4 Results

4.1 Gene expression analysis with cDNA microarrays

To search for genes differentially expressed between SHHF strain and the control of WKY or SHRSP, expression analysis was carried out using rat heart cDNA microarray filters with subtractive suppressive hybridization probes. Sequences from 23 clones were analyzed and genes were identified by homology search using the Blast algorithm at the NCBI database. The chromosomal locations of these genes were located for rat, mouse and human using sequence Blast at Ensembl (Table 4.1).

Table 4.1 **Candidate genes selected from rat heart library (619)**

cDNA clone-ID	Expression	Sequence result and gene description	Chromosomal location		
			Rat	Mouse	Human
M011, M125 [a]	Down	Cytochrome oxidase subunit II gene (COXII)	16	16	5
O1040, H2130, H1653 [b]	Down	Cytochrome oxidase subunit I (COXI)	14	5	17
I0764 [b]	Down	Cd36 antigen	4	5	7
K0331[b]	Down	Phospholamban exon 2	20	10	--
J2312 [b]	Down	Similar to musculus titin (Ttn)	3	2	2
L2350, D0768 [b]	Down	ND4 (NADH-DH subunit4)	16	1	8
H168 [b]	Down	Golgi SNAP receptor complex member 1 (Gosr1)	--	9	10
L0410 [a]	Up	Hybrid protein	6	16	18
D1911 [a]	Up	Clear sequence, part identical to Homo sapiens polymerase I transcription factor RRN3	4	6	2
M2112, P2418 [a]	Up	Heat shock 27 kD protein 2, HSPB2	8	9	11
C095 [a]	Up	Cytochrome c oxidase subunit IV, COX4	3	7	14
J1510, N1222 [b]	Up	Similar to RE70703p (Drosophila melanogaster)	1	16	19
F1749 [b]	Up	Similar to heat shock protein family, HSPB7	5	4	--
J1816 [b]	Up	Laminin receptor 1/40 kDa ribosomal protein	1	4	X
D1745, J114 [b]	Up	Ribosomal protein L14 (Rpl14)	8	9	12
D1754 [b]	Up	Subunit of NADH, ubiquinone oxidoreductase	6	5	13

a, SSH probe was prepared using SHHF strain as tester and SHRSP as driver; b, SHH probe was prepared using SHHF strain as tester and WKY as driver. Genes marked in bold were retested using Real Time PCR. --, No hit on the chromosome.

4.2 Gene expression profiling with Affymetrix chip

Affymetrix RG-U34A and RG-U34B chips, which include 7000 genes, 9000 EST, and control sequences, were used to obtain comprehensive gene-expression analysis. In order to screen candidate genes related to heart failure and cardiomyopathy, five, three and two samples of rat heart tissue from the strains SHHF, WKY and SHRSP were used to prepare targets hybridized with both RG-U34A and RG-U34B chips respectively.

4.2.1 Targets preparation

The quality of the RNA is essential for the success of the analysis. The integrity of total RNA was verified by agarose-formaldehyde gel electrophoresis (Fig. 4.1). RNA yield was quantified by spectrophotometric analysis; all RNA samples were well purified according to the A260/A280 ratio that were between 1.9 and 2.1 for all samples. cRNAs were synthesized and labeled with biotin by *in vitro* transcription reaction. The A260/A280 ratios for all cRNAs were over 2.0. Distribution sizes of fragmented cRNAs ranged from 35 to 200 bases that fit well to the right sizes (Fig. 4.2).

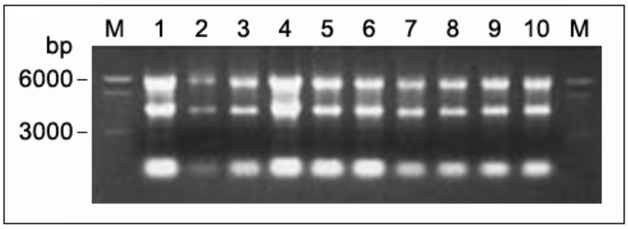

Fig. 4.1 Formaldehyde agarose gel of total RNA isolated from rat heart tissue

1-5 μg of total RNAs isolated from rat heart tissue of SHHF (lane 1-5), WKY (lane 6-8), and SHRSP (lane 9-10) using Trizol method were loaded on 1.2 % formaldehyde agarose gel. M, 1 μg of RNA ladder from MBI.

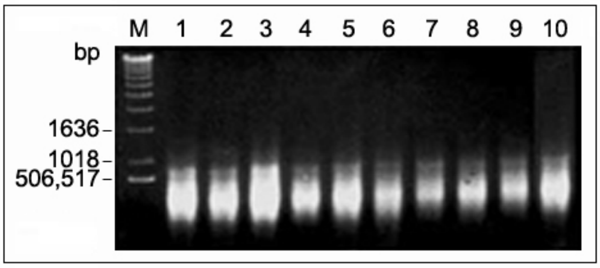

Fig. 4.2 Formaldehyde agarose gel of fragmented cRNAs

1 μg of fragmented cRNA from SHHF (lane 1-5), WKY (lane 6-8), and SHRSP (lane 9-10) was loaded on 1.2% FA gel. M, 0.5 μg of 1kb DNA ladder from Life/Tech.

4.2.2 Hybridization result with Test chips

Hybridization cocktail from each sample was first used to hybridize with the test chip. Test chip hybridization results were scanned after washing and staining (Fig. 4.3). All probe sets were selected as normalization factor and batch analysis was preformed. There are 107 probe arrays including hybridization controls and housekeeping gene sequences on the test chip. About 30-40% of transcripts on the test chip were present in hybridization result and rat housekeeping genes (e.g. *Actin*, *Gapdh*, and hexokinase) were also present. However, the gene *GAPD* from human was absent. Those results suggested that all targets could be further hybridized with working chips.

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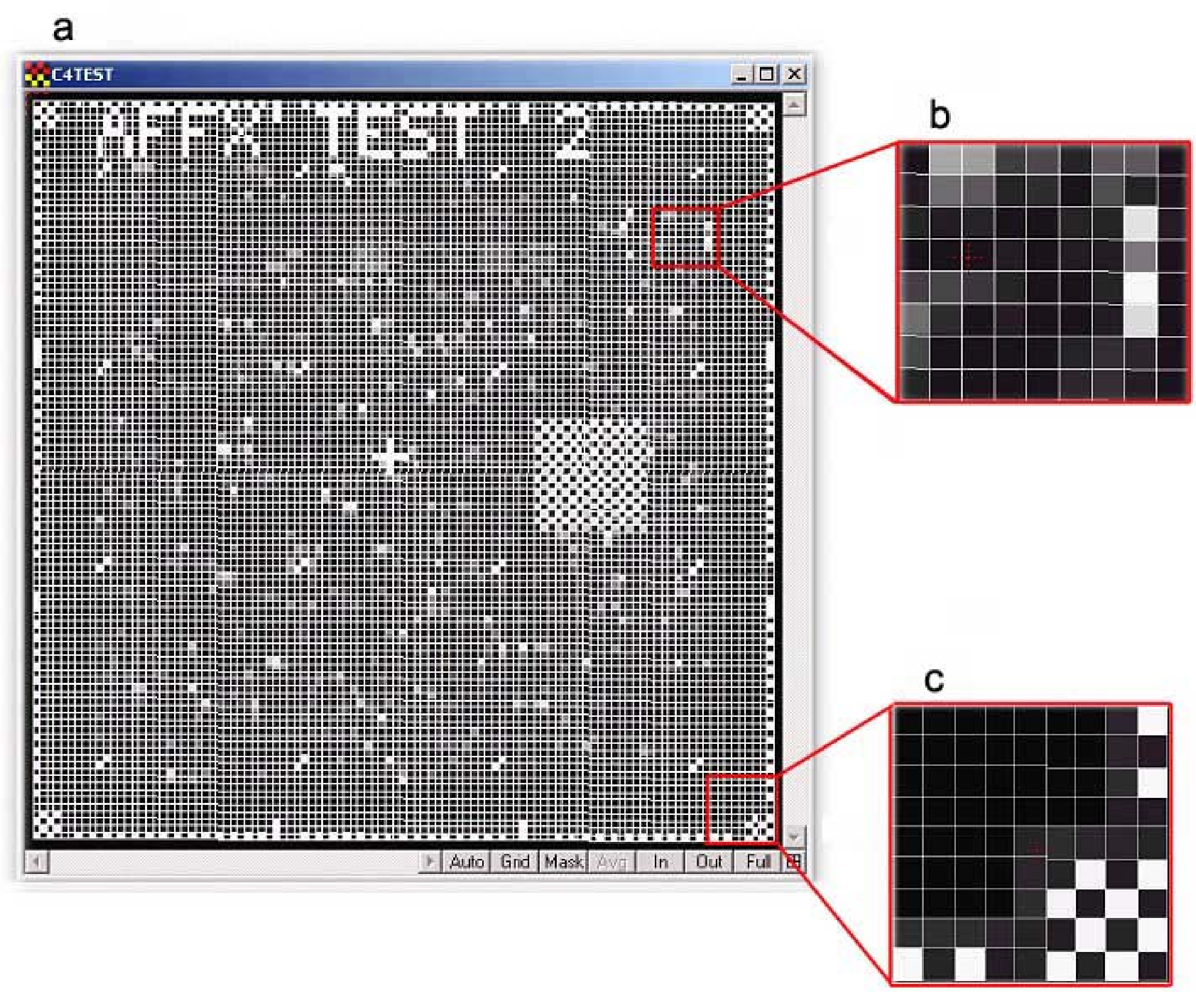

Fig. 4.3 Hybridization result from one of the Affymetrix test chips

The microarray suite software displayed a picture of the image data file (*.dat) and automatically generated the *.cel file from the *.dat file (a). One area that was randomly selected showed white, gray or black squares in different intensity (b). Four corners of the picture showed clearly white and black squares (c).

4.2.3 Hybridization and analysis of RG-U34A and RG-U34B chips

RG-U34A and RG-U34B were separately hybridized with the above hybridization cocktails. Hybridization results were briefly checked on the *.cel file. Global scaling was used to minimize differences of non-biological origin from multiple probe arrays in our study.

An absolute expression analysis was first performed for every chip after hybridization. From absolute analysis, 34.6-41.5% of transcripts on RG-U34A and 29.5-40.8% of transcripts on RG-U34B were present, 57.0-64.1% of transcripts on RG-U34A and 57.3-68.8% on RG-U34B were absent (Table. 4.2). Measured images of one transcript AF072411 on ten chips hybridized with samples from the strains SHHF, WKY and SHRSP showed this gene was downregulated in SHHF strain compared with that in the other two controls (Fig. 4.4).

