTTGGT; 18s reverse = GTCGGCATCGTTTATGGTC; *DUSP-1* forward = CAAGAGCATCCCTGTG

GAGGAC; *DUSP-1* reverse = AGGTAAGC AAGGCAGATGGTGG. PCR expression was normalized to the expression of housekeeping genes using $2^{-\Delta\Delta CT}$ methodology.[15]

Western blotting

NRCMs and rat heart tissue were lysed in RIPA buffer (1% sodium deoxycholate, 50 mm Tris-HCl pH 7.6, 150 mm NaCl, 1% Triton NP-40, 0.1% SDS, 1 mm EGTA) supplemented with phosphatase and protease inhibitor mix (Thermo Scientific, USA). SDS-PAGE was used to resolve proteins followed by transfer and immunoblotting with *DUSP-1* antibody (rabbit; Santa Cruz Biotechnology, USA).

DNA isolation, bisulfite conversion and bisulfite specific PCR

DNA was extracted from rat cardiac tissue and NRCMs according to the manufacturer's instructions using Trizol reagent (Invitrogen, UK). Also, from FFPE tissues, DNA was extracted using the recover all total nucleic acid isolation kit (Ambion, USA). DNA samples were air dried and quantified using an ND1000 spectrophotometer (Thermo Scientific) and stored at −20°C. A total of 500 ng of isolated DNA was bisulfite-converted using Zymo EZ DNA methylation kit (cat no D5005, USA). CpG-rich 5' regions in *DUSP-1* gene methylation status were assessed by bisulfite sequencing PCR (BSP) (Methyl Primer Express v 1.0, Thermo Fisher Scientific, USA): *DUSP-1* (rat) forward CAGGGGAGCAGGGC AGGT-GTCC; *DUSP-1*(rat) reverse CACCA AAGC-CAAAAGCAAAGAC; *DUSP-1* (human) forward AGTTTGGAGTTAAGGTGATAGAA; *DUSP-1* (human) reverse CTATTCCTAATCT-TATAACCCCC. Bisulfite-modified DNA was amplified by PCR using hot start taq DNA polymerase (epitaq, Takara, USA). Followed by analyses of PCR products on 2% agarose gel and gel purification (Qiagen, Cat no 28604, USA). Finally, sequencing of the amplified products was performed (Applied Biosystems, USA) and methylation was determined using BioQ analyser software.[16]

Statistical analysis

All results were shown as mean ± SD. Comparisons between DCM rats and control rats were performed by a Student's *t*-test. A one-way analysis of variance was used followed by the *post hoc* Tukey multiple comparison test to analyze data for NG, HG and osmotic control-treated cardiomyocytes. Probability of <0.05 was considered statistically significant.

Results

Downregulation of DUSP-1 *in DCM*

DUSP-1 expression was examined in the DCM group and in HG-treated NRCMs. We observed significantly decreased expression of *DUSP-1* in the myocardium at both the protein and mRNA level in the DCM group compared with the control group rats (Figure 1a) and in HG-treated NRCMs in comparison with cells treated with NG at 48 h (Figure 1b).[17]

BSP analysis of the DUSP-1 *gene promoter in DCM group and in HG-treated NRCMs*

Previous reports have suggested that hyperglycemia could induce DNA promoter methylation which is often associated with decreased gene expression.[18] So, we hypothesized that increased DNA methylation of the *DUSP-1* promoter region might be contributing to decreased expression of *DUSP-1* in the DCM group and in NRCMs treated with HG. For methylation analysis, we used BSP followed by direct sequencing. We analyzed the *DUSP-1* gene for CpG islands in the promoter region using the United States National Center for Biotechnology Information (NCBI) genome database and observed a CpG island around the transcriptional start site (designated as '0') between the upstream −355 bp and −174 bp for *DUSP-1* using the methylation analysis software, Methyl Primer Express v 1.0 (ABI, USA). Figure 2a shows the structure of the *DUSP-1* gene and the topology of the BSP primers, indicating the CpG island containing 17 CpG sites in the *DUSP-1* promoter. We carried out BSP PCR-based sequencing analysis of myocardial *DUSP-1* in the DCM group, in the control group, and HG and NG-treated NRCMs. An amplicon consisting of 180 bp of *DUSP-1* promoter region was obtained (Figure 2b) which was sequenced for methylation status (Figure 2c). We did not observe any methylation in the *DUSP-1* promoter in the DCM group or in HG-treated NRCMs, as all CpG dinucleotides were found to be converted to TpG suggesting that all CpG sites were un-methylated (Figure 2d).

