Biomarkers, August 2008; 13(5): 535–548

informa
healthcare

Apolipoprotein D as a novel marker in human end-stage heart failure: a preliminary study

YING-JIE WEI[†], YIN-XIA HUANG[†], XIAO-LING ZHANG[†], JUN LI, JIE HUANG, HAO ZHANG, & SHENG-SHOU HU

Key Laboratory of Cardiovascular Regenerative Medicine, Ministry of Health, and Department of Cardiovascular Surgery, Cardiovascular Institute and Fu-Wai Hospital, PUMC and CAMS, 167 Beilishilu, Beijing, 100037, China

Abstract

Apolipoprotein D (Apo D) is reported to be in close association with developing and mature blood vessels, and involved in enhanced smooth muscle cell migration after injury. This study was designed to clarify the expression pattern of Apo D and the possibility of Apo D as a new marker in human end-stage heart failure. Individual RNA samples obtained from independent left ventricular tissue of six heart failure patients derived from cardiomyopathies of different aetiologies during cardiac transplantation and six non-failing control subjects were hybridized to the gene microarray containing, in total, 35 000 well-characterized *Homo sapiens* genes. Apo D was one of the highly expressed genes (3.3-fold upregulated) detected by microarray, which was further confirmed by quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR) (5.88-fold upregulated) in failing hearts compared with non-failing hearts. Both Western blotting and immunohistochemistry analyses also demonstrated the higher levels of Apo D protein in failing hearts. Importantly, we observed elevated levels of plasma Apo D in heart failure patients compared with non-failing control subjects. We demonstrated, for the first time to our knowledge, that Apo D was highly expressed in the mRNA and protein levels in human failing hearts compared with non-failing hearts. Furthermore, our finding of elevated plasma Apo D levels in patients with heart failure provides clues that Apo D may act not only as a cardiac molecular marker but also as a circulating biomarker in patients with heart failure.

Keywords: *apolipoprotein D, heart failure, marker*

(Received 31 October 2007; accepted 29 February 2008)

Introduction

Heart failure is a multifactorial disease with no clear clarification of the underlying mechanism. Despite advances of therapeutic strategies, heart failure remains a major health hazard worldwide with high morbidity and mortality (Greenberg 2007). In the United States alone, more than 1 million people were hospitalized for heart failure (Dunlap 2007), and the 1-year mortality remains high at 25–40% (Vanhoutte et al.

Correspondence: Sheng-Shou Hu, Key Laboratory of Cardiovascular Regenerative Medicine, Ministry of Health, and Department of Cardiovascular Surgery, Cardiovascular Institute and Fu-Wai Hospital, CAMS and PUMC. A 167 Beilishilu, West District, Beijing 100037, P.R. China. Tel: 8610-88398494. Fax: 8610-88396050. E-mail: shengshouhu@yahoo.com
[†]These authors contributed equally to this work.

ISSN 1354-750X print/ISSN 1366-5804 online
DOI: 10.1080/13547500802030363

2006). Cardiomyopathy often results in heart failure; dilated (DCM) and hypertrophic (HCM) cardiomyopathy as well as arrhythmogenic right ventricular cardiomyopathy (ARVC) are the main types of non-ischaemic cardiomyopathy. The left ventricle in DCM is dilated and hypocontractile, and patients often present with easy fatigability, exercise intolerance, dyspnoea and right and left heart failure; in contrast to DCM, the left ventricle in HCM is characteristically hypertrophied and hypercontractile (Hwang et al. 2002). ARVC is characterized by recurrent ventricular tachycardia due to fibrofatty atrophy of the ventricular myocardium accompanied by dilatation of right and/or left ventricles (Kies et al. 2006). Independently of its different aetiologies, the progression of heart failure is often accompanied by changes of some common genes, potentially as markers; for example, the widely recognized B-type natriuretic peptide (BNP), is one of the best characterized markers for diagnosing and managing heart failure (Miller et al. 2007). A great number of gene microarray studies are applied to cardiovascular diseases, heart failure in particular, allowing the recognition of specific-disease molecular mechanisms and improving prognostic and therapeutic assessment (Liew 2005). The microarray expression profiles showed both quantitative and qualitative differences in gene expression between normal and diseased conditions. Therefore, gene microarray technology enables us to examine the differential expression of vast numbers of genes simultaneously, to compare patterns of gene expression between failing hearts and non-failing hearts, and finally to acquire new markers useful for detecting risk, assessing prognosis and guiding management of heart failure, and for understanding the underlying molecular mechanisms leading to heart failure.

Apolipoprotein D (Apo D) is a 29–30-kDa glycoprotein with a lipocalin structure predicting that it binds small hydrophobic ligands (Flower 1994). Subsequently, Apo D was identified as a carrier molecule with high affinity for lipids and also as a component of high-density lipoprotein (Provost et al. 1990). Apo D is present in human serum at concentrations of 47–155 $\mu g\ ml^{-1}$ (Camato et al. 1989). Immunocytochemical localization of Apo D in 12 tissues (liver, kidney, bladder, adrenal, cerebrum, duodenum, testis, lung, spleen, pancreas, heart and skin) showed that a variety of cells contain substantial levels of Apo D (Boyles et al. 1990). Moreover, many of the same cell types varied dramatically in their content of Apo D in different tissues, suggesting that the uptake or secretion of Apo D by cells is regulated (Boyles et al. 1990). Therefore, the broad distribution and precise regulation of Apo D suggest that it may play a general role in cellular metabolism. It has been localized to pericytes in developing blood vessels and is seen in close association with mature blood vessels in a variety of animal tissues (Provost et al. 1991), most recently in human atherosclerotic plaques (Sarjeant et al. 2003). Induction of Apo D occurs in smooth muscle cells (SMCs) after injuries that act as stimuli for SMC migration (Spreyer et al. 1990). Other lipoproteins such as Apo J and extracellular fatty acid binding protein (Ex-FABP) are also associated with cell migration and are prevalent in tissues where active remodelling is taking place (Cermelli et al. 2000). In general, Apo D and its analogues are involved in several pathophysiological processes, including enhanced smooth muscle cell migration and active remodelling consequent to tissue injury. However, the expression pattern of Apo D and its underlying implications in the human failing heart are unclear.