Table 4.2 **Outline of the hybridization result for Affymetrix chips**

Hybridized chips	Present	Absent	Marginal	Total
C2_RG-U34A	3179 (36.1%)	5481 (62.3%)	139 (1.6%)	8799
C3_RG-U34A	3647 (41.5%)	5014 (57.0%)	138 (1.6%)	8799
C4_RG-U34A	3512 (39.9%)	5151 (58.5%)	136 (1.6%)	8799
C5_RG-U34A	3220 (36.6%)	5456 (62.0%)	123 (1.4%)	8799
C6_RG-U34A	3498 (39.8%)	5168 (58.7%)	133 (1.5%)	8799
W18_RG-U34A	3044 (34.6%)	5644 (64.1%)	111 (1.3%)	8799
W19_RG-U34A	3326 (37.8%)	5338 (60.7%)	135 (1.5%)	8799
W22_RG-U34A	3383 (38.5%)	5291 (60.1%)	125 (1.4%)	8799
SP24_RG-U34A	3211 (36.5%)	5484 (62.3%)	104 (1.2%)	8799
SP25_RG-U34A	3313 (37.7%)	5352 (60.8%)	134 (1.5%)	8799
C2_RG-U34B	3008 (34.2%)	5643 (64.2%)	140 (1.6%)	8791
C3_RG-U34B	3396 (38.6%)	5226 (59.5%)	169 (1.9%)	8791
C4_RG-U34B	3586 (40.8%)	5034 (57.3%)	171 (1.9%)	8791
C5_RG-U34B	2712 (30.8%)	5921 (67.4%)	158 (1.8%)	8791
C6_RG-U34B	2764 (31.4%)	5865 (66.7%)	162 (1.8%)	8791
W18_RG-U34B	2834 (32.2%)	5814 (66.1%)	143 (1.6%)	8791
W19_RG-U34B	2597 (29.5%)	6044 (68.8%)	150 (1.7%)	8791
W22_RG-U34B	2798 (31.8%)	5841 (66.4%)	152 (1.7%)	8791
SP24_RG-U34B	2716 (30.9%)	5916 (67.3%)	159 (1.8%)	8791
SP25_RG-U34B	3219 (36.6%)	5414 (61.6%)	158 (1.8%)	8791

C_, Hybridization results of SHHF strain; W_, Hybridization results of WKY strain. SP_, Hybridization results of SHRSP strain.

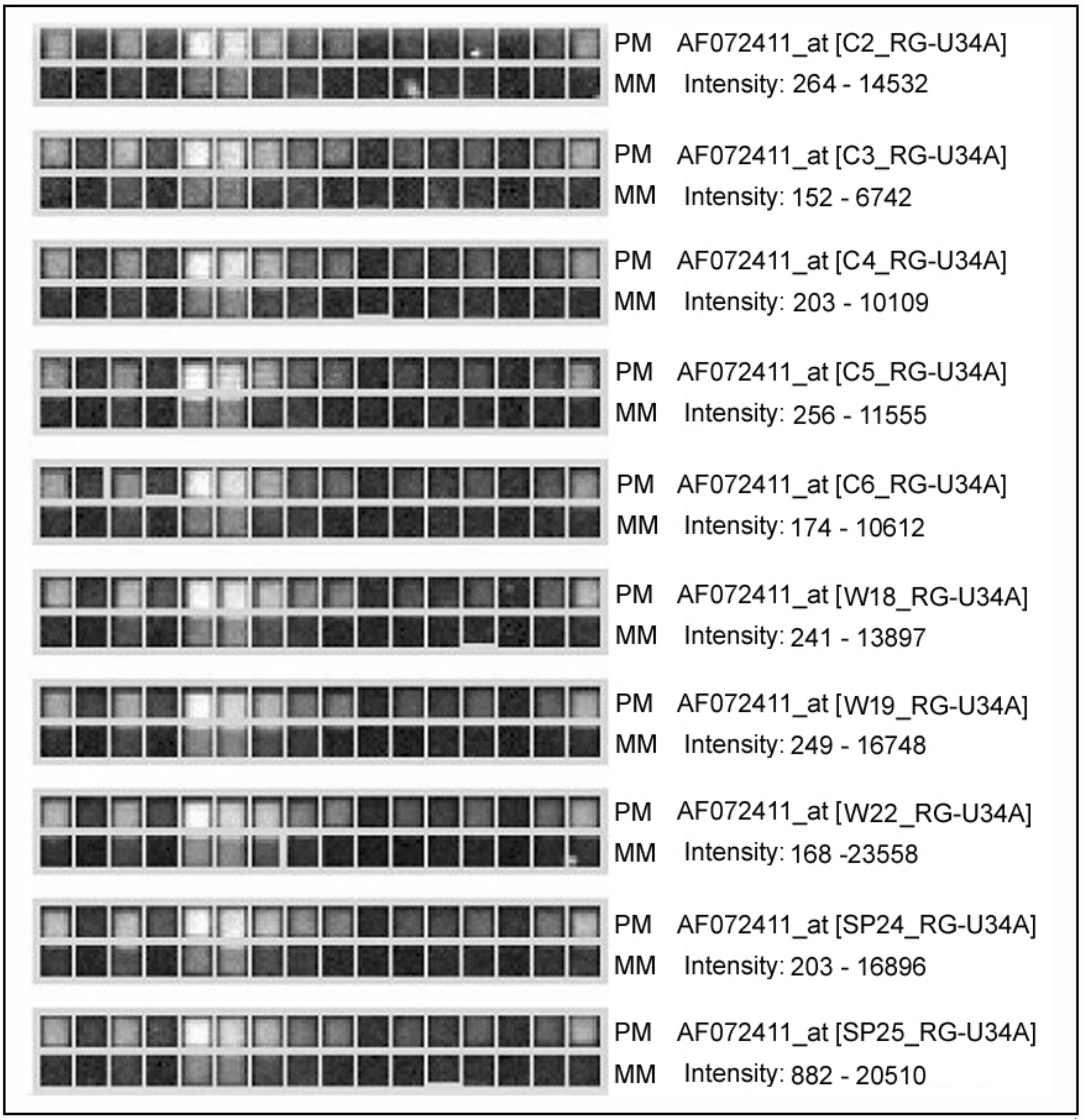

Fig. 4.4 Intensity of hybridization for transcript AF072411 on RG_U34A

The images showed intensity of the transcript AF072411 on RG_U34A chips that hybridized with bio-labeled cRNA from 10 different samples. PM, Perfect match; MM, Mismatch.

A comparison expression analysis was carried out to compare the cell intensity data of an experiment (result from SHHF strain) and a baseline probe array of the same probe array type (result from WKY or SHRSP strain). The hybridization result was statistically analyzed from each chip by making difference call valuable with 1, -1, 0.5, -0.5 or 0 instead of Increased, Decreased, Marginally Increased, Marginally Decreased, and No change. The total value of the difference call for each transcript was summed. The transcripts with an absolute value over half of the total absolute value were selected. Afterwards the genes showing differential expression in SHHF strain compared with WKY (SHHF/WKY) or SHRSP (SHHF/SHRSP), as well as the genes showing differential expression in the SHRSP compared with WKY (SHRSP/WKY) were acquired.

Another comparison expression analysis was performed on mean chip method. Three mean chips of the SHHF, WKY, and SHRSP strains were produced using the cell intensity data. Absolute and comparison analyses were further carried out on mean chips. The transcripts, which were selected using both mean chip and individual chip analyses, were explored by Microsoft Excel Match analysis. Analysis results using the two methods were outlined (Table 4.3). Transcripts showing similar expression change by two comparison methods were 46.2-85.2% in selected transcripts by each comparison analysis. In order to reduce the false positive rates and stand out important genes, the analyzed genes that showed similar differential expression by these two methods were selected as potential genes for further analysis (see in Appendix, Table 11.1-2).

Table 4.3 Overview of genes selected using two different comparison methods

Methods	Total of selected transcripts	Same result using two methods	Differential result using two methods
C/W_U34A_Individual	122	104 (85.2%)	18 (14.8%)
C/W_U34A_mean	147	104 (70.7%)	43 (29.3%)
C/SP_U34A_Individual	115	73 (63.5%)	42 (36.5%)
C/SP_U34A_mean	116	73 (62.9%)	43 (37.1%)
SP/W_U34A_Individual	130	68 (52.3%)	62 (47.7%)
SP/W_U34A_mean	92	68 (73.9%)	24 (26.1%)
C/W_U34B_Individual	59	49 (83.1%)	10 (16.9%)
C/W_U34B_mean	106	49 (46.2%)	57 (53.8%)
C/SP_U34B_Individual	62	47 (75.8%)	15 (24.2%)
C/SP_U34B_mean	88	47 (53.4%)	41 (46.6%)
SP/W_U34B_Individual	87	43 (49.4%)	44 (50.67%)
SP/W_U34B_mean	75	43 (57.3%)	32 (42.7%)

C/W_U34A_individual or C/W_U34A_mean, Comparison analysis of SHHF strain versus WKY using RG_U34A individual chip or mean chip; C/SP_U34A_individual or C/SP_U34A_mean, Comparison analysis of SHHF versus SHRSP; SP/W_U34A_individual or SP/W_U34A_mean, Comparison analysis of SHRSP vs. WKY.

4.2.4 Gene expression profiling in rat heart tissue

The genes showing differential expression in SHHF/WKY, SHHF/SHRSP and SHRSP/WKY were set up after the above comparison analyses. Of the126 genes (153 probe sets) showing differential expression in the SHHF/WKY comparison, 44, or 34.9%, were downregulated and 82, or 65.1%, were upregulated (Appendix, Table 11.1). Among of the 104 genes (120 probe sets) exhibiting differential expression in the SHHF/SHRSP comparison, 46 genes, or 42.2%, were downregulated and 58, or 55.8%, were upregulated (Appendix, Table 11.2). Many genes tested with more than one probe set on the Affymetrix chips were found similar expression change in the study (Marked in bold in Appendix, Table 11.1-2).

To identify genes related to heart failure and cardiomyopathy in SHHF strain, genes showing the same expression changes in the groups SHHF/WKY and SHHF/SHRSP were selected (blue and red area in Fig 4.5), SHRSP strain was used to balance hypertensive background. Twenty-eight genes tested in 38 probe sets were found significantly differential expression in SHHF strain compared with the two controls (Table 4.4). Among of them, genes involved in fatty acid metabolism, e.g., *Cd36*, pyruvate dehydrogenase, were exclusively downregulated. However, genes related to glucose metabolism, such as fructose bisphosphatase 2, uncoupling protein 1, and retinoid X receptor were upregulated. Sequences of these selected genes were downloaded from the Genbank at NCBI and were further located on the chromosome of rat, mouse and human using Blast algorithm.

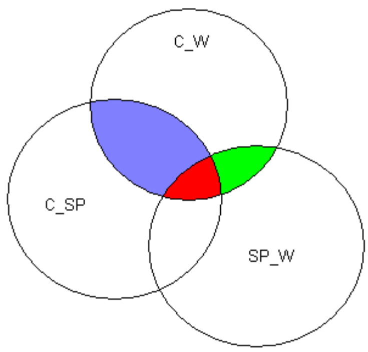

Fig. 4.5 Groups of genes selected from Affymetrix chips

Three circles represent selected genes from the groups SHHF/WKY, SHHF/SHRSP, or SHRSP/WKY after two comparison analyses with individual or mean chips. The areas in blue and red show genes exhibited differential expression in the comparisons of SHHF/WKY and SHHF/SHRSP. The areas in green and red show the genes that were differential expression in the comparisons of SHHF/WKY and SHRSP/WKY.

