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Identification of a novel gene, HCR2, in aorta of hypercoagulable rats using suppression subtractive hybridization

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Abstract

Screening and identification of novel genes involved in hypercoagulable state (HCS) is important in understanding the underlying mechanisms of development of atheroslerosis, which implicated in critical vascular diseases such as coronary heart disease and stroke. HCS was induced in rats with high carbohydrate diet. Subtractive hybridization experiments between aorta tissues from hypercoagulable rats and controls showed that a novel cDNA (GenBank Accession No. AY234417), designated as hypercoagulability related gene-2 (HCR2), was highly expressed in aorta tissue. The predicted protein encoded by HCR2 contains 78 amino acids, which has a theoretical isoelectric point (p*I*) of 8.59 and molecular weight (MW) of 8841.7 based on sequence analysis. Our data suggest that HCR2 may be involved in HCS and identification of the gene may contribute to our understanding of the mechanisms for the production of a hypercoagulable state of several critical clinical disorders.

Keywords: Hypercoagulable state; Rat model; Aorta; Screening of novel genes; Suppression subtractive hybridization; Rapid amplification of cDNA ends; Hypercoagulability related gene-2

Coronary heart disease (CHD) and stroke are the main forms of what is known as cardiovascular disease. They are the leading causes of morbidity, disability, and mortality in the world. Hypercoagulable state (HCS) has been implicated as risk factor for premature-onset atherosclerosis, which involved in above disorders. As to the underlying mechanism, hypercoagulability is considered one of the most important contributing factors in these diseases. Normally, the body maintains a homeostatic balance between coagulation and fibrinolysis. When the balance is disrupted under certain conditions and the activity of coagulation is enhanced, the blood becomes more prone to clotting. HCS can be influenced by some factors, such as damage of blood vessel wall, reduction of blood flow, and changes of blood components. HCS can induce an enhanced intravascular coagulation, and play a significant role in the pathophysiology of CHD and stroke [1].

Several reports indicated that HCS may be induced by mutation and unusual expression of several genes, such as those of coagulation factor V, prothrombin, methylene tetrahydrofolate reductase [2], thrombodulin [3], antithrombin, protein C, protein S [4], plasminogen activator inhibitor-1 (PAI-1) [5], and angiotensin- converting enzyme (ACE) genes [6]. However, full understanding of the molecular mechanism of HCS development in diseases is still lacking. Screening of HCS-related novel genes will be of value in: (a) understanding the molecular mechanism of underlying the HCS in cardiovascular diseases and (b) identifying

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potential new molecular targets for clinical diagnosis and therapeutic intervention.

Techniques currently used to characterize many of the differentially expressed transcripts include differential display RT-PCR (DDRT-PCR) [7], representational difference analysis (RDA) [8], DNA chip technique [9], and serial analysis of gene expression (SAGE) [10]. However, the high percentage of false positives generated by DDRT-PCR strongly limits the usability of the method. DNA chip technique is limited to detect previously characterized genes on the chip. In 1996, Diatchenko et al. [11] established a polymerase chain reaction-based cDNA subtractive strategy termed suppression subtractive hybridization (SSH), which is characterized an efficient method for identification of differentially expressed genes. SSH is similar to RDA but has a number of advantages [11]. The SSH technique allows normalization of abundant and rare cDNAs, subtraction of common cDNAs, isolation of previously unknown cDNAs. The suppression PCR technique allows selective amplification of differentially expressed cDNAs, while concurrently suppressing amplification of those equally expressed genes. Although several studies using SSH have successfully identified the differentially expressed genes in a variety of disease states [12–14], this study, for the first time, identified the altered gene expression in hypercoagulable state in a model animal. We have successfully identified a hypercoagulability related novel gene (HCR2) based on the method and proved an effectiveness of this method. The results of current study could provide clues to the potential role of new gene in disease processes and may be helpful in understanding the molecular mechanism underlying the HCS in cardiovascular diseases.

