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SHORT COMMUNICATION

ASB1 differential methylation in ischaemic cardiomyopathy: relationship with left ventricular performance in end-stage heart failure patients

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Abstract

Aims Ischaemic cardiomyopathy (ICM) leads to impaired contraction and ventricular dysfunction, causing high rates of morbidity and mortality. Epigenomics allows the identification of epigenetic signatures in human diseases. We analyse the differential epigenetic patterns of the *ASB* gene family in ICM patients and relate these alterations to their haemodynamic and functional status.

Methods and results Epigenomic analysis was carried out using 16 left ventricular (LV) tissue samples, eight from ICM patients undergoing heart transplantation and eight from control (CNT) subjects without cardiac disease. We increased the sample size up to 13 ICM and 10 CNT for RNA sequencing and to 14 ICM for pyrosequencing analyses. We found a hypermethylated profile (cg11189868) in the *ASB1* gene that showed a differential methylation of 0.26$\Delta\beta$ (P = 0.016). This result was validated by a pyrosequencing technique (0.23$\Delta\beta$, P = 0.048). Notably, the methylation pattern was strongly related to LV ejection fraction (r = −0.849, P = 0.008), stroke volume (r = −0.929, P = 0.001), and end-systolic and diastolic LV diameters (r = −0.743, P = 0.035 for both). *ASB1* showed a down-regulation in messenger RNA levels (−1.2-fold, P = 0.039).

Conclusions Our findings link a specific *ASB1* methylation pattern to LV structure and performance in end-stage ICM, opening new therapeutic opportunities and providing new insights regarding which is the functionally relevant genome in the ischaemic failing myocardium.

Keywords Ischaemic cardiomyopathy; Epigenomics; Heart failure; Left ventricular dysfunction; Stroke volume; *ASB1*

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Introduction

There are several large-scale studies deciphering the alterations in human heart failure (HF) proteome and genome, to elucidate the molecular mechanisms involved in the pathophysiology of this syndrome.[1,2] However, apart from these global changes, there are other omic approaches revealing insights in the regulation of expression patterns in the disease. Epigenomics has allowed the identification of epigenetic signatures in human diseases including different types of cancer,[3–5] neurological disorders,[6,7] or infections.[8] There are also some evidences of their role in cardiovascular diseases.[9–11] Moreover, the development of the novel approach MethylationEPIC BeadChip (Infinium) microarray has improved greatly the coverage, reaching 850 000 CpG methylation sites (850K).[12]

Our group has extensively studied different pathophysiological factors in HF, including apoptosis processes[13] and cytoskeletal and cell adhesion molecular alterations,[14,15] that

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are important to the clinical status of patients. Interestingly, the gene family *ASB* codifies ankyrin repeat and SOCS box proteins that mediate, through their specific domains, protein–protein interactions, protein synthesis, and myogenesis and proteasomal degradation processes.[16–18] Moreover, members of this family have been related to skeletal muscle mass regulation.[19]

Taking into account these previous data and the increasing evidence of the influence of epigenetic changes in the pathophysiology of human diseases, we hypothesize that the epigenetic changes in the *ASB* gene family may relate to the ischaemic left ventricular (LV) performance. We analysed specific gene methylation changes in patients with end-stage ischaemic cardiomyopathy (ICM) compared with those in control (CNT) subjects. Furthermore, we related these alterations with LV function and with invasively calculated stroke volume.

Methods

Cardiac tissue samples

Epigenomic experiments were conducted with eight LV tissue samples from ICM patients undergoing cardiac transplantation and eight non-diseased donor hearts (CNT), increasing the sample size for RNA sequencing (13 ICM) and pyrosequencing (14 ICM). Clinical history, haemodynamic study, electrocardiogram, and Doppler echocardiography data were available. These data were collected by physicians blind to the subsequent analysis of LV function that was carried out. Patients were functionally classified according to the New York Heart Association criteria and were receiving medical treatment following the guidelines of the European Society of Cardiology.[20]

All CNTs had normal LV function (LV ejection fraction $> 50\%$), and none had any history of cardiac disease. Samples were obtained from non-diseased donor hearts that had been rejected for cardiac transplantation owing to size or blood type incompatibility. Donors died of either cerebrovascular or motor vehicle accidents.

Tissue samples were collected from near the apex of left ventricle, maintained in 0.9% NaCl, and preserved at 4°C for a maximum of 4.4 ± 3 h after the coronary circulation loss. Then, samples were stored at −80°C until used. Appropriate handling and rapid sample collection and storage, by our on-call (24 h) team over the last 10 years, led to the obtaining of high-quality samples (RNA integrity number ≥ 9 and DNA ratios 260/280 ~ 1.8 and 260/230 ~ 2.0).