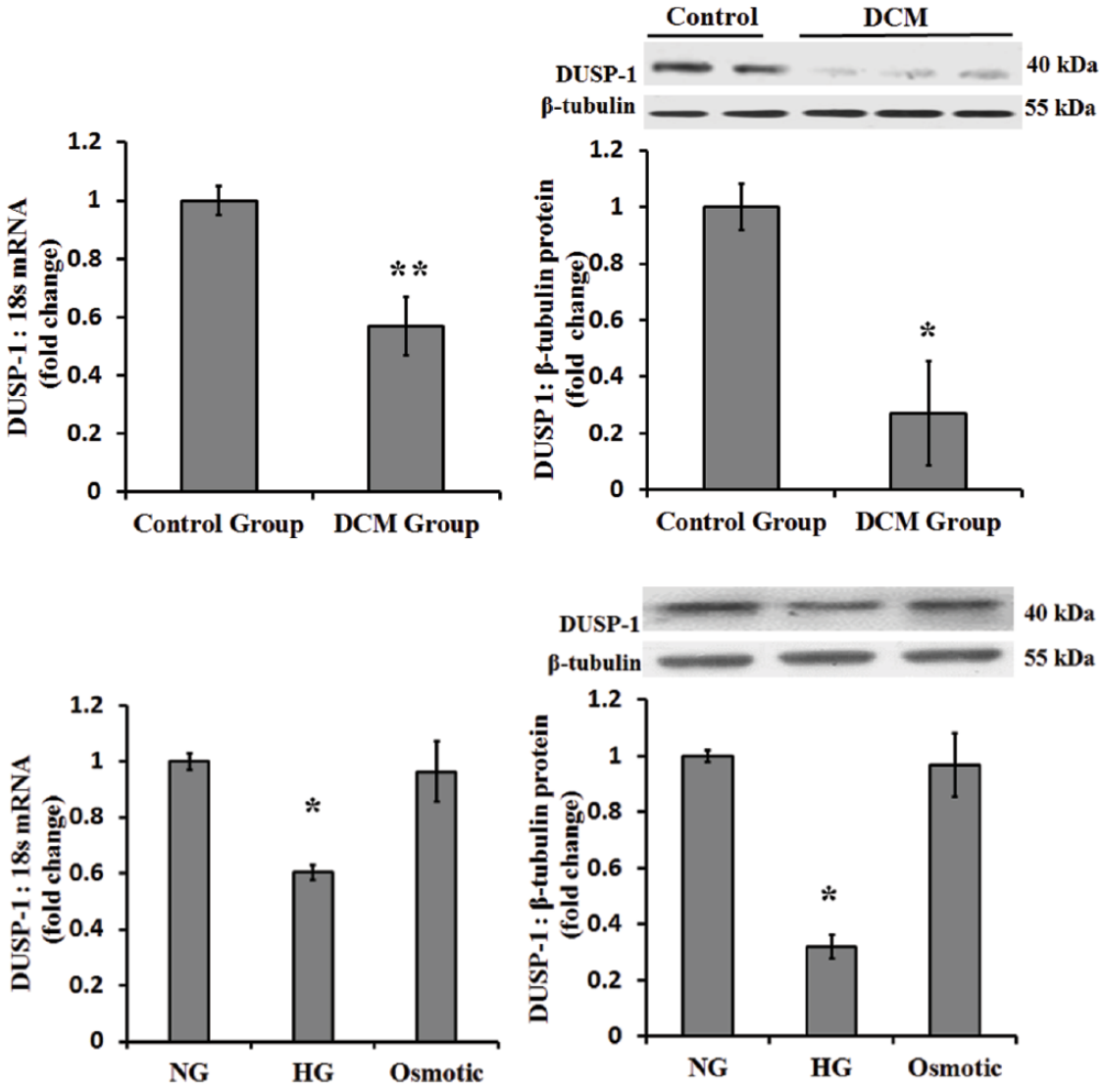

Figure 1. *DUSP-1* expression in DCM rats and in HG-treated NRCMs. (a) *DUSP-1* mRNA (left panel) and protein levels (right panel) in cardiac tissue of DCM rats ($n = 6$) and control group ($n = 6$) rats. (b) *DUSP-1* mRNA (left panel) and protein levels (right panel) in HG, NG and mannitol (osmotic) treated NRCMs. For a and b, bar graph (right panels) shows expression ratio of *DUSP-1* over β-tubulin. Data are mean ± SD from at least three independent sets of experiments carried out in triplicate. *$p < 0.05$, **$p < 0.001$ compared with control group or NG.
DCM, diabetic cardiomyopathy; *DUSP-1*, dual specific phosphatase-1; HG, high glucose; NG, normal glucose; NRCM, neonatal rat cardiomyocyte; SD, standard deviation.

BSP analysis of the DUSP-1 *gene promoter in DCM patients*

Using the BSP, we also examined the methylation status of *DUSP-1* gene in archived myocardial tissue of DCM patients and of normal healthy controls. Using the NCBI genome database, we searched 'human *DUSP-1* gene' and found a CpG island around the transcriptional start site (designated as '0') between the upstream −274 bp and −73 bp for *DUSP-1* using the methylation analysis software, Methyl Primer Express v1.0 (ABI). Figure 3a illustrates the structure of the *DUSP-1* gene and the topology of the BSP primers, indicating the position of the CpG island containing 15 CpG sites in the *DUSP-1* promoter. We followed this with BSP PCR-based sequencing analysis to assess the methylation status of the *DUSP-1* gene in myocardium of DCM and control autopsy tissues. Figure 3b shows amplified 201bp DNA sequence of *DUSP-1* promoter for methylation analysis. Figure 3c shows representative BSP