Materials and methods

Induction of hypercoagulable state in rats. Twelve male healthy Wistar rats, weighing at 244 ± 64 g, were purchased from Sichuan University Animal Center. The rats were housed in cages and kept at room temperature at 20–25 °C with free access to rat food and water. After 7 days on a normal diet, the rats were divided into two groups. The control group ($n = 6$) was fed with a normal diet (ratio of total calories: 62% carbohydrate, 17% protein, and 21% fat). A HCS group ($n = 6$) was fed with a prepared high carbohydrate diet (HCD, ratio of total calories: 79.2% carbohydrate, 16% protein, and 4.8% fat) for 3 days. The levels of plasma triglyceride (TG), prothrombin time (PT), activated partial thromboplastin time (APTT), tissue-plasminogen activator (t-PA), and plasminogen activator inhibitor-1 (PAI-1) were determined as described previously [15]. The data were analyzed with Statistical Package for Social Services (SPSS version 10.0). All values are presented as means ± SD. Differences between mean values were assessed by an independent sample t test. A P value of <0.05 was considered statistically significant. Then, all rats were killed and their aorta tissues (aortic arch, aorta pectoralis, and aorta ventralis) were surgically dissected and immediately transferred to a tube containing cold RNA Stabilization Reagent (Qiagen, Valencia, CA, USA). The aortas were stripped of the endothelium and periadventitial fatty tissues in the Stabilization Reagent at 4 °C, snap frozen in liquid nitrogen, and stored at −80 °C for subsequent RNA isolation.

Isolation of total RNA. The removed aorta tissues of six rats in hypercoagulable state induced by HCD were mixed together as tester and the aorta tissues of six rats fed with normal diet mixed together as driver. Total RNA was isolated from tester and driver using the RNeasy Midi Kit (Qiagen, Valencia, CA, USA), respectively, and quantitated by ultraviolet spectrometry (UV-265, Kyoto, Japan). The ratio of A_{260} to A_{280} was in the range of 2.0–2.1. About 10 μg of total RNA was electrophoresed on a 1.0% denaturing agarose gel to check its integrity.

Establishing cDNA libraries by suppression subtractive hybridization. SMART PCR cDNA Synthesis Kit (Clontech, Palo Alto, CA, USA) was used to reverse transcribe total RNA extracted from tester and driver according to the manufacturer's protocol. Suppression subtractive hybridization (SSH) was performed with the PCR-Select cDNA Subtraction Kit (Clontech, Palo Alto, CA, USA) as described by the manufacturer with some modifications. Briefly, tester cDNA was divided into two groups and ligated separately with two different adaptors, adaptor 1 and adaptor 2R. The adaptor 1-ligated and adaptor 2R-ligated tester cDNAs were separately hybridized at 68 °C for 9 h with an excess of driver cDNA after denaturation at 98 °C for 90 s. The two hybridized samples were then mixed together without denaturation and hybridized at 68 °C overnight with excess of driver cDNA. The resulting mixture was added with 200 μl dilution buffer and amplified by two suppression PCRs. The primary PCR was performed at 94 °C for 30 s, 66 °C for 30 s, and 72 °C for 90 s for 30 cycles in a reaction volume of 25 μl. The amplified product was diluted to 10-fold and 1 μl diluted primary PCR product was used as template in the secondary nested PCR for 15 cycles at 94 °C for 30 s, 68 °C for 30 s, and 72 °C for 90 s using two nested primers, 1 and 2R, provided in the kit. A second PCR-based analysis was performed according to the instruction in the kit user manual to test for the efficiency of subtraction (see below). The subtracted PCR products were cloned into the pMD18-T vector (TaKaRa Biotech, Dalian, China) for screening.

Evaluation of the subtraction efficiency. To evaluate the efficiency of the subtraction, the relative amount of G3PDH cDNA present in the subtracted and unsubtracted cDNA populations after SSH was examined by PCR amplification using G3PDH 3′ primer (5′-TCCAC CACCCTGTTGCTGTA-3′) and 5′ primer (5′-ACCACAGTCCATG CCATCAC-3′).