Table 4.4 **Genes with differential expression in the SHHF/WKY and SHHF/SHRSP**

No.	Probe set ID	C	C/W C/SP	C/W	C/SP	Chromosomal location			Gene description[2]
		AC	DC	FC	FC	Rat	Mouse	Human	
1	AF072411_g_at	P	D	-3.4	-3.2	4	5	7	cd36 antigen (***Cd36***)
2	L22654_at	A	D	-5	-6	4	6	2	Anti-acetylcholine receptor antibody gene
3	M20131cds_s_at	A	D	-3.3	-2.4	1	7	10	Cytochrome P450 2E1 (***Cyp2e1***)
4	Rc_AA955251_at	P	D	-2.5	-3.1	14	15	10	Interferon-induced protein 6-16 precursor
5	U44948_at	P	D	-2.1	-2.5	20	10	3	Smooth muscle cell LIM protein (***Csrp2***)
6	Rc_AA998152_at	P	D	-3.5	-2.4	10	11	17	Insulin receptor tyrosine kinase substrate protein p53.
7	M12822cds_f_at[1]	A	D	-9.1	-4.9	4	6	2	Ig kappa-chain gene C-region
8	Rc_AA945750_at	A	D	-15.3	-23.1	13	1	--	Dimethylaniline monooxygenase (FMO 1)
9	AF034577_at	P	D	-3.1	-4.6	4	6	7	Pyruvate dehydrogenase (***Pdk4***)
10	Rc_AA942718_at	P	D	-2.1	-1.9	3	2	20	Bcl2-like 1 (***Bcl2l1***)
11	Rc_AI639060_at[1]	P	I	13.5	3.4	7	10	19	Unknown EST
12	X03894_at	P	I	10.5	5.8	13	8	4	uncoupling protein 1 (***Ucp1***)
13	M64795_f_at	A	I	8.8	5.1	20	17	6	MHC class I antigen
14	AJ005046_g_at	P	I	5.2	3.5	17	13	9	Fructose-1,6-bisphosphatase (***Fbp2***)
15	Rc_AI009603_at	P	I	3.7	3.5	5	4	--	HRPAP20 short form
16	Rc_AA849518_at	P	I	3.4	2.7	2	3	4	Similar to group XII-1 phospholipase protein
17	Rc_AA946457_g_at	P	I	2.7	2.6	6	17	--	Hypothetical protein KIAA0846
18	AF037272_at	P	I	2.6	3.1	19	8	16	WAP four-disulfide core protein (***Wfdc1***)
19	Rc_AA849841_at	P	I	2.5	2.5	5	4	4	Syncoilin
20	Rc_AA859805_at	P	I	2.5	2	8	9	15	Mus musculus, Similar to lysyl oxidase-like 1
21	Rc_AI043601_at	P	I	2.4	1.8	8	9	15	Hypothetical WD-repeat protein alr3466
22	K01934mRNA#2_at	P	I	4.2	2.8	1	7	11	Hepatic product spot 14 (***Thrsp***)
23	M69246_at	P	I	2.1	4.3	1	7	9,11	Collagen-binding protein (***Serpinh1***)
24	Rc_AI060197_at	P	I	2	2.2	11	8	21	Membrane protein C21or f4
25	U02553cds_s_at	P	I	2.9	5.8	10	17	5	Protein tyrosine phosphatase (***Ptpn16***)
26	Z49858_at	P	I	1.8	2.8	19	8	X	Plasmolipin
27	D26393exon_s_at	P	I	-4.8	2.9	4	6	2	HK2 gene for type II hexokinase
28	M25804_g_at	P	I	3.1	3.1	10	11	17	Thyroid hormone receptor alpha

1, The genes also showed differential expression in the SHRSP/WKY comparison. 2, Gene symbols were marked in bold. AC, Absolute call; DC, Difference call; FC, Fold change; C, SHHF; W, WKY; SP, SHRSP. --, No hit on the chromosome.

The SHRSP strain has some similar syndromes as SHHF, like hypertension. Thus genes exhibited the same expression change in the groups SHHF/WKY and SHRSP/WKY were also selected (Area in green and red in Fig. 4.5). These genes maybe play roles in hypertension and other similar phenotypes in SHHF and SHRSP (Appendix, Table 11.3).

4.3 Comparison analysis of candidate genes using Real Time PCR

To verify the differential expression analysis from the microarrays**,** quantitative Real Time PCR was performed using primers specific to the selected genes (Table 2.5). In order to get rid of any effect from the contamination of genomic DNA, cDNA quality was approved by PCR amplification of the *Gapdh* gene (Fig. 4.6 a), and the purity of cDNAs was confirmed by PCR amplification using angiotensinogen intron primers (Fig. 4.6 b). Relative quantification of gene expression was calculated from C_T. All quantitations were normalized to an endogenous control (18s rRNA) to account for variability in the initial concentration of the different samples. Relative quantification of gene expression in the comparisons of SHHF/WKY or SHHF/SHRSP was calculated.

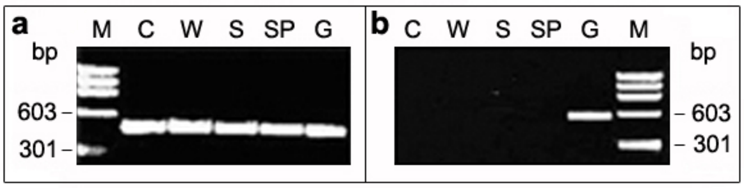

Fig. 4.6 Analysis of cDNA using PCR with *Gapdh* and angi_intron specific primers

PCRs were amplified from cDNA acquired from SHHF (C), WKY (W), SHR (S), SHRSP (SP), and rat genomic DNA (G) using the rat housekeeping gene *Gapdh* specific primer (a), or using primers designed from two close introns of the rat angiotensinogen gene sequence (b). M, 0.2 µg of PhiX174 *Bsu*RI (*Hae*III) DNA marker.

Sixteen genes selected from the Affymetrix chip were further detected using Real Time PCR with SYBR Green (Fig. 4.7). Except for the gene cytochrome P450 2E1 (*Cyp2e1*), other nine genes, *Cd36*, pyruvate dehydrogenase (*Pdk4*), smooth muscle cell LIM protein (*Csrp2*), protein tyrosine phosphatase (*Pnpn16*), uncoupling protein 1 (*Ucp1*), fructose-1, 6-bisphosphatase (*Fbp2*), WAP four-disulfide core protein (*Wfdc1*), collagen-binding protein (*Serpinh1*), and anti-acetylcholine receptor were found similar expression change in SHHF strain compared with control WKY and SHRSP both in Affymetrix arrays and using Real Time PCR analysis. The genes, nuclear protein 1 (*Nupr1*), sulfotransferase family 1A

(*Sult1a1)*, alpha 1 collagen III (*Col3a1*), interleukin 2 receptor (*Il2rb*), and cyclin D2 (*Ccnd2*) showed consistent changes in the SHHF/WKY comparison with Real Time PCR and Affymetrix chips. The upregulation of the gene synaptic glycoprotein (*SC2*) in the SHHF/SHRSP comparison was also confirmed using Real Time PCR.

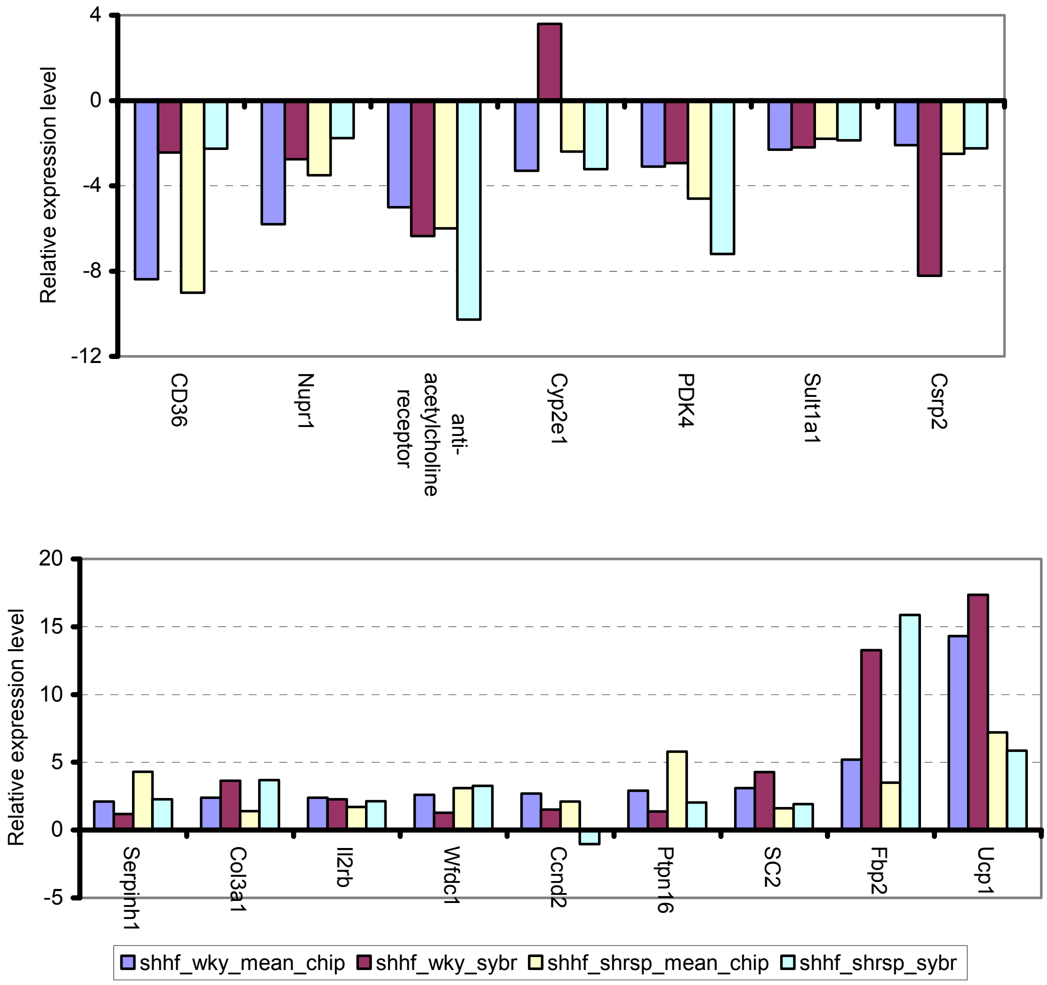

Fig. 4.7 Verification of differential expression of candidate genes using Real Time PCR

The upper part of the diagram shows downregulated genes detected using Real Time PCR. The lower part shows upregulated genes detected using Real Time PCR.

4.4 Molecular basis of *Cd36* in SHHF model

4.4.1 Expression level of *Cd36* in SHHF strain

In the SHHF/WKY and SHHF/SHRSP comparison, *Cd36* showed downregulation detected from 7 probe sets on the Affymetrix chips. These probe sets represent different parts of the *Cd36* cDNA sequence (Fig. 4.8). The first five probe sets were close on the side of the 5' end of *Cd36* cDNA. Expression level of *Cd36* in SHHF was 2.8 to 7.5 times downregulated compared with that in WKY and SHRSP. The sequence of the probe set rc_AA925752 locates between the end of the open reading frame (ORF) and the beginning of the 3' untranslated region, where a large cDNA fragment in SHHF *Cd36* cDNA sequence was different compared with WKY cDNA sequence from our later study (Fig. 4.11). Therefore, the result on the Affymetrix chips of the probe set rc_AA925752 reflected not only the expression level of *Cd36* but also its mutation in SHHF. The last probe set rc_AA946398 locates at the 3' end of *Cd36* gene, where SHHF *Cd36* cDNA sequence (long transcript) shows about 92.7% identity to that of WKY. The decreased expression of *Cd36* in SHHF was also found with Real Time PCR (Fig. 4.7) and cDNA array analysis (Table 4.1).