The aim of this study was to investigate the changes of Apo D in mRNA, protein and plasma multilevels in failing hearts derived from cardiomyopathies of different

aetiologies in order to explore the feasibility of Apo D as a new marker for human end-stage heart failure.

Materials and methods

Patients and heart samples

Individual left ventricular samples were obtained from six patients with end-stage heart failure (age 26 ± 10 years, male) undergoing heart transplantation and six non-failing control subjects (age 30 ± 8 years, male). All patients had end-stage heart failure without any other organ failures or detected diseases. The comparable clinical and haemodynamic characteristics of the heart failure patients are shown in Table I, including two patients with ARVC, two patients with DCM and two patients with HCM. The six non-failing control hearts from donors were not used for transplantation because of subtle coronary calcification or slight valvular abnormality, which made them not suitable for cardiac transplantation. All the non-failing control subjects were healthy and the corresponding clinical and haemodynamic characteristics were not examined. All patients and control subjects gave written informed consent for this investigation, which was approved by the Institutional Ethical Review Board of Fu Wai Hospital.

Each of the individual samples was taken from the left ventricular free wall of independent failing hearts immediately after transplantation and independent non-failing hearts which were removed from donors within 1–2 h. Part of each sample was snap frozen and stored in liquid nitrogen for gene microarray, real-time reverse transcriptase polymerase chain reaction (RT-PCR) and Western blotting analyses, and part was fixed in 10% neutral buffered formalin for pathological examination and immunohistochemical analyses.

Table I. Clinical and haemodynamic characteristics.

Group	Sex	Age (years)	HF	CI	PAP	PVR	CVP	PAWP	LVEF
Failing hearts									
1. ARVC	M	37	IV	1.46	67/44	810	9	28	27
2. ARVC	M	16	IV	2.50	24/15	64	15	15	15
3. DCM	M	29	IV	2.05	60/39	286	11	35	20
4. DCM	M	40	IV	1.73	64/33	294	12	31	20
5. HCM	M	24	IV	2.11	42/22	127	11	23	30
6. HCM	M	15	III	3.40	22/9	84	−2	7	22
Non-failing hearts	M								
7. NC	M	46							
8. NC	M	30							
9. NC	M	27							
10. NC	M	25							
11. NC	M	24							
12. NC	M	28							

ARVC, arrythmogenic right ventricle cardiomyopathy; DCM, dilated cardiomyopathy; HCM, hypertrophic cardiomyopathy; NC, non-failing control; HF, heart function; CI, cardiac index; PAP, pulmonary artery pressure; PVR, pulmonary vascular resistance; CVP, central vein pressure; PAWP, pulmonary artery wedge pressure; LVEF, left ventricular ejection fraction.

RNA isolation

Total RNA was isolated independently from each sample of the individual left ventricular free walls of six heart failure patients undergoing transplantation, and six non-failing control subjects using a TRIzol reagent (Gibcol BRL Life Technologies, New York, NY, USA) and clean-up with Qiagen RNeasy mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. RNA quantity was determined by A_{260} measurement, and RNA integrity was checked by formaldehyde gel electrophoresis. High quantity RNA with A_{260}/A_{280} ratio above 1.8 and intact ribosomal 28S and 18S bands was used for microarray and real-time RT-PCR analyses.

Microarray analysis

Each of the individual heart failure samples was obtained from the left ventricular free wall of explanted hearts of six patients during heart transplantation. Each of the individual not-failing control heart tissues from six donors were obtained from the left ventricular free wall of hearts not used for heart transplantation. We used a human genome 70-mer oligonucleotide microarray from CapitalBio Corporation (Product No. 220011, Version 2.0, Beijing, China). A total of 35 000 well-characterized *Homo sapiens* genes purchased from Qiagen were printed on amino-silaned glass slides. Arrays were fabricated using an OmniGrid microarrayer (Genomic Instrumentation Services, San Carlos, CA, USA). Fluorescent dye (Cy5 and Cy3-dCTP; Amersham Pharmacia Biotech, Piscataway, NJ, USA)-labelled DNA was produced through Eberwine's linear RNA amplification method (Van Gelder et al. 1990) and subsequent enzymatic reaction. In detail, double-stranded cDNA containing T7 RNA polymerase promoter sequence (5′-AAACG ACGGC CAGTG AATTG T AATA CGACT CACTA TAGGC GC-3′) was synthesized with 5 μg of total RNA from individual heart failure samples as test and normal adult hearts as control using a cDNA synthesis system kit according to the protocol recommended by the manufacturer (TaKaRa, Dalian, China). A T7-(oligo)dT primer (5′-AAACG ACGGC CAGTG AATTG TAATA CGACT CACTA TAGGC GC TT TTT TTT TTT TTT TTT-3′) was used instead of the poly(T) primer provided in the kit. The synthesized double-stranded cDNA was purified with a PCR purification kit (Qiagen), and the final cDNA was eluted in 60 μl of elution buffer. Half of the double-stranded cDNA product was concentrated by vacuuming to 8 μl and was subjected to an *in vitro* transcription reaction in a 20-μl reaction system using the T7 RiboMAX Express large-scale RNA production system (Promega Corporation, Madison, WI, USA). The reaction was continued for 3 h at 37°C, and the amplified RNA was purified with an RNeasy Mini kit (Qiagen). cDNA was fluorescently labelled with Klenow enzyme after reverse transcription. Briefly, 2 μg of amplified RNA was mixed with 2 μg of random hexamer, denatured at 70°C for 5 min, and cooled on ice. Then, 4 μl of first-strand synthesis buffer, 2 μl of 0.1 M dithiothreitol, 1 μl 10 mM deoxynucleoside triphosphate, and 1.5 μl SuperScript II (Invitrogen Ltd, Carlsbad CA, USA) were added. Tubes were incubated at 25°C for 10 min and then at 42°C for 60 min. The products were purified using a PCR purification kit (Qiagen) and vacuumed down to 10 μl. The cDNA was mixed with 2 μg random nonamer, denatured at 95°C for 3 min, and immediately cooled on ice. The deoxynucleoside triphosphates and Cy5-dCTP or Cy3-dCTP were added at a final concentration of 120 μM each dATP,