Screening of cDNA clones with reverse dot-blot analysis. Following the cloning of the subtracted PCR product, a total of 192 individual recombinant clones were picked and used to seed into two 96-well microtiter plates containing 100 μl LB medium and 50 μg/ml ampicillin at 37 °C for 4 h with shaking. One microliter of the growing culture was transferred to 0.5 ml of PCR tube and used as the PCR template. PCR was performed with nested primers (NP1: 5′-TCGAGCGGCC GCCCGGGCAGGT-3′; NP2R: 5′-AGCGTGGTCGCGGCCGAG GT-3′). After PCR amplification, the PCR products were analyzed by gel electrophoresis to confirm that each recombinant had the proper insert. For denaturing purposes, each PCR product was mixed with an equivalent volume of 0.6 N NaOH. Two microliters of denatured PCR products was dotted onto nylon membranes (Roche Applied Science, Mannheim, Germany) in duplicate and fixed by baking in an oven for 2 h at 80 °C. Unsubtracted cDNA probes from tester and driver were DIG-labeled using DIG High Prime DNA Labeling and Detection Starter Kit II (Roche Applied Science, Mannheim, Germany). Membranes were hybridized at 50 °C for 16 h in DIG Easy Hyb. The hybridization signals were detected by chemiluminescence with CSPD (Roche Applied Science, Mannheim, Germany). Clones that hybridized to the tester probe alone or showed differences in signal intensity of greater than fivefold as assessed by densitometry were selected for further analysis.

DNA sequence analysis. Twenty positive clones confirmed by reverse dot-blot analysis were selected for sequencing. Plasmids were extracted and the inserts were used for automated sequencing in an

ABI 3700 automated sequencer (GeneCore Biotechnology, Shanghai, China). The sequences obtained were compared against GenBank database using the BLAST program.

Analysis of mRNA differential expression by semi-quantitative reverse transcriptase-polymerase chain reaction. Expression of five clones of interest from SSH, namely HC002, HC019, HC039, HC057, and HC067, as well as G3PDH as control, was evaluated and compared in tester and driver by semi-quantitative RT-PCR as described previously [16]. The primer sequences used in RT-PCR of these clones are listed in Table 1. Briefly, total RNAs of tester and driver were isolated as described above. The total RNAs were subjected to semi-quantitative RT-PCR using TaKaRa one step RNA PCR Kit (TaKaRa Biotech, Dalian, China). Two microliters of total RNA was reverse transcribed and PCR amplified in 50 μl of reaction volume containing 10× one-step RNA PCR buffer, 5 U AMV reverse transcriptase XL, 5 U AMV-Optimized *Taq*, 5 mM $MgCl_2$, 1 mM dNTP, and 0.4 μM gene-specific primer (Table 1). The reverse transcription was carried out at 50 °C for 30 min and followed by 2 min at 94 °C to inactivate the AMV transcriptase XL. The PCR conditions were 28–36 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min. The PCR products were separated on 2.0% agarose gel. The images were quantified by densitometry scanning followed by analysis with GrabIt version 3.0 (GDS-800, USA). Intensities of bands were normalized and expressed relative to the intensity of the band of G3PDH. The mean ± SD of three independent experiments were compared and analyzed in each clone.

Rapid amplification of cDNA ends. Rapid amplification of cDNA ends (RACE) of clone HC002 was performed in both directions using SMART RACE cDNA amplification kit (Clontech, Palo Alto, CA, USA). To obtain the 5′ region of HC002, we used gene-specific primer-1 (GSP1, 5′-TCACATTTAGCACCACTCCTGCCATCC-3′) and nested gene primer-1 (NGSP1, 5′-CAGACTGTGGATGTGAAGG TCCGA -3′). To determine the 3′-end, we used gene-specific primer-2 (GSP2, 5′-GGT AGGAGTAGCAGGACAGCCCACAGG-3′) and nested gene primer-2 (NGSP2, 5′-GCTGGATGGCAGGAGTGGTG CTAAATG-3′). The PCR products were subcloned into the pMD18-T vector and sequenced.

The DNA sequence of the cDNA insert, and 3′- and 5′-RACE products were combined, yielding a 1275 bp cDNA—a complete coding sequence of a gene, designated as hypercoagulability related gene-2 (HCR2).

Bioinformatic analysis of HCR2. The cDNA sequence and deduced protein sequence of HCR2 were compared against GenBank database using the BLAST program. The genomic structure of HCR2 was identified by aligning the cDNA sequence with corresponding genomic sequence (GenBank Accession No. NW_043724.1). The chromosome location of HCR2 was determined by aligning the cDNA sequence with rat genome. Analysis of the predicted protein was performed by the ExPASy search program (http://cn.expasy.org/tools).