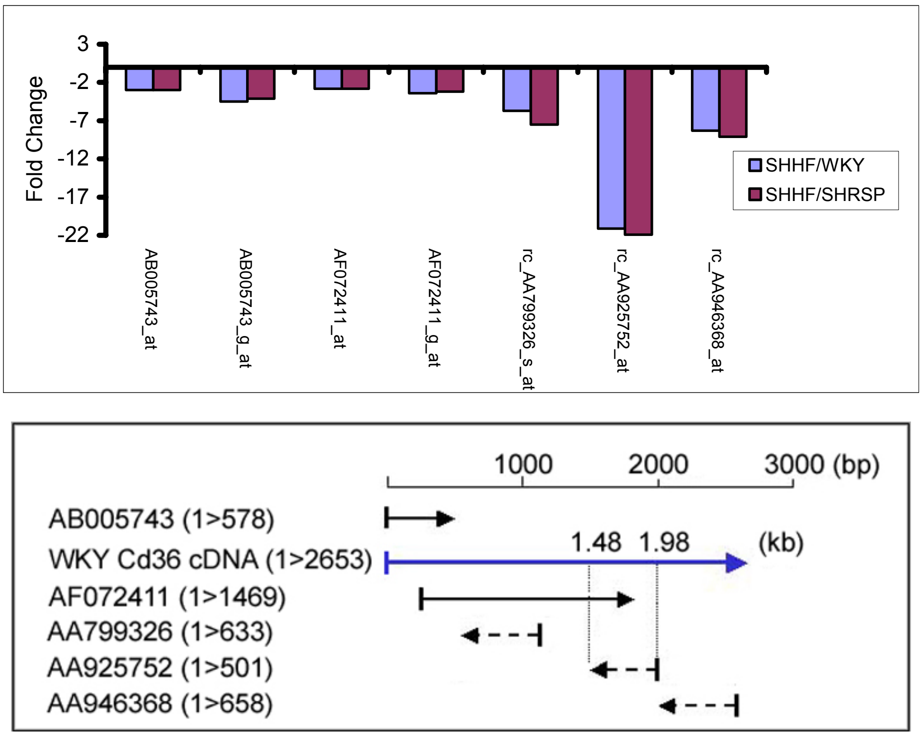

Fig. 4.8 Expression level of *Cd36* in SHHF strain compared with WKY or SHRSP

The upper part of the diagram, Expression of *Cd36* in the SHHF/WKY or SHHF/SHRSP comparisons by expression profiling using Affymetrix chips. The lower part, Relative positions for sequences of *Cd36* probe sets on WKY *Cd36* cDNA sequence.

4.4.2 Different transcripts of *Cd36* in SHHF strain

To determine the basis of the differential hybridization signals for *Cd36* from the microarrays, expression of *Cd36* in SHHF and control strains was analyzed using northern-blot with its 5' and 3' probes (Fig. 4.9 a). In SHHF and SHR/mol rat heart tissue, two major transcripts (about 3.5 kb and 5.0 kb) were seen, which were not present in the controls. However, the 2.8-kb transcript observed in WKY and SHRSP rat heart tissue was not detected in SHHF and SHR/mol. The two major transcripts in SHHF heart tissue were also detected using Smart Race method and were confirmed as specific fragments for *Cd36* with southern blot analysis (Fig. 4.9 b, c).

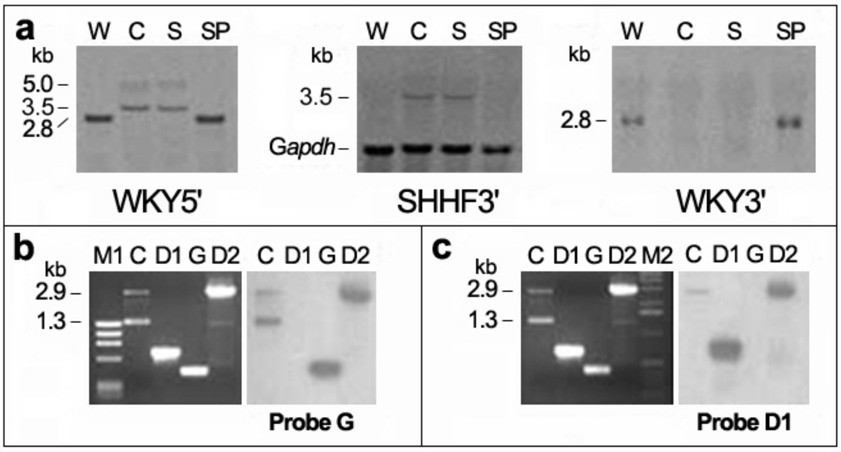

Fig. 4.9 Analysis of *Cd36* transcripts by northern blot and southern blot

a, Northern blots of total RNA from SHHF (C), SHR/mol (S), WKY (W) and SHRSP (SP) rats. Each filter was hybridized with α-P^{33} labeled PCR products from near 5'end of WKY cDNA, 3' end of WKY or SHHF cDNA. G*apdh* was used as endogenous control. b and c, *Cd36* 3' RACE PCR products using SHHF *Cd36* gene specific primer GSP3 and UPM (The left part, lane C). Both of the two fragments were confirmed as specific transcripts for *Cd36* in SHHF heart tissue using southern blot with α-P^{33} labeled PCR products G and D1 (The right part). G, PCR products from SHHF cDNA (nt 2061-2483) using *Cd36* G primers; D1 and D2, PCR products from WKY (nt 1626-2305) or from SHHF (nt 1579-4617) using *Cd36* D primers; M1, 0.2 µg of PhiX174 *Bsu*RI (*Hae*III) DNA marker; M2, 0.5 µg of 1kb DNA ladder from Life/Tech.

4.4.3 SHHF and WKY *Cd36* cDNA sequences

To obtain the *Cd36* cDNA sequence, five primer pairs were designed to amplify the entire *Cd36* cDNA from SHHF and WKY by RT-PCR in five overlapping segments. One large unexpected fragment D (3039 bp) was found in SHHF and SHR/mol, instead of the 680 bp fragment observed in WKY and SHRSP (Fig 4.10). Then the fragments D from SHHF and

WKY were cloned and sequenced respectively. The two different cDNA draft sequences from SHHF and WKY were acquired by assembling all sequenced fragments with Seqman from DNAStar program.

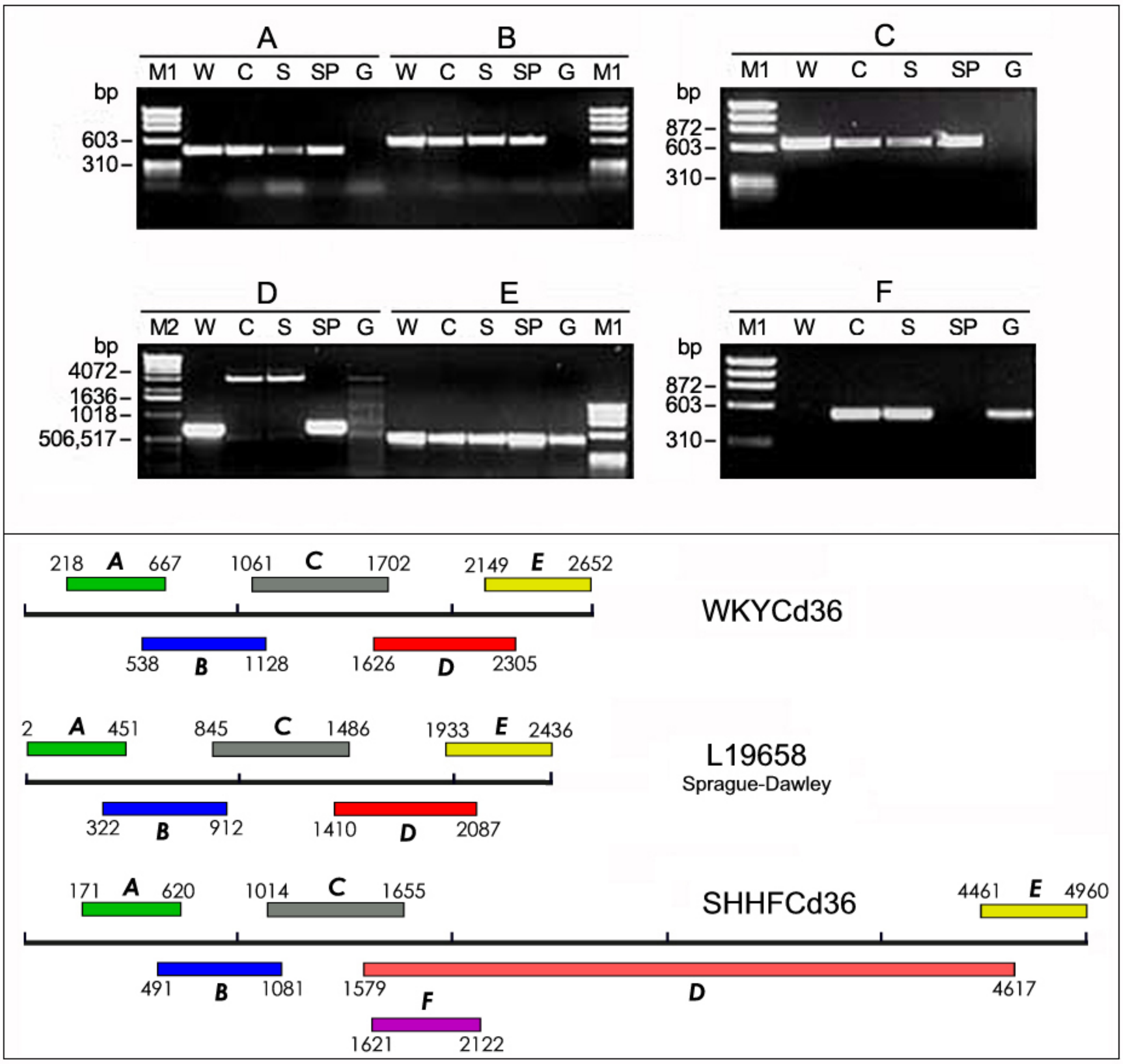

Fig. 4.10 PCR products amplified from SHHF and WKY cDNA

The upper part of the diagram, PCR products using *Cd36* primers A (A), B (B), C (C), D (D), E (E), and F (F). PCR products were absent in WKY and SHRSP using primer F. W, WKY; C, SHHF; S, SHR; SP, SHRSP; G, genomic DNA from WKY; M1, PhiX174 *Bsu*RI (*Hae*III) DNA marker; M2, 1kb ladder marker. The below part, *Cd36* primers A, B, C, D and E were designed from the published *Cd36* cDNA sequence (GenBank accession number L19658). Primer F was designed from the SHHF *Cd36* cDNA sequence (acquired in this study, unpublished, nt 1621-2122). Expected PCR products amplified using above primers from WKY and SHHF cDNA were compared.