dGTP, and dTTP and 60 μM dCTP and 40 μM Cy5-dCTP (or Cy3-dCTP), respectively. Klenow enzyme (1 μl) from Takara was added, and the reaction was performed at 37°C for 60 min. The labelled DNA was purified with a PCR purification kit and then resuspended in elution buffer and quantified by UV spectrophotometry. Labelled control and test samples were quantitatively adjusted based on the efficiency of Cy5-dCTP or Cy3-dCTP incorporation and mixed into 30 μl hybridization solution (3×SSC (1 ×SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.2% sodium dodecyl sulfate, 25% formamide, and 5′ Denhart's solution). DNA in hybridization solution was denatured at 95°C for 3 min before loading onto a microarray. The array was hybridized at 42°C overnight and washed with two consecutive washing solutions (0.2% sodium dodecyl sulfate, 2 SSC at 42°C for 5 min, and 0.2% SSC for 5 min at room temperature). Arrays were scanned with a ScanArray Express scanner (Parckard Bioscience, Kanata, OT, USA), and the obtained images were analyzed with GenePix Pro 4.0 (Axon Instruments, Foster City, CA, USA). A space- and intensity-dependent normalization based on a LOWESS program (Yang et al. 2002) was employed. For each test and control sample, two hybridizations were performed by using a reverse fluorescence strategy. Only genes whose alteration tendency was consistent (both above 1.5-fold) in both microarrays were selected as differentially expressed genes.

Real-time RT-PCR

To confirm further the expression pattern of upregulated and downregulated genes obtained by microarray analysis, genes of interest were subject to quantitative real-time RT-PCR. Triplicate aliquots of each RNA sample were used in the same reaction, with a minimum of two independent experiments. As an internal control, GAPDH (glyceraldehydes-3-phosphate dehydrogenase) was amplified in parallel with the genes of interest. Five micrograms total RNA from 10 heart samples (five from ARVC-failing hearts and five from non-failing control hearts which were used in microarray experiments) was incubated with 10 U RNase-free DNase I (Invitrogen Ltd) at 37°C for 30 min. RNA was further purified with an RNeasy Mini kit (Qiagen) for subsequent use. The purified total RNAs (500 ng) were reverse transcribed with 200 U M-MLV reverse transcriptase (Invitrogen Ltd). Gene-specific primers were designed using the Primer3 program (http://www.genome.wi.mit.edu) and verified for base complementarity (http://www.basic.nwu.edu/biotools/oligocalc.html). The primer sequences were: (1) Apo D forward: 5′-TTG AGA GCT GAT GGA ACT GTG AA-3′, reverse: 5′-TGG CAG GCT CTG TGA GGT TA-3′; (2) GAPDH forward: 5′-TGG GTG TGA ACC ATG AGA AG-3′, reverse: 5′-GTT CGC TGT TGA AGT CAG A-3′. Real-time PCRs were performed by employing a DNA Master SYBR green I kit and a LightCycler (both from Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. The results were analyzed using LightCycler software version 3.5 (Roche Diagnostics). The real-time PCR amplification product was analyzed by melting curve analysis and 1.2% agarose gel electrophoresis, respectively. The relative amount of individual target mRNA normalized to GAPDH was calculated according to the method described by Pfaffl (2001). Fold differences were calculated by dividing each heart failure sample by the amount of product generated in the mean non-failing heart samples.

Western blotting

Protein samples obtained from left ventricle free wall of failing hearts ($n=6$) and non-failing hearts ($n=6$) were analyzed using a 15% SDS-PAGE in a Bio-Rad Miniprotean III electrophoresis unit. After SDS-PAGE, the proteins were electro-transferred to a nitrocellulose membrane under a constant voltage of 15 V for 20 min. The membranes were then blocked with PBS containing 7.5% non-fat dry milk powder and 0.1% Tween 20 for 1 h. The membranes were then incubated overnight with the primary antibody in PBS-T containing 5% non-fat dry milk, and finally incubated with the specific horseradish peroxidase (HRP)-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, Inc., PA, USA). Peroxidase activity was visualized by enhanced chemiluminescence according to manufacture's instructions (ECL kit, Amersham Bioscience, NJ, USA).

Rabbit anti-human Apo D polyclonal antibody (Catalog No. 10520-1-AP) was purchased from Proteintech Group Inc. (Chicago, IL, USA), and mouse anti-human GAPDH monoclonal antibody (Catalog No. ab9484) was purchased from Abcam Inc., (Cambridge, MA, USA).

Histological and immunohistochemical analyses

Heart samples from left ventricle free wall from failing hearts ($n=6$) and non-failing hearts ($n=6$) were isolated and fixed in 10% neutral buffered formalin. Dehydration was accomplished through alcohol and xylene gradients, followed by embedding in paraffin. Sections (5μm) were prepared and stained with haematoxylin and eosin to assess morphological features of the diseased and control hearts.