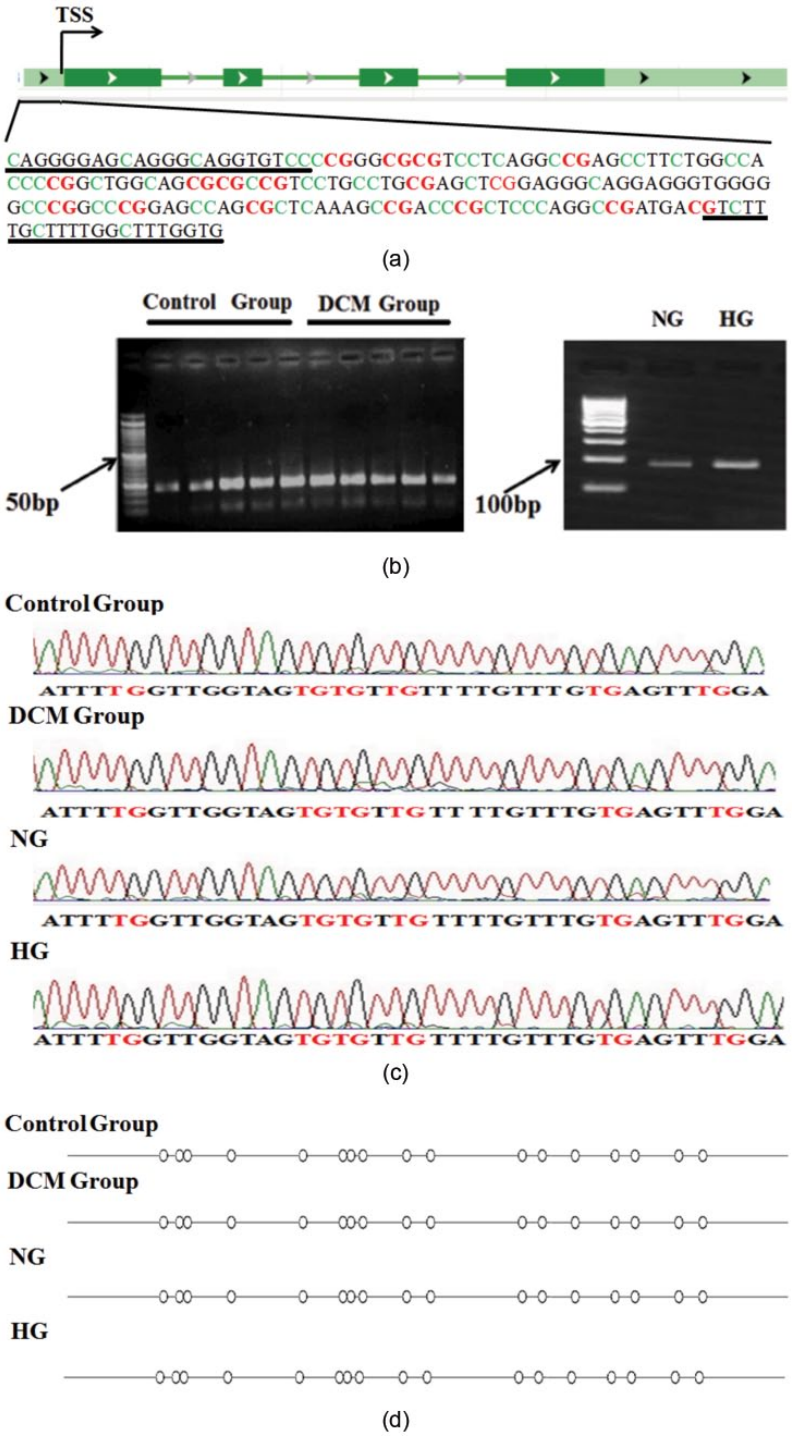

Figure 2. Detection of *DUSP-1* gene methylation in DCM rats and in HG-treated NRCMs. (a) Illustration of the *DUSP-1* gene and topology of the BSP primer. The black bar indicates the analyzed region. CpG dinucleotides are shown in red and cytosines located in regions other than CpG dinucleotides are shown in green. The latter is used as internal controls for monitoring bisulfite treatment efficiency. The underlined sequence indicates the primers for BSP. (b) Agarose gel showing representative product of bisulfite-specific PCR analysis of *DUSP-1* gene in control, DCM, NG-treated NRCMs and HG-treated NRCMs. (c) Representative sequencing data of the *DUSP-1* gene promoter in control, DCM, NG-treated cardiomyocytes and HG-treated cardiomyocytes obtained using direct BSP PCR-based sequencing analysis. (d) BSP analysis of the methylation status of *DUSP-1* in control, DCM, NG-treated NRCMs and HG-treated NRCMs.
BSP, bisulfite sequencing PCR; DCM, diabetic cardiomyopathy; *DUSP-1*, dual specific phosphatase-1; HG, high glucose; NG, normal glucose; NRCM, neonatal rat cardiomyocyte; PCR, polymerase chain reaction.