To get full length of the cDNA sequences, 5' and 3' RACEs were performed. Two 3' PCR fragments with different length were detected in SHHF heart tissue (Fig. 4.9 b). The sequences of the 5' and 3' ends of SHHF and WKY *Cd36* cDNA were acquired by sequencing 5 to 10 positive clones for every RACE PCR fragment (Fig. 4.11). WKY *Cd36* cDNA sequence (2653 bp) and two cDNA sequences of SHHF (SHHF *Cd36*_cDNA_long, 4960 bp and *Cd36*_cDNA_short, 3366 bp) were obtained after being assembled with the two draft cDNA sequences respectively. The lengths of two SHHF *Cd36* cDNA sequences were similar to the size of the two transcripts detected in this strain by northern blot (Fig 4.9 a). Except for part of the last exon (nt 3367-4960) of SHHF *Cd36*_cDNA_long, SHHF *Cd36*_cDNA_short was 100% identical to it at nt 1-3366. WKY *Cd36* cDNA and SHHF *Cd36*_cDNA_long were also compared (Fig. 4.12). The gene *Cd36* open reading frame (ORF) was complete in SHHF *Cd36* cDNA, followed by a long untranslated sequence divergent from WKY *Cd36* cDNA sequence. Compared with WKY, the first upstream ORF was absent in SHHF *Cd36* cDNA.

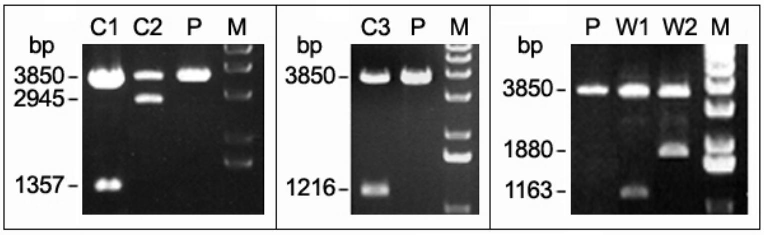

Fig. 4.11 Positive clones including *Cd36* 5' and 3' RACE PCR products

Plasmids were digested using *Eco*RI and loaded on 0.8% agarose gel. C1, C2 and C3, Positive clones include SHHF *Cd36* 3′ RACE PCR products (C1: 1357 bp and C2: 2945 bp:) amplified using GSP2 and UPM and 5′ RACE PCR product (C3: 1216 bp) amplified using GSP1 and UPM. W1 and W2, Positive clones include WKY *Cd36* 3' RACE PCR product (W1: 1163 bp) amplified using GSP4 and UPM and WKY *Cd36* 5′ RACE PCR product (W2: 1880 bp) amplified using GSP4 and UPM. P, Digested pDriver cloning vector as negtive control; M, 0.5 µg of 1kb DNA ladder from Life/Tech.

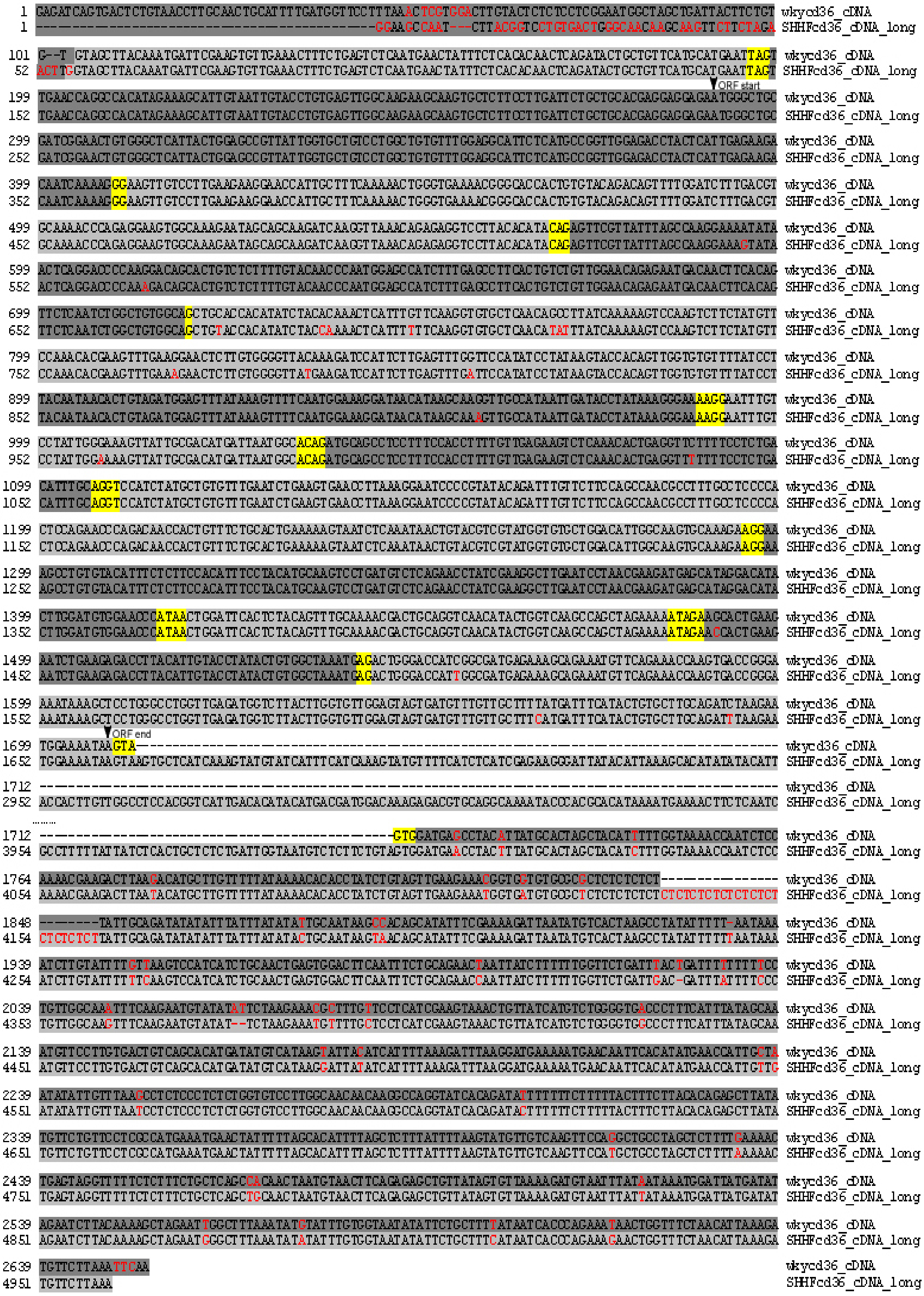

Fig. 4.12 Alignment of *Cd36* cDNA sequence

The *Cd36* cDNA sequences from WKY (wkycd36_cDNA) and SHHF (SHHFcd36_cDNA_long) were aligned using MegAlign of the DNAStar program. About 3.3 kb of SHHF Cd36 cDNA sequences are omitted, where do not match to WKY cDNA sequence. Exons are labeled as intervals using dark gray and light gray; sequences marked in yellow are shared by two close exons. The mismatches between two sequences are marked in red.

4.4.4 Blast analysis of *Cd36*

Blast analysis of WKY and SHHF *Cd36* cDNA sequences was performed against the rat genome database at NCBI. Three and a half quite similar homologue fragments at the *Cd36* locus on rat genome chromosome 4q11 were matched. Thirty-three exons and 3 genes were found in this genomic region spanning 248794 bp (Fig 4.13). Three genes are LOC360376 (hypothetical gene), LOC362310 (similar to fatty acid translocase/*Cd36*) and LOC296786 (similar to fatty acid transport protein). All these three model genes carry a Cd36 family domain. WKY *Cd36* cDNA sequence showed higher homology with the first two model genes than with SHHF *Cd36* cDNA sequence (Fig 4.14), especially the sequences of exons 2 to 4 were 100% identical to the exons at the beginning of the first model gene. The sequence nt 521-4960 in SHHF *Cd36* cDNA (including exons 5 to 14) is 99.6% identical to the exons of the gene LOC296786. However the cDNA from nt 56 to nt 523 (exons 2 to 4), which is identical with WKY *Cd36* cDNA sequence, is only 95% identical to the exons of the gene LOC296786. WKY *Cd36* exon 1, which is 92% identical to mouse musculus *Cd36* gene upstream promoter, is quite different from SHHF exon 1 sequence.

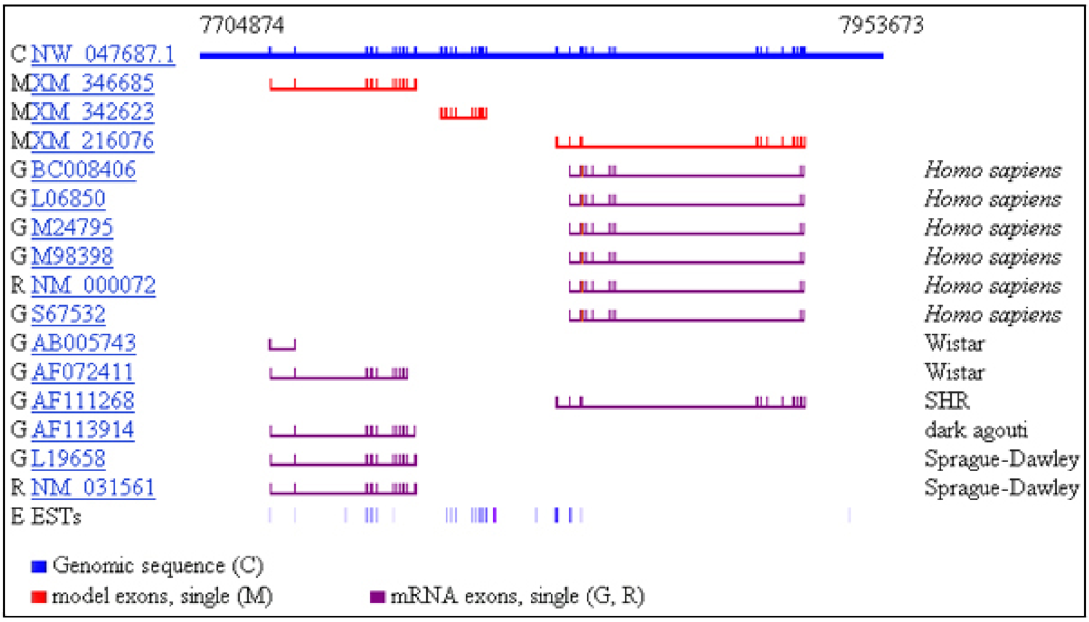

Fig 4.13 Evidence viewer Rattus norvegicus NW 047687.1 acquired from NCBI

C, contig; M, model mRNA; R, RefSeq mRNA; G, GenBank mRNA. XM 346685, model mRNA for hypothetical gene LOC360376; XM 342623, model mRNA for gene LOC362310; XM 216076, model mRNA for gene LOC296786. BC008406, L06850, M24795, M98398, NM_000072, and S67532, homo sapiens CD36 antigen. AB005743, mRNA for fatty acid transporter; AF07241, mRNA fatty acid translocase/CD36; AF111268, fatty acid transport protein (CD36/FAT); AF113914, cell surface protein *Cd36* mRNA; L19658, FAT mRNA. NM_031561, Cd36 antigen.