Slides were fixed for 10 min in 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 for 5 min, and blocked in 5% BSA. Sections were then incubated with primary antibodies against human Apo D polyclonal antibody (Catalog No. 10520-1-AP) for 1 h at room temperature and washed in PBS buffer for 10 min. Sections were then incubated with IgG-peroxidase-conjugated secondary antibody (Sigma, St Louis, MO, USA) for 1 h at room temperature, washed in PBS buffer for 10 min, and incubated with 0.5 mg ml^{-1} diaminobenzidine tetrahydrochloride 2-hydrate plus 0.05% H_2O_2 for 5 min. Negative control sections were incubated with secondary antibody alone. All the slides were stained with haematoxylin, dehydrated, mounted and viewed by light microscope.

ELISA assay of plasma Apo D

Venous blood from heart failure patients (both retrospectively on the initial group that was tested, and prospectively on a novel cohort of patients) (six patients from ARVC, eight from DCM, and four from HCM, $n=18$) and the matched non-failing control subjects ($n=18$) was collected into EDTA-containing Vacuette tubes (Greiner Bio-One) and centrifuged at 3000*g* (15 min, 4°C). Plasma samples were stored at −70°C until batch analysis was performed.

Apo D was assessed using an enzyme-linked immunosorbent assay (ELISA). The commercial Apo D ELISA Assay Kit for measuring human Apo D was purchased from Sun Biomedical Technology, Co. Ltd. (Beijing, China). The ELISA assay was performed according to the previously described method of Crowther (1995). In detail, purified anti-Apo D capture antibody was diluted to 1–4 μl ml^{-1} in binding

solution and 100 µl of diluted antibody was added to the wells of an enhanced protein-binding ELISA plate. Plates were sealed to prevent evaporation and incubated overnight at 4°C. The plate was brought to room temperature (RT) and the capture antibody solution removed. Non-specific binding was blocked by adding 200 µl of blocking buffer per well. The plate was sealed and incubated at RT for 1–2 h. It was then washed ≥ 3 times with PBS/Tween. Standards and samples were added (diluted in blocking buffer/Tween) at 100 µl per well. The plate was sealed and incubated for 2–4 h at RT or overnight at 4°C then washed ≥ 4 times with PBS/Tween. The biotinylated anti-Apo D detection antibody was diluted to 0.5–2 µl ml^{-1} in blocking buffer/Tween and 100 µl of diluted antibody were added to each well. The plates were sealed and incubated for 1 h at RT then washed ≥ 4 times with PBS/Tween. The streptavidin–HRP conjugate was diluted to its pre-titred optimal concentration in blocking buffer/Tween and 100 µl were added to each well. The plate was sealed and incubated it at RT for 30 min then washed ≥ 5 times with PBS/Tween. The TMB was used according to directions or ABTS was used as a substrate. ABTS substrate solution was thawed within 20 min of use; 100 ml of 3% H_2O_2 were added per 11 µl of substrate and vortexed. Immediately, 100 µl were dispensed into each well and incubated at RT (30 min) for the colour to develop. The optical density (OD) was read for each well with a microplate reader set to 405 nm.

Data analysis

All data from gene microarray, real-time RT-PCR and Western blotting were normalized to the stable expressed gene-GAPDH and statistically analyzed using the Student's *t*-test. All values are presented as mean $\pm$ standard deviation (SD). A *p*-value less than 0.05 was regarded as statistically significant.

Results

Highly expressed genes and Apo D mRNA level in human failing hearts

Each RNA sample from individual hearts with heart failure ($n = 6$) and non-failing control hearts ($n = 6$) was analyzed using human gene microarray containing 35 000 well-characterized *Homo sapiens* genes. Compared with non-failing hearts, the upregulated genes included well-documented marker genes of heart failure, such as pro-ANP and pro-BNP (Hwang et al. 2002, Barth et al. 2006), ACE2 (Kittleson 2005), TIMP1 (Spinale 2007) and CARP (Zolk et al. 2002). Some genes previously not associated with heart failure were also found to be upregulated in failing hearts. For example, Apo D, which can be taken up by smooth muscle cells and regulates their mobility in response to growth factors; FABP4, which is involved in fatty acid uptake, transport and metabolism; LTBP2, which functions as a structural component of microfibrils and participates in cell adhesion; DPT, involved in cell–matrix interaction and matrix assembly; EFEEMP1, which regulates the binding of TIMP-3; COMP, which acts as a non-collagenous extracellular matrix protein; and TIMP1, which is involved in degradation of the extracellular matrix (Table II).

Apo D (3.3-fold upregulated) was one of the highly expressed genes detected by this microarray. To validate the microarray expression values, Apo D was further analyzed by quantitative real-time RT-PCR. As a result, Apo D expression was increased by 5.88-fold in failing hearts compared to non-failing hearts (Table II and Figure 1).

Table II. Validation of selected upregulated genes in microarray analysis by quantitative real-time RT-PCR (failing hearts/non-failing hearts).

Gene	Accession no.	Fold change	SD	*p*-Value	Fold change in microarray
pro-ANP	BC005893	116.8	28.09	<0.01	30.4
pro-BNP	NM_002521	89.9	17.42	<0.01	7
APOD	**NM_001647**	**5.88**	**1.57**	**<0.01**	**3.3**
UCHL1	NM_004181	15.5	5.67	<0.01	11.2
ACE2	NM_021804	26.95	5.45	<0.01	3.8
RASL11B	NM_023940	12.29	2.13	<0.01	5.9
LTBP2	NM_000428	9.24	1.27	<0.01	7.7
SNCA	NM_000345	6.37	1.81	<0.01	4
PHLDA1	AK26181	4.85	0.65	<0.01	4.5
GADD45A	L24498	4.56	0.58	<0.01	5.1
COMP	NM_000095	3.82	0.47	<0.01	1.9
CSPG2	U16306	2.91	0.61	<0.01	4.5
EFEMP1	NM_004105	2.76	0.81	<0.01	4.4
SLC25A27	NM_004277	2.74	0.41	<0.01	2
SOCS2	NM_003877	2.36	0.71	<0.01	3.6
CARP	NM_014391	2.4	0.52	<0.01	3.3
FABP4	NM_001442	1.67	0.43	<0.05	1.9
DPT	NM_001937	1.59	0.63	<0.05	3.5
TIMP1	NM_003254	1.54	0.34	<0.05	2.4

A strong concordance was found for the Apo D expression ratios between microarray and quantitative real-time RT-PCR.