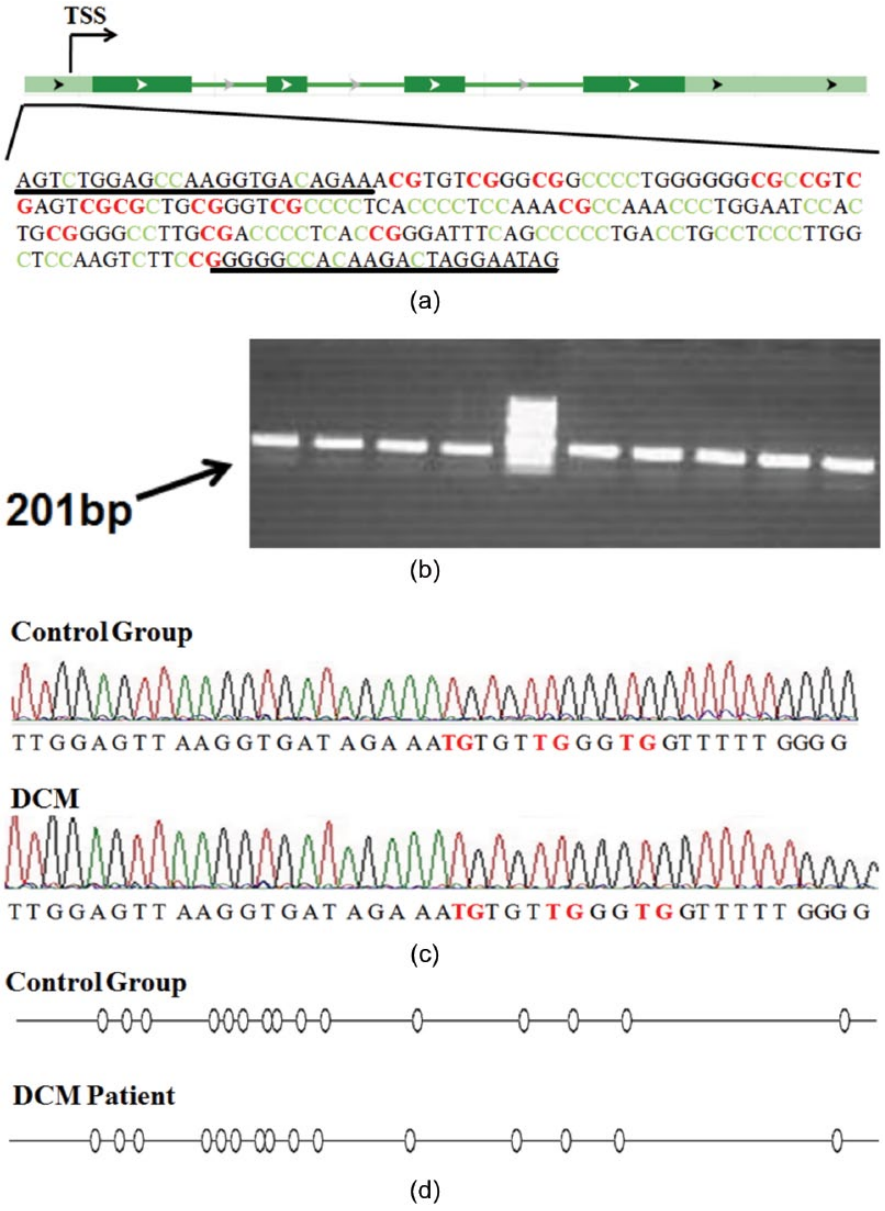

Figure 3. Detection of *DUSP-1* gene methylation in archived myocardial tissues of DCM patients. (a) Illustration of the human *DUSP-1* gene and topology of the BSP primer. The black bar indicates the analyzed region. CpG dinucleotides are shown in red and cytosines located in regions other than CpG dinucleotides are shown in green. The latter is used as internal controls for monitoring bisulfite treatment efficiency. The underlined sequence indicates the primers for BSP. (b) Agarose gel showing representative product of bisulfite-specific PCR analysis of the *DUSP-1* gene. (c) Representative sequencing data of the *DUSP-1* gene promoter in DCM patients and in the control group obtained using direct BSP PCR-based sequencing analysis from both sides. (d) BSP analysis of the methylation status of *DUSP-1* in control and DCM patients group.
BSP, bisulfite sequencing PCR; DCM, diabetic cardiomyopathy; *DUSP-1*, dual specific phosphatase-1; PCR, polymerase chain reaction.

PCR-based sequencing analysis results for the *DUSP-1* promoter. Similar to the *DUSP-1* promoter methylation status of DCM rat and HG-treated NRCMs, we did not observe any methylation in promoter region of *DUSP-1* gene in DCM patients and the control group (Figure 3d).