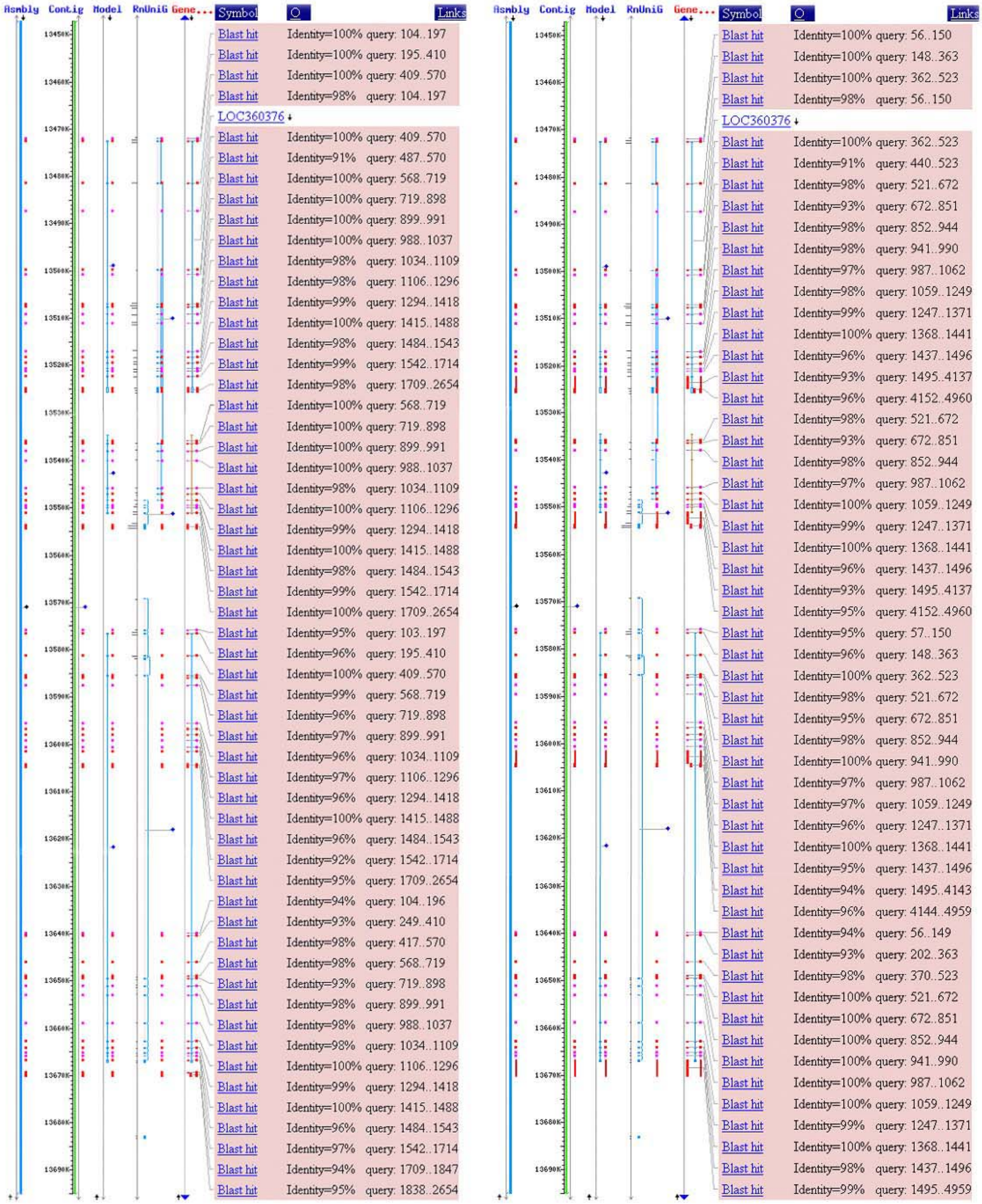

Fig. 4.14 Blast analysis of the *Cd36* cDNA sequence against the Rat genome

The left part of the diagram shows the blast result of WKY *Cd36* cDNA sequence against the rat genome at NCBI. The right part shows the blast result of SHHF *Cd36* cDNA sequence (the long transcript).

4.4.5 *Cd36* copy number and deletion breakpoints in SHHF strain

The sequences of *Cd36* intron 4 and intron 3 acquired from the genomic sequence NW_047687.1 from Genbank were compared. Five repeat regions within exon 4 and its boundary were found. Seven primer pairs (Table 2.3) were designed to amplify the boundary of exon 4, intron 3, and intron 4. One or two PCR fragments observed in WKY amplified with intron 3 IV or intron 4 III primers respectively were not detected from SHHF genomic DNA (Fig. 4.15, Fig. 4.16). Only one fragment was found from SHHF genomic DNA when amplified with other primers (Fig. 4.15). PCR fragments of intron 3 I, intron 4 I, and II from SHHF genomic DNA were sequenced and compared with corresponding parts of published genomic sequences. A deletion of about 165.4 kb from the beginning of the first intron 4 to the last repeated intron 4 was identified in SHHF *Cd36* DNA sequence (Fig. 4.16). The upstream and downstream limits of the breakpoint were designated as the region where the pattern of sequence variants observed in intron 4 of SHHF *Cd36* gene changed from being identical to LOC360376 and different from LOC296786, to always being different from LOC360376 and always identical to LOC296786. Comparison of the sequence data from the first 900 bp of intron 4 in SHHF indicated that breakpoint was between nt 9 and nt 630 away from the first repetition exon 4/intron 4 boundary (Fig. 4.17).

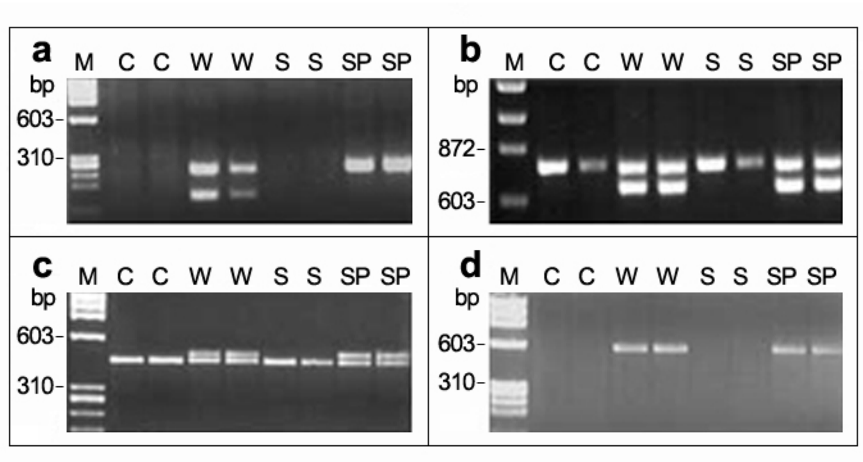

Fig. 4.15 Analysis of *Cd36* deletion in SHHF strain

PCRs were performed from rat genomic DNA from SHHF (C), WKY (W), SHR (S), SHRSP (SP) with primers intron 4 III (a), intron 3 I (b), intron 3 III (c) or intron 3 IV (d).

NW047687.1		Expected PCR products from WKY DNA		Primers	PCR products from SHHF DNA
Exon 2	24801_24894				
Exon 3	25362_25577				
		33219_33652	434 bp	Intron 3 III	~434 bp
		33489_33807	319 bp	Intron 3 II	~319 bp
		33622_34387	766 bp	Intron 3 I	771 bp
Exon 4	34248_34409				
		34357_35133	777 bp	Intron 4 I	773 bp
		35080_35662	583 bp	Intron 4 II	No
Exon 2	40205_40298				
		51550_51977	428 bp	Intron 3 III	
		51814_52132	319 bp	Intron 3 II	
		51947_52711	765 bp	Intron 3 I	
Exon 4	52572_52733				
		52681_53456	776 bp	Intron 4 I	
Exon 4	53591_53750				
		53698_54473	776 bp	Intron 4 I	
		59322_59570	249 bp	Intron 4 III	No
Exon 5	59887_60038				
Exon 6_15					Deletion
		88108_88355	248 bp	Intron 4 III	No
Exon 5	88669_88820				
Exon 6_15					
Exon 2	128769_128863				
Exon 3	129336_129551				
		133209_133669	461 bp	Intron 3 III	
		133639_134296	658 bp	Intron 3 I	
Exon 4	134157_134318				
		134266_135042	777 bp	Intron 4 I	
		134989_135526	538 bp	Intron4 II	
		137828_137979	152 bp	Intron 4 III	No
Exon 5	138301_138452				
Exon 6_15					
Exon 2	192683_192776				
Exon 3	193257_193440				
		197715_198261	547 bp	Intron 3 IV	No
		198231_199065	835 bp	Intron 3 I	
Exon 4	198926_199087				
		199035_199810	776 bp	Intron 4 I	773 bp
		199757_200291	535 bp	Intron4 II	534 bp
Exon 5	201827_201964				
Exon 6_15					