Levels of cardiac Apo D protein in human failing hearts

Western blotting analysis revealed apparent increase in Apo D protein levels in failing hearts compared with non-failing control hearts ($p < 0.01$) (Figure 2).

In the light microscope examination, compared with non-failing hearts (Figure 3, Non-failing), a varying degree of interstitial fibrosis in two DCM failing hearts and two HCM failing hearts (Figure 3, DCM and HCM) and fibrofatty or fatty replacement in two ARVC failing hearts (Figure 3, ARVC) was observed.

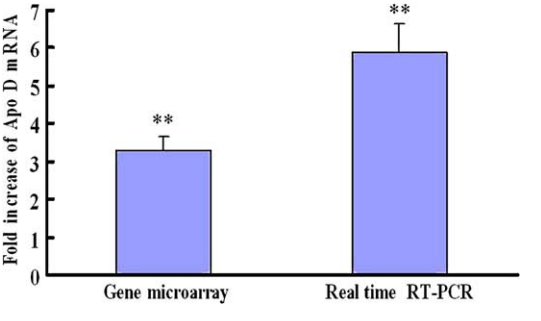

Figure 1. Fold increase of Apo D mRNA expression in failing hearts ($n = 6$) relative to non-failing control hearts ($n = 6$) by gene microarray (3.3-fold upregulated) and quantitative real-time RT-PCR (5.88-fold upregulated) analyses. Statistical comparison was carried out by Student's *t* test. $^{**}p < 0.01$ vs non-failing hearts.

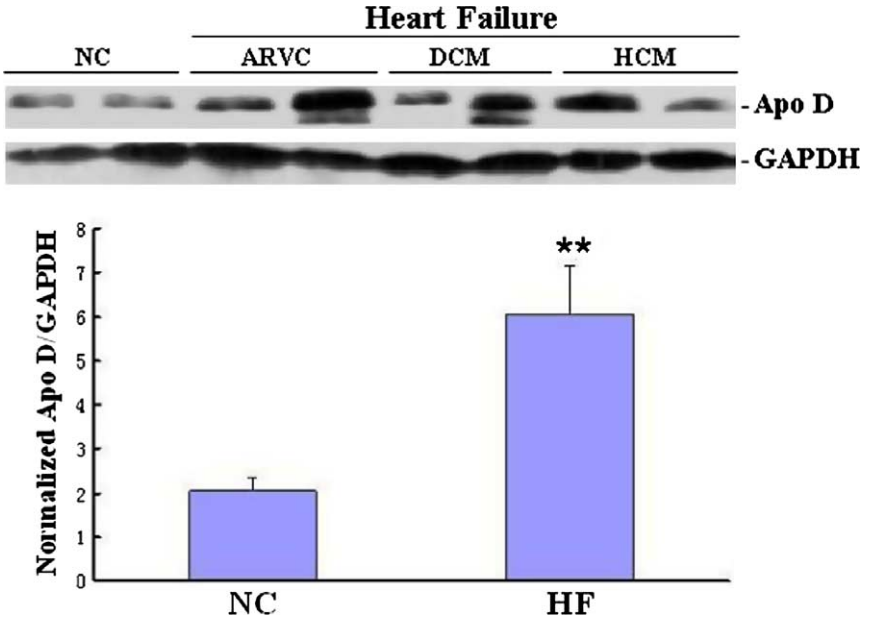

Figure 2. Western blotting analysis of Apo D protein levels in failing hearts ($n=6$) versus non-failing hearts ($n=6$). The internal standard GAPDH was used to normalize for equal protein loading. Apo D protein concentrations are significantly upregulated in failing hearts compared with non-failing control hearts ($p<0.01$). HF, heart failure patients; NC, non-failing control subjects; ARVC, arrythmogenic right ventricular cardiomyopathy; DCM, dilated cardiomyopathy; HCM, hypertrophic cardiomyopathy.

In the immunohistochemical analysis, the control specimens from non-failing hearts ($n=6$) showed no or weak Apo D immunoreactivity (Figure 4, Non-failing). In contrast, strong Apo D immunoreactivity was found in all the failing hearts ($n=6$) (Figure 4, DCM, HCM and ARVC) showing a fine granular pattern in residual myocytes.

Plasma levels of Apo D in heart failure patients

As shown in Figure 5, plasma Apo D level was higher in heart failure patients ($n=18$, 153.52 ± 44.46 μg ml^{-1}) than in non-failing control subjects ($n=18$, 127.35 ± 25.46 μg ml^{-1}) ($p<0.05$).