This study was approved by the Ethics Committee (Biomedical Investigation Ethics Committee of La Fe University Hospital of Valencia, Spain) and was conducted in accordance with the guidelines of the Declaration of Helsinki.[21] Signed informed consent was obtained from each patient or relative in the case of CNT subjects.

DNA extraction, quality assessment, and Infinium MethylationEPIC BeadChip

DNA was extracted using a modified phenol–chloroform protocol. The epigenomic study (Infinium MethylationEPIC BeadChip platform, Illumina) used the HumanMethylation450 BeadChip protocol.[12] All purified DNA samples were randomly distributed into 96-well plates. Bisulfite conversion of 600 ng of genomic DNA was performed using the EZ-96 DNA Methylation Kit (Zymo Research Corp., Irvine, CA, USA) following the manufacturer's recommendations. About 200 ng of bisulfite-converted DNA was used for hybridization on the Infinium MethylationEPIC BeadChip (Illumina). Briefly, samples were whole-genome-amplified followed by an enzymatic endpoint fragmentation, precipitation, and resuspension. The resuspended samples were hybridized onto the BeadChip for 16 h at 48°C and washed. A single-nucleotide extension with labelled dideoxynucleotides was performed, and repeated rounds of staining were applied with a combination of labelled antibodies differentiating between biotin and 2,4-dinitrophenol (DNP). Colour balance adjustment and quantile normalization were performed in order to normalize the samples between the two colour channels. Methylation level was displayed as beta values ranging from 0 to 1. Beta values with detection $P > 0.01$ were removed from the analysis. The raw data were normalized and background corrected. The resulting raw data (IDATs) were normalized and background corrected using the methylation module (1.9.0) in GENOMESTUDIO (v2011.1) software.

Validation by pyrosequencing

Five hundred nanograms of DNA were converted using the EZ DNA Methylation Gold (Zymo Research) bisulfite conversion kit, following the manufacturer's recommendations. Specific sets of primers for PCR amplification and sequencing were designed (PyroMark assay design v2.0.01.15) to hybridize with CpG-free sites, ensuring methylation-independent amplification. PCR was performed with biotinylated primers, and the PyroMark Vacuum Prep Tool (Biotage, Sweden) was used to prepare single-stranded PCR products. Reactions were performed in a PyroMark Q24 System version 2.0.6 (QIAGEN), and the methylation value was obtained from the average of the CpG dinucleotides included in the sequence analysed.

RNA sequencing and computational analysis

These protocols were performed as described in the work of Ortega *et al*.[14] The data presented in this manuscript have been deposited in the National Center for Biotechnology

Information's Gene Expression Omnibus[22] (GSE55296) (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE55296).

Statistical methods

Data were analysed as previously described.[14] $P < 0.05$ was considered statistically significant. CpGs with $\Delta\beta \geq \pm 0.1$ were considered differentially methylated. All graphics have been performed with the SIGMAPLOT V10.0 software.

Results

Clinical characteristics of patients

For epigenomic studies, we analysed eight ICM samples, all men with a mean age of 53 ± 5 years, and eight CNT samples, 63% men with a mean age of 59 ± 20 years. Sample size was increased for RNA sequencing (13 ICM, all men with a mean age of 54 ± 7 years, and 10 CNT, 80% men with a mean age of 47 ± 16 years) and also for pyrosequencing (14 ICM, all men with a mean age of 53 ± 6 years). Patients had a New York Heart Association classification of III and IV and were diagnosed with co-morbidities including hypertension and diabetes mellitus. Co-morbidities and other echocardiographic data were not available for the CNT group, in accordance with the Spanish Organic Law on Data Protection 15/1999. Clinical characteristics of patients are shown in *Table 1*.

Methylation profile of *ASB* family and gene expression analysis of *ASB1* gene

We analysed the methylation status (beta values) of CpGs belonging to the *ASB* gene family between eight ICM patients and eight CNT donors using the 850K methylation array. Analysis of CpG differential methylation revealed the presence of only one hypermethylated CpG site of all *ASB* family, located in the *ASB1* gene (chr2:239344401–239344627) with a $\Delta\beta > 0.1$. The hypermethylated CpG site (cg11189868) displayed a differential methylation profile of $0.26\Delta\beta$ (P = 0.016) (*Figure 1A*). We also validated these results through pyrosequencing, observing a $0.23\Delta\beta$ (P = 0.048) (*Figure 1B*).