Fig. 4.16 Analysis of *Cd36* deletion in SHHF strain

NW047687.1, Rattus norvegicus chromosome 4 WGS supercontig from 7705272 to 7953265.

```
     GGAAGTTGTCCTTGAAGAAGGAACCATTGCTTTCAAAAACTGGGTGAAAACGGGCACCACTGTGTACAGACAGTTTTGGATCTTTGACGT Majority

1    .......................................................................................... NW_047687.1_34248_35358
1    .......................................................................................... NW_047687.1_52572_53682
1    -NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN.........NNNNNNNNNNNNN.N.-........... NW_047687.1_53591_54701
1    .......................................................................................... NW_047687.1_134157_135267
1    NNNNNNNN.................T................................................................ NW_047687.1_198926_200036
1    .......................................................................................... sequence from SHHF

     GCAAAACCCAGAGGAAGTGGCAAAGAATAGCAGCAAGATCAAGGTTAAACAGAGAGGTCCTTACACATACAGGTGAGTGGGCTCTTCAGG Majority

91   .......................................................................................... NW_047687.1_34248_35358
91   .......................................................................................... NW_047687.1_52572_53682
89   ................NNNNN..................................................................... NW_047687.1_53591_54701
91   ................................................................................A......... NW_047687.1_134157_135267
91   ............T...................................................................A......... NW_047687.1_198926_200036
91   ................................................................................A......... sequence from SHHF

     AGTAGTGGTCTCACTCTGTTTCTGAGAACTCTTCCTCCTAAGGAATTCCATGGCATTGAAGTGTACTTAAT-CTTGCCAACAAAATTTAC Majority

181  .............................G.................................C.......-.................. NW_047687.1_34248_35358
181  .............................G.........................................-.................. NW_047687.1_52572_53682
179  .............................G.........................................-.................. NW_047687.1_53591_54701
181  .....................................................................T.T.................. NW_047687.1_134157_135267
181  ............................................-..........................-.................. NW_047687.1_198926_200036
181  ............................................-........................G.-.................. sequence from SHHF

     ATCAAAATGTTTTCCCTACAAGTAAAGGATCCTAGTCTACAGATTTAGGTTCTACCAATATATGCTACCCAAATGTTACCATATATTATA Majority

270  .......................................................................................... NW_047687.1_34248_35358
270  .......................................................................................... NW_047687.1_52572_53682
268  .......................................................................................... NW_047687.1_53591_54701
271  .......................................................................................... NW_047687.1_134157_135267
269  .......................................................................................... NW_047687.1_198926_200036
269  ......................................................T................................... sequence from SHHF

     TCAAAAAGAGCTGTAGAATTTATTGCAAGTTTAAAGCTACTAAGTAGCTCCCCTACTAAAAACTTTATGGATGGTT-GTTTGACACTTGA Majority

360  ............................................................................T............. NW_047687.1_34248_35358
360  .G..........................................................................-............. NW_047687.1_52572_53682
358  .G..........................................................................-............. NW_047687.1_53591_54701
361  ............................................................................-............. NW_047687.1_134157_135267
359  ............A...........ATC.T...............................................T............. NW_047687.1_198926_200036
359  ............................................................................-............. sequence from SHHF

     GTCATGTTCCAAAATGTCCAGTTGGCATACTGAGAGATTTATGAATACATGATGAACATCTTAGAAGGAAACAGGTATCTTACTTCTGTG Majority

450  .......................................................................................... NW_047687.1_34248_35358
449  ................................................................................G......... NW_047687.1_52572_53682
447  ................................................................................G......... NW_047687.1_53591_54701
450  ..............................................G........................................... NW_047687.1_134157_135267
449  ................................................................................G......... NW_047687.1_198926_200036
448  ............--............................................................................ sequence from SHHF

     ATTATCCTGGTGTGGCCTCTAGGTCAGAAACAACCGTAACACCCGTCAACTGCTACAACAAGTGGATGGAACAGACTCTACCTGGTTATT Majority

540  ...........T....T......C....................A...........................G................. NW_047687.1_34248_35358
539  .......................................................................................... NW_047687.1_52572_53682
537  .......................................................................................... NW_047687.1_53591_54701
540  ............................................A............................................. NW_047687.1_134157_135267
539  ............................................A............................................. NW_047687.1_198926_200036
536  ..............................................................................G........... sequence from SHHF

     TCAAACTCCTTTGTGGAAATCAGATATGAGTTCAGTGTTGAGAACCACAGAAATGAAATGATTGTGCATAGCTGCAGCCCCTGCTAAGTG Majority

630  .......................................................................................... NW_047687.1_34248_35358
629  .......................................................................................... NW_047687.1_52572_53682
627  .......................................................................................... NW_047687.1_53591_54701
630  .......................................................................A.................. NW_047687.1_134157_135267
629  .......................................................................A.................. NW_047687.1_198926_200036
626  ...............................................................T..............T........... sequence from SHHF
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Fig 4.17 continued to next page

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    TCGAAGTCAGATATGCATTGAGATTTAAATAGCAATTTAAAGCTTACAATGTTTCACTCTCTTATACTTTTAAGTATTTTAATAGTTTAT Majority

720 .....................C......................................G.........C................... NW_047687.1_34248_35358
719 .........................................................G............C................... NW_047687.1_52572_53682
717 .........................................................G............C................... NW_047687.1_53591_54701
720 .........................................................................A................ NW_047687.1_134157_135267
719 .........................................................G...............A................ NW_047687.1_198926_200036
716 ............................................................G............A................ sequence from SHHF

    TTAATAAAGTTATAAATAAAATTTGAAATAAGTGCATCTAGGGGAAATACTAATTCAGCACCTTTTGTTGGAAGAAAAAGAAAGTGGAAG Majority

810 ..............................T..................A........................................ NW_047687.1_34248_35358
809 .....................A.................................................................... NW_047687.1_52572_53682
807 .....................A.................................................................... NW_047687.1_53591_54701
810 ..............................T..................A........................................ NW_047687.1_134157_135267
809 .......................................................................................... NW_047687.1_198926_200036
806 .......................................................................................... sequence from SHHF

    AAGCAGAAGGAGAAGGAGA----GAGGAGGAAGGAGGAGGAGGAGGAGGAAG--------AGGAGGGGAGGGAGAGGAGGAGGAGGGGG Majority

900 .....A............----------ACG..........A.-.A......--------....C.A...A.A...A..A.......... NW_047687.1_34248_35358
899 ...................ATGA.GA.GA.......A...............TAAGGAAG....C.A...A.A...NNNNN......... NW_047687.1_52572_53682
897 ...................ATGA.GA.GA.......A...............TAAGGAAG....C.A...A.A...A..A.......... NW_047687.1_53591_54701
900 .....A.............CGAG............A...........A.G..--------.......A...................... NW_047687.1_134157_135267
899 ................TT-----............A..........G.....--------.......A...................... NW_047687.1_198926_200036
896 ................TT-----............A................--------.......A...................... sequence from SHHF

    G-CACAAAATGATACCACCAAATGTTGTCCACATTCATATAGAGATAGATAGGAGCTGGTAGTCATATTTCTACATACAGTTTTTTTATA Majority

971 .-...........................A...............................................T.-C......... NW_047687.1_34248_35358
989 .--NNNNNNNNNNNNNNNNNGT..NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNGC.CC NW_047687.1_52572_53682
987 .-...........................A...............................................T.-C......... NW_047687.1_53591_54701
982 .-................................C....................................................... NW_047687.1_134157_135267
976 .G............................................T........................................... NW_047687.1_198926_200036
973 .-............................................T........................................... sequence from SHHF
```

Fig. 4.17 SHHF *Cd36* exon 4 and intron 4 sequence compared with published sequence

The sequence of SHHF *Cd36* exon 4 and intron 4 (1-900 bp) were aligned with the corresponding parts of the sequence NW_047687.1 using MegAlign of DNAStar program. The sequences (1-162 bp) are exon 4. The limits of the breakpoint are marked with arrows. Other SHHF sequence variants that were observed different from the others may be sequence variations between SHHF and BN, since the genome sequences are derived from the BN rat. Decoration 1, Hide (as '.') residues that match the Majority exactly. Decoration 2, box residues that differ from the majority.

4.4.6 Western blots of Cd36 protein in SHHF heart tissue

To determine whether the SHHF transcripts translate to a normal Cd36 protein. The protein Cd36 was analyzed by western blots. Cd36 was detected in plasma membrane from WKY and SHRSP heart tissue but not detectable from SHHF and SHR/mol heart tissue (Fig.4.18).

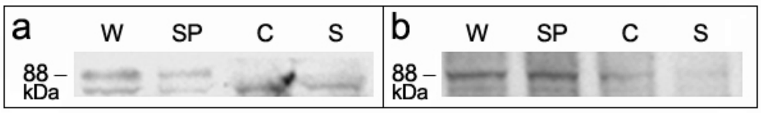

Fig. 4.18 Western blot of Cd36 protein

40 µg of microsomal and plasma membrane proteins in WKY (W), SHRSP (SP), SHHF (C) and SHR/mol (S) were separated on 8% SDS-PAGE gel, Cd36 was visualized using a polyclonal rabbit anti- human Cd36 antibody (a) and monoclonal human Ig M anti-human Cd36 antibody (b).

4.4.7 Comparison of predicted protein sequence of Cd36

Cd36 coding sequence (cds) was 1419 bp, with 472 amino acids in length, and about 52.73 KDa of its predicted protein. Cd36 had multiple polymorphisms among the different rats from their predicted amino acids sequence (Fig. 4.19), SHHF Cd36 ORF that was consistent with SHR/NCrlBR had 11 amino acids variation compared with WKY. For the SHRSP, there's only a point mutation (G/A, Arg262/Glu) compared to WKY (Collison *et al*., 2000). From comparisons of amino acids sequences, WKY Cd36 was more similar to the XP_346685 (from model gene LOC 360376), however SHHF Cd36 fit better to the XP_216076 (From model gene LOC296786). These further confirmed that WKY *Cd36* cDNA was transcribed from the gene LOC360376 and SHHF *Cd36* from the gene LOC296786. Although Cd36 amino acid sequence of WKY also had 9 amino acids variation compared with that of Sprague-Dawley, it might cause by the strain difference.

```
     MGCDRNCGLITGAVIGAVLAVFGGILMPVGDLLIEKTIKREVVLEEGTIAFKNWVKTGTTVYRQFWIFDVQNPEEVAKNSSKIKVKQRGPYTYRV Majority

1    ............................................................................................... Wistar-darkagouti
1    ............................................................................................... XP_346685
1    ............................................................................................... SHHF-SHR
1    ....L........I................................................................................. XP_216076
1    ..................................................................V..................I......... Sprague-Dawley
1    -------------------------------------------------------------------------MN.P..--------------.. XP_342623

     RYLAKENITQDPKDSTVSFVQPNGAIFEPSLSVGTENDNFTVLNLAVAAAPHIYTNSFVQGVLNSLIKKSKSSMFQTRSLKELLWGYKDPFLSLV Majority

96   ............................................................................................... Wistar-darkagouti
96   ............................................................................................... XP_346685
96   ......S..........................................V....Q...F.....IF.....................E......I SHHF-SHR
96   ..........................................P......V....Q...F.....IF.....................E......I XP_216076
96   ............................................................................................... Sprague-Dawley
9    ............................................................................................... XP_342623

     PYPISTTVGVFYPYNNTVDGVYKVFNGKDNISKVAIIDTYKGKRNLSYWESYCDMINGTDAASFPPFVEKSQTLRFFSSDICRSIYAVFESEVNL Majority

191  ............................................................................................... Wistar-darkagouti
191  .......................................................................R.................G..... XP_346685
191  .................................................K............................................. SHHF-SHR
191  .................................................K............................................. XP_216076
191  ........................S.........................................LG...R....................... Sprague-Dawley
104  .......................................................................R....................... XP_342623

     KGIPVYRFVLPANAFASPLQNPDNHCFCTEKVISNNCTSYGVLDIGKCKEGKPVYISLPHFLHASPDVSEPIEGLNPNEDEHRTYLDVEPITGFT Majority

286  ............................................................................................... Wistar-darkagouti
286  ............................................................................................... XP_346685
286  ............................................................................................... SHHF-SHR
286  ............................................................................................... XP_216076
286  .......................................................N.....................T................. Sprague-Dawley
199  ............................................................................................... XP_342623

     LQFAKRLQVNILVKPARKIEALKNLKRPYIVPILWLNETGTIGDEKAEMFRNQVTGKIKLLGLVEMVLLGVGVVMFVAFMISYCACRSKNGK    Majority

381  ............................................................................................    Wistar-darkagouti
381  ............................................................................................    XP_346685
381  ....................P..................................................................F....    SHHF-SHR
381  ....................P..................................................................F....    XP_216076
381  ...S........................................................................................    Sprague-Dawley
294  ............................................................................................    XP_342623
```

Fig 4.19 Comparison of Cd36 amino acid sequence

Wistar-dark agouli, from WKY Cd36 cDNA (from this study), AF072411 (Wistar), AF113914 (dark agouti); XP_346685, from XM346685; SHHF-SHR, from cds of SHHF Cd36 cDNA (from this study) and AF111268 (SHR); XP_216076, from XM216076; Sprague-Dawley, from L19658. XP342623, from XM342623.

4.5 Functional study of Cd36 in SHHF rat using linkage analysis

4.5.1 Genotype of F2 animals

The cross of SHHF with WKY strain was set up to further characterize the phenotype and to facilitate gene identification. (SHHF×WKY) F2 animals were genotyped by *Cd36 Hin*fI RFLP analysis. The animals were scored as SHHF homozygotes if the complete *Hin*fI digestion showed only the 77-bp fragment, and as either SHHF/WKY heterozygotes or WKY homozygotes if both the 77 bp and 107 bp bands were present (Fig. 4.20, Fig. 4.21).