Discussion

The advantage of comparing gene expression profiles between human failing hearts and non-failing hearts is the potential to identify heart failure-specific molecular events and the accompanied markers. Gene microarray technology can provide a useful tool to sieve new biomarkers to detect risk, assess prognosis and guide management of heart failure. Cardiac markers also play an important role in and provide better pathophysiological understanding of heart failure. Natriuretic peptides have moved the field forwards in the last 20 years but they still fail to comply with all the properties of an ideal marker for heart failure. Besides high sensitivity, specificity and reproducibility, an ideal marker should have little biovariability, and be

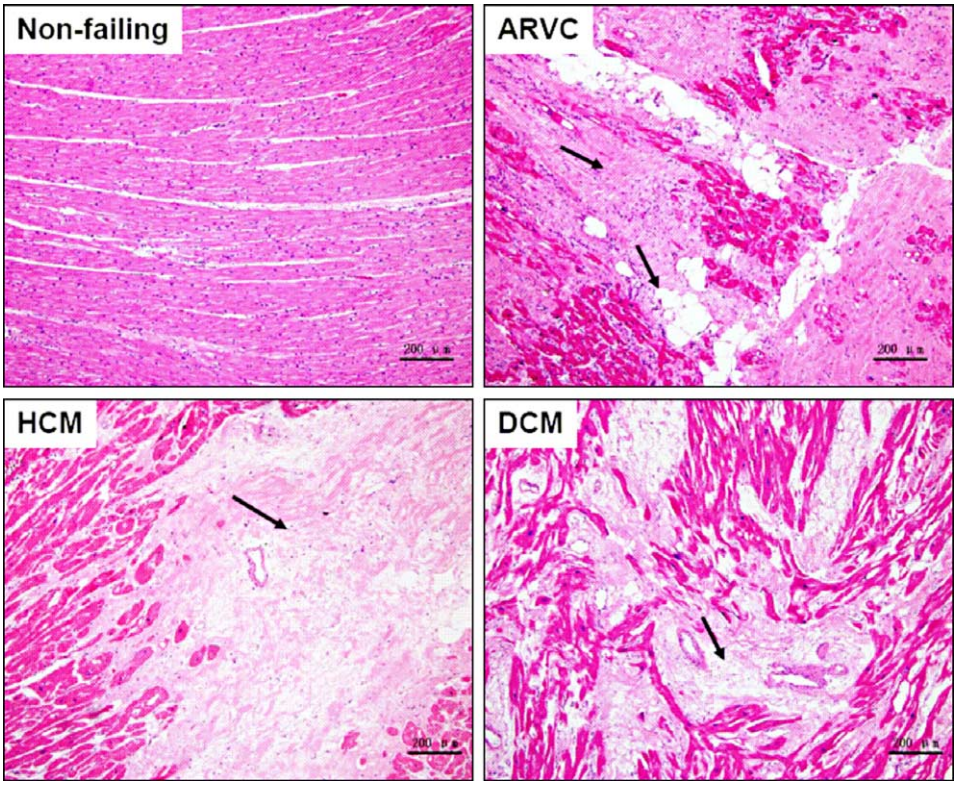

Figure 3. Representative light microscopic findings in haematoxylin-eosin staining. The normal appearance of myocardial fibres with central nuclei is seen in non-failing hearts. Fatty or fibrofatty replacement is found in ARVC failing hearts. Fibrosis is observed in DCM and HCM failing heats. ARVC, arrythmogenic right ventricular cardiomyopathy; DCM, dilated cardiomyopathy; HCM, hypertrophic cardiomyopathy. Original magnification $\times 100$. Scale bar $= 200$ μm.

independent of demographic characteristics, widely available and cost-effective. Currently, we still do not have a single marker or a combination of markers to answer all the clinically relevant questions in heart failure (Lainscak et al. 2007). Therefore, the search for ideal markers in heart failure is still ongoing.

A great number of gene microarray studies are applied to cardiovascular diseases, heart failure in particular, allowing the recognition of specific-disease molecular mechanisms and useful markers to improve prognostic and therapeutic assessment (Tan et al. 2002, Liew 2005, Nanni et al. 2006). The differentially expressed genes in human end-stage heart failure we identified included known marker genes of heart failure such as pro-ANP and pro-BNP as well as potentially possible marker genes we identified, such as Apo D.

In a published expression microarray study involving 60 medulloblastomas, Apo D was one of the four markers most useful in predicting the prognosis of medulloblastomas (Pomeroy et al. 2002). Apo D expression measured by quantitative RT-PCR may also be useful in the differential diagnosis of primary brain tumours, particularly pilocytic astrocytomas and gangliogliomas (Hunter et al. 2005). It has been reported that Apo D is seen in close association with developing and mature blood vessels, and is involved in enhanced smooth muscle cell migration after injury