Analysis of mRNA expression in various rat tissues by Northern blot. Multiple Tissue Northern (MTN) blot (Clontech, Palo Alto, CA, USA) with mRNA from 8 tissues and DIG High Prime DNA Labeling and Detection Starter Kit II (Roche Applied Science, Mannheim, Germany) were used to determine the tissue expression pattern of HCR2 gene. The blotting membrane was incubated in DIG Easy Hyb at 50 °C for 30 min, and then hybridization was preformed using a 567-bp cDNA fragment of HCR2 labeled with DIG-11-dUTP by random priming at 50 °C for 16 h. After washing, the hybridization signals were detected by chemiluminescence with CSPD (Roche Applied Science, Mannheim, Germany). Then, the HCR2 probe was stripped and the membrane was rehybridized with β-actin probe for internal loading control.

Results

Plasma triglyceride and haemostatic parameters of model animals

Table 2 shows the levels of plasma TG, PT, APTT, t-PA, and PAI-1 in normal and hypercoagulable rats. The plasma TG levels in the HCS group were 2.43 times of the control group. No significant difference of t-PA activity was found between the normal group and the HCS group. Both PT and APTT in the HCS group were significantly shorter than that in the control group

Table 2
Contents of TG, PT, APTT, t-PA, and PAI-1 in hypercoagulable state and control groups ($\bar{x} \pm SD$)

	Control group ($n = 6$)	HCS group ($n = 6$)
TG (mmol/L)	0.44 ± 0.19	1.07 ± 0.31***
PT (s)	16.40 ± 2.60	9.85 ± 0.89***
APTT (s)	27.55 ± 3.78	20.60 ± 4.56**
t-PA (IU/ml)	0.30 ± 0.03	0.35 ± 0.11
PAI-1 (IU/ml)	0.34 ± 0.07	0.43 ± 0.05**

TG, triglyceride; PT, prothrombin time; APTT, activated partial thromboplastin time; t-PA, tissue-plasminogen activator; and PAI-1, plasminogen activator inhibitor-1.
** $P < 0.01$ as compared to control group.
*** $P < 0.001$ as compared to control group.

Table 1
Primers used for reverse transcription-polymerase chain reaction

Clone	Primer sequence (forward and reverse)	Size (bp)
HC002	5′-AATGGAGGGAATAGAGGG-3′ 5′-CAGCCAAGGATACAGACC-3′	546
HC019	5′-TATGATGTTATGTGAACCGAAAGAC-3′ 5′-ATGTCTGTGGACCTCACCT-3′	371
HC039	5′-ACCTCAACAAGAATTTTTCAC-3′ 5′-ACATGTAGAAGTTTATTTCAGG-3′	498
HC057	5′-CAGTAGAGGCAGCCGTGGC-3′ 5′-TTTGAGACTCAAAAAGGGAC-3′	380
HC067	5′-ACCAGTCGCTGTTCTGGG-3′ 5′-AGCGGATCCAGATGCCTG-3′	462
G3PDH	5′-ACCACAGTCCATGCCATCAC-3′ 5′-TCCACCACCCTGTTGCTGTA-3′	452

($P < 0.001$, $P < 0.01$). PAI-1 activity in the HCS group was significantly higher than that in the control group ($P < 0.01$). The results indicated that hypercoagulable state could be induced in rats with high carbohydrate diet.

Suppression subtractive hybridization

The suppression subtraction efficiency was evaluated by PCR, using a housekeeping gene (G3PDH) as internal reference. As showed in Fig. 1, the abundance of G3PDH was reduced 60-fold after subtraction (Fig. 1). After ensuring that G3PDH was extensively removed in the subtracted pools, the subtracted cDNA was cloned into the pMD18-T vector and the products were used in further screening procedures.

192 positive clones from the subtractive library were randomly picked and arrayed in duplicate by spotting onto nylon membranes and screened by reverse hybridization using probes taken from unsubtracted tester and driver cDNA samples (Fig. 2). Among them 42 (22%) clones were detected as differentially expressed transcripts corresponding to genes with greater than fivefold average expression difference. Only 20 positive clones from reverse dot-blot analysis were sequenced. BLAST similarity searches against the GenBank nucleotide sequence database showed nine sequences with >90% identity to known genes and 11 putative novel sequences with less than 70% nucleotide sequence homology (Table 3).