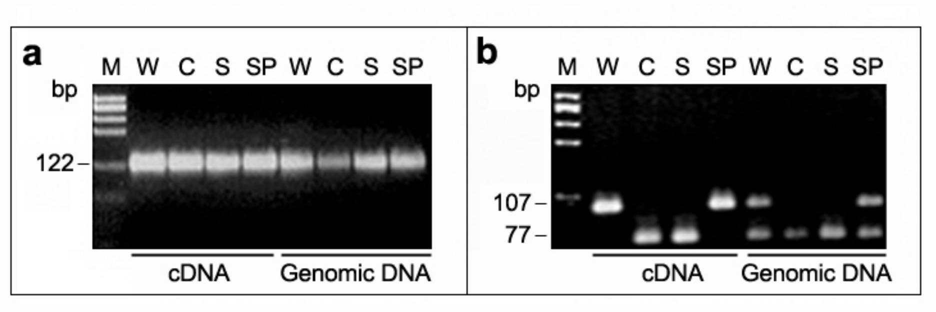

Fig. 4.20 Analysis of PCR products amplified from cDNA and genomic DNA

a, PCR products were amplified from cDNA and genomic DNA of strains SHHF (C), WKY (W), SHR/mol (S), and SHRSP (SP) with *Cd36* Hinf primer. b, PCR products were further analyzeded by *Hin*fI digestion on 4% agarose gel (for small DNA from Biozym).

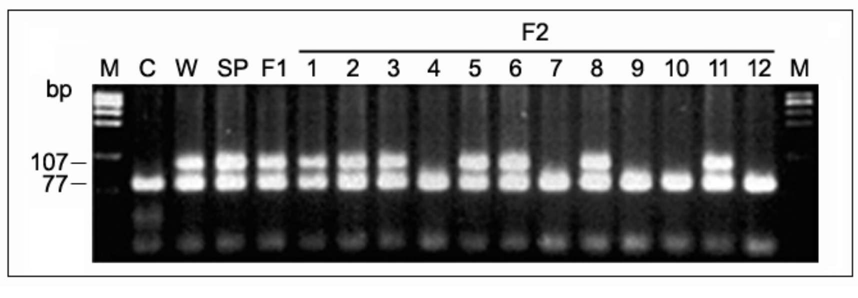

Fig. 4.21 Genotype results of F2 animals by *hin*fI RFLP analysis (example)

PCR products amplified from genomic DNA of (SHHF×WKY) F2 (F2, 1-12), SHHF (C), WKY (W), SHRSP (SP) and (SHHF×WKY) F1 with *Cd36* Hinf primer were analyzed on 4% agarose gel after *Hin*fI digestion. M1, PhiX174 *Bsu*RI (*Hae*III) DNA marker.

4.5.2 Expression of *Cd36* in F2 animals

The expression of *Cd36* in F2 heart tissue was detected. The normal transcript of *Cd36* was confirmed absent in SHHF *Cd36* homozygotes, and present in both WKY homozygotes and

SHHF/WKY heterozygotes by *Hin*fI RFLP analysis (Fig. 4.22). *Cd36* showed 2.2-fold downregulation in SHHF homozygotes compared with WKY homozygotes and two-fold downregulation compared with SHHF/WKY heterozygotes with TaqMan quantitative PCR.

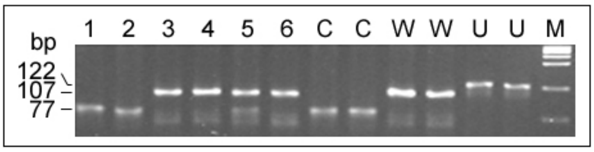

Fig. 4.22 Expression of *Cd36* in (SHHF×WKY) F2 animals

PCR products amplified from cDNA of SHHF *Cd36* homozygotes (Lane 1, 2), WKY *Cd36* homozygotes (Lane 3, 4), SHHF/WKY heterozygotes (Lane 5, 6), SHHF (C), and WKY (W) with *Cd36* Hinf primer were analyzed on 4% agarose gel after *Hin*fI digestion. U, Undigested PCR products; M1, PhiX174 *Bsu*RI (*Hae*III) DNA marker.

4.5.3 linkage analysis by MapMaker

The F2 animals were also genotyped by the markers D4Rat6, D4Rat221 and D4Bro1 on either side of *Cd36* (Table 4.6). The calculated hemodynamic parameters were used as indicators of cardiovascular performance in response to heart failure. The linkage analysis with MapMaker was performed on the original data following a normal distribution or transformed data that more closely fit the normal distribution (Table 4.8). The LOD scores above 1.5 were listed in Table 4.7. The highest LOD score of 2.07 was detected at marker D4rat 221 in male rats for the cardiac output, which indicates the locus on chromosome $4q^{11\text{-}12}$ showing suggestive linkage to heart failure (Fig. 4.23) (Lander and Kruglyak, 1995). *Cd36* is less than 1 cM away from this marker.

Table **4.6 Positions of *Cd36* and three markers on chromosome 4**

Marker	Chr_start (bp)	Chr_end (bp)	Expected size	Estimated distance to *Cd36*
D4bor1	6115283	6115445	162	7,331,555
D4rat6	10298232	---[1]	173	3,148,595
Cd36	***13447000***	***13695000***		
D4rat221	90925354 [2]	14388573	158	693,573

1, primer 2 of D4rat6 could not be mapped. 2, primer 1 of D4rat221 hits for a different contigs on chromosome 4.

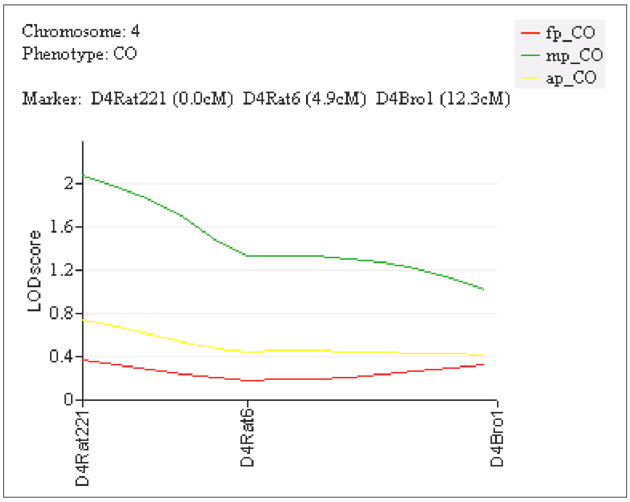

Fig 4.23 MapMaker result for Cardiac output

Table **4.7 MapMaker results of the LOD scores above 1.5**

Gender	Markers	MDP	Tau	SV	CO	HGmg	LUNGmg	RNmg	LNmg
	D4Rat221 (0 cM)[1]	1.148	0.569	1.652	2.071	0.347	0.475	2.025	1.399
Male	D4Rat6 (4.9 cM)	1.605	1.529	0.613	1.333	0.689	0.178	2.038	1.448
	D4Bro1 (12.3 cM)	1.42	1.174	0.826	1.016	1.085	0.049	2.002	1.556
	D4Rat221 (0 cM)	0.2	0.004	0.117	0.367	0.758	1.026	0.157	0.261
Female	D4Rat6 (4.9 cM)	0.101	0.207	0.196	0.184	1.03	1.568	0.682	0.771
	D4Bro1 (12.3 cM)	0.023	0.102	0.412	0.326	1.155	2.53	0.418	0.479
	D4Rat221 (0 cM)	0.463	0.081	1.117	0.737	1.017	0.085	1.328	0.885
All	D4Rat6 (4.9 cM)	0.407	0.537	0.267	0.451	1.534	0.419	2.039	1.568
	D4Bro1 (12.3 cM)	0.301	0.354	0.66	0.41	2.052	0.452	1.805	1.412

1. Relative position of the three markers.

4.5.4 Comparison analyses by t-test and anova

From t-test analysis that performed between SHHF *Cd36* homozygotes and WKY *Cd36* homozygotes or SHHF/WKY *Cd36* heterozygotes, combined anova analysis that performed among SHHF homozygotes, WKY homozygotes and SHHF/WKY heterozygotes divided by genotype result of Marker D4Rat221, Cardiac output showed significant difference among SHHF homozygotes, SHHF/WKY heterozygotes and WKY homozygotes in F2 male rats (Table 4.8, $P < 0.05$). Time constant of relaxation and Mean diastolic pressure also showed significantly difference among two homozygotes and heterozygotes in male rats according genotype result of *Cd36* combined with Marker D4Rat 6 (Table 4.8, $P < 0.05$).

Table **4.8 Comparison analyses of (SHHF×WKY) F2 animals by t-test and anova**

Parameters	Data transformation			Values of t-test analysis[1]			Values of anova analysis[2]		
	All	Female	Male	All	Female	Male	D4Rat221	D4Rat6	D4Bro1
PMAX	1div	1div	1div	0.311	0.606	0.294	0.764	0.327	0.324
PMIN	sqrt	sqrt	sqrt	0.033	0.157	0.069	0.211	0.055	0.074
PBD	sqrt	sqrt	sqrt	0.109	0.252	0.169	0.458	0.21	0.254
PED	1div	1div	orig	0.439	0.844	0.053	0.066	0.038	0.061
PES	1div	1div	1div	0.14	0.612	0.168	0.521	0.174	0.253
DP	1div	1div	1div	0.41	0.63	0.486	0.954	0.584	0.425
SEP	1div	log	log	0.884	0.159	0.201	0.347	0.263	0.675
DFP	sqrt	sqrt	sqrt	0.826	0.207	0.406	0.8	0.375	0.328
MSP	1div	1div	1div	0.253	0.596	0.25	0.633	0.278	0.345
MDP	log	log	sqrt	0.198	0.641	0.025	0.083	0.025	0.051
CT	1div	1div	-	0.1	0.213	-	-	-	-
RT	orig	orig	orig	0.322	0.739	0.099	0.266	0.08	0.079
DPMAX	orig	orig	orig	0.593	0.953	0.44	0.997	0.757	0.727
DPMIN	orig	orig	orig	0.686	0.904	0.626	0.866	0.877	0.779
CI	-	orig	-	-	0.275	-	-	-	-
TAU	orig	orig	orig	0.254	0.88	0.035	0.281	0.0302	0.087
VMAX	orig	orig	orig	0.607	0.81	0.338	0.063	0.343	0.228
VMIN	orig	orig	orig	0.352	0.607	0.093	0.162	0.324	0.218
EDV	orig	orig	orig	0.776	0.688	0.437	0.061	0.396	0.28
ESV	orig	orig	orig	0.356	0.63	0.114	0.149	0.361	0.206
SV	orig	orig	orig	0.334	0.43	0.129	0.027	0.257	0.163
CO	orig	orig	orig	0.295	0.208	0.014	0.011	0.051	0.109
EF	log	log	log	0.418	0.723	0.106	0.286	0.435	0.256
SW	orig	orig	orig	0.757	0.255	0.302	0.041	0.318	0.218
EA	1div	1div	1div	0.109	0.805	0.047	0.051	0.173	0.122
HR	log	log	log	0.725	0.324	0.172	0.546	0.125	0.147
ANIMALG	n_log	log	log	0.253	0.642	0.048	0.079	0.16	0.502
HGMG	n_log	log	log	0.432	0.227	0.987	0.456	0.215	0.084
LUNGMG	n_log	log	log	0.242	0.267	0.527	0.339	0.676	0.897
RNMG	n_log	log	log	0.004	0.618	0.001	0.012	0.01	0.022
LNMG	n_log	log	log	0.024	0.803	0.004	0.045	0.034	0.045

1, t-test results of all rats (191), male rats (105) or female rats (86) were analyzed between SHHF *Cd36* (-/-) homozygotes and WKY *Cd36* (+/+) homozygotes or WKY/SHHF *Cd 36* (+/-) heterozygotes. 2, Anova results of the male rats were analyzed among SHHF homozygotes, WKY homozygotes and SHHF/WKY heterozygotes divided separately according the genotype result of Marker D4Rat221, D4Rat6 or D4Bro1. -, The transformation is not allowed.