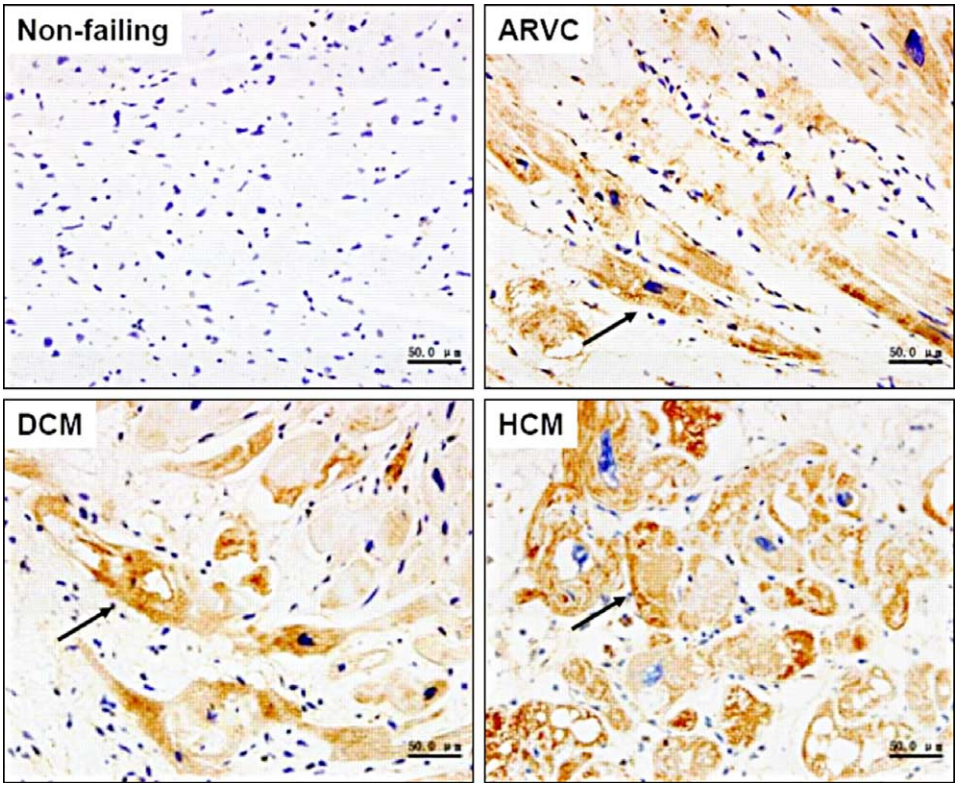

Figure 4. Representative light microscopic Apo D immunoreactivity. Very little immunoreactivity of Apo D is observed in non-failing hearts, but there is strong BNP immunoreactivity in residual cardiomyocytes of ARVC, DCM and HCM failing hearts. ARVC, arrythmogenic right ventricular cardiomyopathy; DCM, dilated cardiomyopathy; HCM, hypertrophic cardiomyopathy. Original magnification $\times 400$. Scale bar = 50 μm.

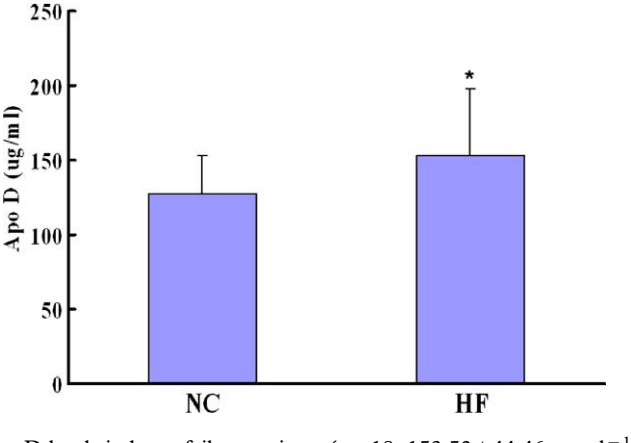

Figure 5. Plasma Apo D levels in heart failure patients ($n = 18$, 153.52 ± 44.46 μg ml^{-1}) compared to the non-failing control subjects ($n = 18$, 127.35 ± 25.46 μg ml^{-1}). Values are mean $\pm$ SD. HF, heart failure patients; NC, non-failing control subjects. *$p < 0.05$.

(Spreyer et al. 1990, Provost et al. 1991). However, the expression pattern and the underlying implications of Apo D is not clear in human heart failure consequent to cardiomyopathies of different aetiologies.

In this study, for the first time to our knowledge, we observed that Apo D mRNA is highly expressed in human failing hearts derived from three types of cardiomyopathy, compared with non-failing hearts, by microarray and further by real-time RT-PCR. Elevated levels of Apo D protein were also observed by Western blotting and immunohistochemistry in human failing hearts. Therefore, Apo D is the common cardiac marker with high expression in cardiomyopathies of different aetiologies. We deduced that the elevated levels of Apo D protein are mainly produced, but not taken up by myocardial cells, because significantly increased Apo D mRNA was observed in cardiomyocytes. We hypothesized that Apo D may act as not only a cardiac marker, but also a plasma biomarker in heart failure patients compared with non-failing control subjects. In fact, we did observe elevated plasma Apo D level in heart failure patients. Compared with the well-documented biomarkers pro-ANP and pro-BNP, a lower increase of Apo D might be because of multiple tissue sources of Apo D involving in the maintenance of plasma Apo D concentration (Boyles et al. 1990) and high concentration of Apo D existing in the plasma (Hunter et al. 2005); therefore, there is a slight elevation of Apo D levels released by damaged cardiomyocytes in patients with heart failure, and changes in plasma Apo D may not sensitively reflect changes within the myocardium.

The heart, like all other organs, requires several forms of lipids, which supply calories and are required for esterification reactions used in the creation of a number of structural cellular lipids. However, in certain diseased conditions, excess lipids can lead to heart muscle dysfunction either because they impair cellular contractility or because they cause apoptotic death of cardiomyocytes (Park et al. 2007). Apo D, as a transport protein, can carry lipid to the heart. In our study, we found elevated levels of Apo D in failing hearts compared with non-failing hearts, which suggests that too much lipid might be transitioned into the heart by Apo D, which could be involved in heart dysfunction.

We acknowledge some limitations in this study. First, we cannot exclusively establish a cause and effect relationship between increased Apo D and human end-stage heart failure. Second, we were limited by small sample sizes and the complexity of molecular mechanisms involved in heart failure derived from different aetiologies of cardiomyopathy. Third, the non-failing hearts we used as control were relatively normal because of subtle coronary calcification or slight valvular abnormality which might compromise the Apo D synthesis, although we could not find any pathological changes in the myocardium itself. Lastly, Apo D does not comply with all the properties of an ideal marker for heart failure. For example, the specificity of Apo D as a marker is not ideal, like the well-documented marker – the natriuretic peptides for heart failure.

In conclusion, we demonstrated, for the first time, highly expressed Apo D in the mRNA and protein levels as well as high plasma Apo D in human heart failure compared with non-failing control subjects. Apo D was identified to be not only a cardiac marker but also a plasma biomarker in patients with heart failure, which may be useful in risk stratification and prognostic prediction of human heart failure. The underlying implications of Apo D involvement in human heart failure merit further studies.

Acknowledgements

This work was supported by grants from National '863' Project (2006AA02Z4B8). The authors have no financial disclosures.

References

Barth AS, Kuner R, Buness A, Ruschhaupt M, Merk S, Zwermann L, et al. 2006. Identification of a common gene expression signature in dilated cardiomyopathy across independent microarray studies. Journal of the American College of Cardiology 48:1610–1617.