Further, we performed an analysis of *ASB1* messenger RNA levels through RNA sequencing, and we found a down-regulation of the *ASB1* gene expression of -1.2-fold (P = 0.039) (*Figure 1C*).

Relationships between *ASB1* differential methylation and left ventricular function and performance

We sought to investigate the potential relationships between *ASB1* differential methylations and expression and haemodynamic and echocardiographic parameters of ICM patients. Interestingly, the differential methylation pattern of *ASB1* cg11189868 was strongly linked to stroke volume ($r = -0.929$, P = 0.001) and LV ejection fraction ($r = -0.849$, P = 0.008) (*Figure 2*). This *ASB1* methylation profile also related to end-systolic and end-diastolic LV diameters ($r = -0.743$, P = 0.035 for both).

Discussion

In this study, we analysed the methylation profile of the *ASB* gene family in ICM patients, showing the presence of a differentially methylated CpG site located at the *ASB1* gene. None of the other *ASB* family genes showed methylation changes. This analysis demonstrates the presence, not previously

Table 1 Clinical characteristics of ischaemic cardiomyopathy patients

	ICM (n = 8) Epigenomics	ICM (n = 13) RNA sequencing	ICM (n = 14) Pyrosequencing
Age (years)	53 ± 5	54 ± 7	53 ± 6
Gender male (%)	100	100	100
NYHA class	III–IV	III–IV	III–IV
BMI (kg/m^2)	28 ± 3	26 ± 4	28 ± 4
Haemoglobin (mg/dL)	14 ± 2	14 ± 3	14 ± 2
Haematocrit (%)	44 ± 4	41 ± 6	42 ± 5
Total cholesterol (mg/dL)	152 ± 43	162 ± 41	171 ± 46
Prior hypertension (%)	25	30	39
Prior smoking (%)	88	84	92
Diabetes mellitus (%)	63	38	54
LVEF (%)	24 ± 6	24 ± 4	23 ± 5
LVESD (mm)	57 ± 8	55 ± 7	56 ± 7
LVEDD (mm)	65 ± 7	64 ± 7	64 ± 7

BMI, body mass index; ICM, ischaemic cardiomyopathy; LVEF, ejection fraction; LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; NYHA, New York Heart Association.

Figure 1 Differentially methylated profile of *ASB1* and gene expression in ICM patients. (A) Methylation pattern of the *ASB1* gene in ICM patients showing the expansion of the differentially methylated CpG sites between ICM and CNT. (B) Validation of DNA methylation CpG island by pyrosequencing. (C) Gene expression analysis of *ASB1* gene through RNA sequencing. CNT, control; ICM, ischaemic cardiomyopathy; TSS, transcription start site. In box-and-whiskers plot, boxes show the third quartile (Q3) and first quartile (Q1) range of the data, whiskers are the 5% to 95% percentiles, and dots are outliers. Data are represented as median value ± standard deviation.