Discussion

Diabetic patients continue to show long-lasting detrimental effects in spite of good glycemic control, which has been attributed to hyperglycemia-mediated 'metabolic memory'.[19] This metabolic memory has been attributed partly to

hyperglycemia-mediated epigenetic changes in the genes of key pathways involved in diabetes-induced complications, which persist even after a return to normo-glycemic state and were suggested to play important role in pathophysiology of diabetes and associated vascular complications.[9] We examined the methylation status of a MAPK phosphatase, *DUSP-1* in the heart tissue from diabetic patients with DCM and in the hearts of an animal model of DCM and also in HG-treated NRCMs. *DUSP-1* dephosphorylates the p38, JNK and ERK after localizing to the nucleus *in vitro*.[20] Decreased cardiac expression of *DUSP-1* and increased expression of all three MAPKs has been shown in the STZ-induced diabetic rats.[8] Bueno and colleagues have shown that decreased expression of *DUSP-1* induces cardiac hypertrophy in a transgenic mice model, suggesting a role of DUSPs and MAPKs in the pathophysiology of DCM.[5] However, the epigenetic regulation of *DUSP-1* has not been investigated thus far. Our results showed no change in the promoter methylation status of this gene in cardiac tissues from rat models of DCM or in HG-treated NRCMs. Similarly, we found no significant changes in promoter methylation profile in cardiac tissues from archived human myocardial DCM autopsies. These results indicate that promoter DNA methylation of the *DUSP-1* gene is not altered in a diabetic or hyperglycemic milieu. These results suggest that hyperglycemia does not induce promoter methylation of *DUSP-1* and thus does not play any role in altered expression of *DUSP-1* in DCM hearts. Our findings contrast with results previously reported in cancer studies, in which aberrant promoter methylation of *DUSP-1* has been shown to regulate the gene's expression in various types of tumors; for example, promoter methylation of the *DUSP-1* gene was shown to play a role in silencing *DUSP-1* expression in primary oral squamous cell carcinoma.[21] *DUSP-1* promoter methylation has been also reported in breast cancer and was suggested to be a potential biomarker for malignancy of breast cancer.[22] The observed difference between our results and those reported in cancer may be due to different disease etiologies. Epigenetic modifications are reported to play a central role in progression of cardiovascular diseases including diabetes-associated cardiac diseases. Hyperglycemia is an important epigenetic modifier resulting in long-lasting epigenetic modifications that lead to differential expression of genes involved in diabetes-induced pathological changes. There are no reports on promoter methylation analysis of *DUSP-1* in cardiovascular diseases including DCM. To our knowledge, this is the first report analyzing the promoter methylation pattern in DCM. Our results suggest that DNA methylation does not seem to regulate the myocardial expression of *DUSP-1* gene in DCM. Therefore, our results suggest that promoter DNA methylation of the *DUSP-1* gene is not altered in a diabetic or hyperglycemic milieu. Apart from the promoter methylation role of other epigenetic mechanisms like chromatin modifications (acetylation or methylation), regulation by non-coding RNAs could possibly play a role in the regulation of *DUSP-1* expression.

Limitations and future perspectives

In this study, we only analyzed the promoter region of *DUSP-1* gene, which is the main limitation of our study. Furthermore, the DNA methylation analyses were carried out in human samples in a relatively small number of individuals; to fully understand the mechanism of promoter methylation of the *DUSP-1* gene, a larger population would need to be explored. A functional study which includes the epigenetic mechanisms in the upstream signaling pathways of *DUSP-1* gene regulation or other areas of *DUSP-1* region would also have to be carried out. Finally, we cannot exclude the role of other nonmethylation factors like histone modifications and microRNAs in downregulation of *DUSP-1* expression.

Conclusion

In conclusion, we found no evidence that promoter methylation-mediated silencing is responsible for the reduced expression of the *DUSP-1* gene in DCM. However, the role of other nonmethylation epigenetic factors cannot be excluded in the regulation of *DUSP-1* gene expression. Histone modifications could possibly play a role in the reduction of *DUSP-1* expression. On the other hand, microRNA-based regulation might be involved in the regulation of *DUSP-1* expression. Further, studies are clearly warranted to explore the underlying mechanisms leading to decreased *DUSP-1* expression in DCM.

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Conflict of interest statement

The authors declare that there is no conflict of interest.

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