Boyles JK, Notterpek LM, Wardell MR, Rall SC, et al. 1990. Identification, characterization, and tissue distribution of apolipoprotein D in the rat. Journal of Lipid Research 31:2243–2256.

Camato R, Marcel YL, Milne RW, Lussier-Cacan S, Weech PK. 1989. Protein polymorphism of a human plasma apolipoprotein D antigenic epitope. Journal of Lipid Research 30:865–275.

Cermelli S, Zerega B, Carlevaro M, Gentili C, Thorp B, Farquharson C, et al. 2000. Extracellular fatty acid binding protein (Ex-FABP) modulation by inflammatory agents: 'physiological' acute phase response in endochondral bone formation. European Journal of Cell Biology 79:155–164.

Crowther JR. 1995. ELISA. Theory and Practice. Methods in Molecular Biology 42:1–223.

Dunlap ME. 2007. New studies influencing treatment of heart failure. Pharmacotherapy 27:3S–11S.

Flower DR. 1994. The lipocalin protein family: a role in cell regulation. FEBS Letters 354:7–11.

Greenberg B. 2007. Can We IMPROVE-CHF management by measuring natriuretic peptides? Circulation 115:3045–3047.

Hunter SB, Varma V, Shehata B. 2005. Apolipoprotein D expression in primary brain tumors: analysis by quantitative RT-PCR in formalin-fixed, paraffin-embedded tissue. Journal of Histochemistry & Cytochemistry 53:963–969.

Hwang JJ, Allen PD, Tseng GC, Lam CW, Fananapazir L, Dzau VJ, et al. 2002. Microarray gene expression profiles in dilated and hypertrophic cardiomyopathic end-stage heart failure. Physiological Genomics 10:31–44.

Kies P, Bootsma M, Bax J. 2006. Arrhythmogenic right ventricular cardiomyopathy: screening, diagnosis, and treatment. Heart Rhythm 3:225–234.

Kittleson MM, Minhas KM, Irizarry RA, Ye SQ, Edness G, Breton E, Conte JV, Tomaselli G, . Garcia JGN, Hare JM. 2005. Gene expression analysis of ischemic and nonischemic cardiomyopathy: shared and distinct genes in the development of heart failure. Physiological Genomics 21:299.

Liew CC. 2005. Expressed genome molecular signatures of heart failure. Clinical Chemistry & Laboratory Medicine 43:462–469.

Miller WL, Hartman KA, Burritt MF, Grill DE, Rodeheffer RJ, Burnett JC, et al. 2007. Serial biomarker measurements in ambulatory patients with chronic heart failure: the importance of change over time. Circulation 116:249–257.

Nanni L, Romualdi C, Maseri A. 2006. Differential gene expression profiling in genetic and multifactorial cardiovascular diseases. Journal of Molecular & Cellular Cardiology 41:934–948.

Park TS, Yamashita H, Blaner WS, Goldberg IJ. 2007. Lipids in the heart: a source of fuel and a source of toxins. Current Opinions in Lipidology 18:277–282.

Pfaffl MW. 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Research 29:e45.

Pomeroy SL, Tamayo P, Gaasenbeek M, Sturla LM, Angelo M, McLaughlin ME, et al. 2002. Prediction of central nervous system embryonal tumour outcome based on gene expression. Nature 415:436–442.

Provost PR, Weech PK, Tremblay NM, Marcel YL, Rassart E. 1990. Molecular characterization and differential mRNA tissue distribution of rabbit apolipoprotein D. Journal of Lipid Research 31:2057–2065.

Provost PR, Villeneuve L, Weech PK, Milne RW, Marcel YL, Rassart E. 1991. Localization of the major sites of rabbit apolipoprotein D gene transcription by in situ hybridization. Journal of Lipid Research 32:1959–1970.

Sarjeant JM, Lawrie A, Kinnear C, Yablonsky S, Leung W, Massaeli H, et al. 2003. Apolipoprotein D inhibits platelet-derived growth factor-BB-induced vascular smooth muscle cell proliferated by preventing translocation of phosphorylated extracellular signal regulated kinase 1/2 to the nucleus. Arteriosclerosis. Thrombosis & Vascular Biology 23:2172–2177.

Spinale FG. 2007. Myocardial matrix remodeling and the matrix metalloproteinases: influence on cardiac form and function. Physiological Reviews 87:1285–1342.

Spreyer P, Schaal H, Kuhn G, Rothe T, Unterbeck A, Olek K, et al. 1990. Regeneration-associated high level expression of apolipoprotein D mRNA in endoneurial fibroblasts of peripheral nerve. EMBO Journal 9:2479–2484.

Tan FL, Moravec CS, Li J, Apperson-Hansen C, McCarthy PM, Young JB, Bond M. 2002. The gene expression fingerprint of human heart failure. Proceedings of the National Academy of Sciences USA 99:11387–11392.

Van Gelder RN, von Zastrow ME, Yool A, Dement WC, Barchas JD, Eberwine JH. 1990. Amplified RNA synthesized from limited quantities of heterogeneous cDNA. Proceedings of the National Academy of Sciences USA 87:1663–1667.

Vanhoutte D, Schellings M, Pinto Y, Heymans S. 2006. Relevance of matrix metalloproteinases and their inhibitors after myocardial infarction: a temporal and spatial window. Cardiovascular Research 69:604–613.

Yang YH, Dudoit S, Luu P, Lin DM, Peng V, Ngai J, et al. 2002. Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. Nucleic Acids Research 30:e15.

Zolk O, Frohme M, Maurer A, Kluxen F-W, Hentsch B, Zubakov D, Hoheisel D, Zucker IH, Pepe S, Eschenhagena T. 2002. Cardiac ankyrin repeat protein, a negative regulator of cardiac gene expression, is augmented in human heart failure Biochemical and Biophysical Research Communications 293: 1377–1382.