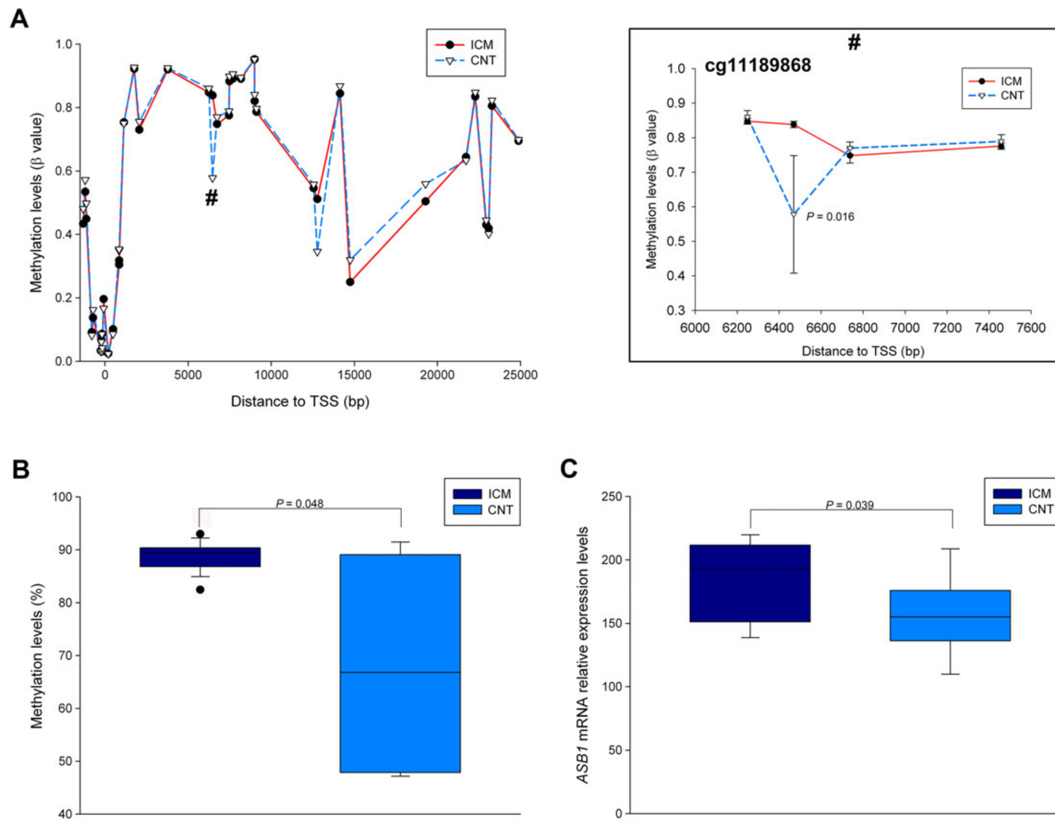
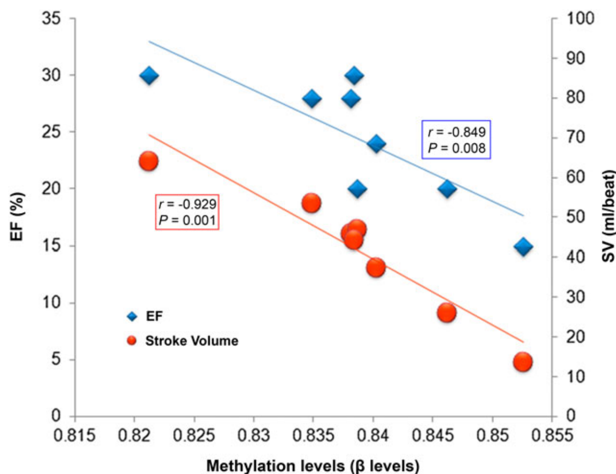


Figure 2 Relationships of the differentially methylated CpG site with the invasive-calculated SV and with eco-Doppler-based EF. CNT, control; EF, ejection fraction; ICM, ischaemic cardiomyopathy; SV, stroke volume.



reported, of an association between a differentially methylated pattern, validated by pyrosequencing, of the *ASB1* gene in ICM subjects with a haemodynamic status, LV performance, and cardiac function of these patients.

In previous studies, we have analysed the transcriptomic changes in cytoskeletal components of HF patients,[15] showing important alterations and links with LV dysfunction. Importantly, the gene coding of ankyrin repeat domain 1, *ANKRD1*, showed relationships with functional status of these patients, indicating a relevant role of this ankyrin gene in HF.

The *ASB* gene family codifies ankyrin repeat and SOCS box proteins, being involved in protein–protein interactions acting as adaptors that target proteins for proteasomal degradation.[18] Scant data are available about the specific function of *ASB1*, relating it with alterations in spermatogenesis[23]; moreover, no studies have been conducted in cardiac tissues, but its protein superfamily has relevant implications in controlling the skeletal muscle contractile apparatus structural fixation and adequate regulation of differentiation steps.[17] *ASB2* has

been implicated as a negative regulator of skeletal muscle mass through the transforming growth factor beta pathway, indicating that increased levels prevents hypertrophy.[19] The limited data available in the literature on the specific function of *ASB1*, together with our results, suggest that this gene could be involved in the pathology of cardiac function. As demonstrated, gain of methylation of *ASB1* CpG island closely relates to LV function, dimensions, and output, and none of the other 18 *ASB* family genes show such change, indicating that an increased degree of methylation may be an indicator of deteriorating haemodynamic and cardiac function. In contrast, the *ASB1* gene expression calculated by means of the RNA sequencing technique did not show any LV significant relationships, suggesting a prominent role for this DNA methylation, maybe related to an unknown specific function in coding.

Conclusions

Our findings link a specific *ASB1* methylation pattern to LV morphology and performance in end-stage ICM and provide new insight and raise questions regarding which is the functionally relevant genome for the ischaemic failing myocardium.

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Conflict of interest

None declared.

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