Confirmation of SSH results by semi-quantitative RT-PCR

To confirm the results of SSH, five different clones that gave strong positive signals on reverse dot-blot analysis were selected for semi-quantitative RT-PCR. The primer sequences used in RT-PCR are summarized in Table 1. Fig. 3 shows results of RT-PCR. The signals were normalized with housekeeping gene G3PDH for comparisons (Fig. 3). It was found that the mRNA level of HC019 in the tester was 8.8% higher than in the

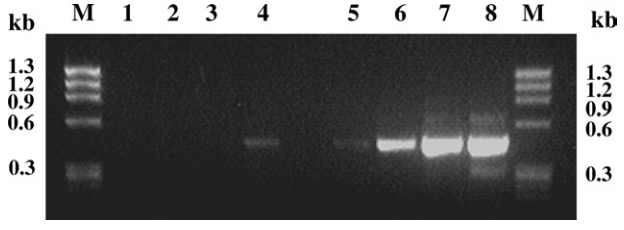

Fig. 1. Evaluation of subtraction efficiency. PCR was performed using G3PDH 3′ primer and 5′ primer. Relative amount of G3PDH products were shown in subtracted (lanes 1–4 for 18, 23, 28, and 33 cycles, respectively) and unsubtracted (lanes 5–8 for 18, 23, 28, and 33 cycles, respectively) secondary PCR product. M, φ × 174-*Hae*III digest DNA marker.

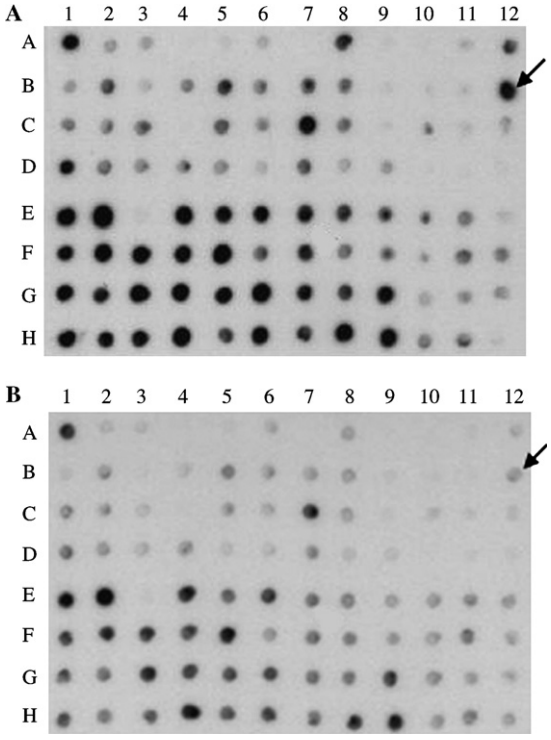

Fig. 2. Representative results of reverse dot analysis using unsubtracted cDNA as probes. Two identical membranes were hybridized with DIG-labeled probes prepared from (A) tester cDNA and (B) driver cDNA. One of the differentially expressed clones was marked with arrows.

Table 3
List of clones isolated by SSH and reverse dot-blot hybridization

Clone	Size (bp)	Gene	Accession No.
HC001	420	ATP-binding cassette 1, sub-family A, member 1	AF287263
HC008	481	Mus musculus similar to nuclear matrix transcription factor	XM_145115
HC010	633	Chinese hamster provirus	M73970
HC021	424	Rattus norvegicus ATPase synthase subunit 6 mRNA	AF504920
HC025	440	Mus musculus tubby like protein 1	AF105711
HC031	329	Rat genes for vasopressin, oxytocin	X59496
HC039	498	Rattus norvegicus alkaline phosphodiesterase	U78788
HC067	462	Mus musculus similar to Ras GTPase-activating-like protein	XM_124937
HC002	567	Novel sequence	BQ901227
HC017	755	Novel sequence	BQ901228
HC019	371	Novel sequence	BQ901229
HC022	872	Novel sequence	BQ901230
HC040	571	Novel sequence	BQ901231
HC042	284	Novel sequence	BQ901232
HC051	636	Novel sequence	BQ901233
HC057	421	Novel sequence	BQ901234
HC058	475	Novel sequence	BQ901235
HC059	822	Novel sequence	BQ901236
HC063	357	Novel sequence	BQ901237

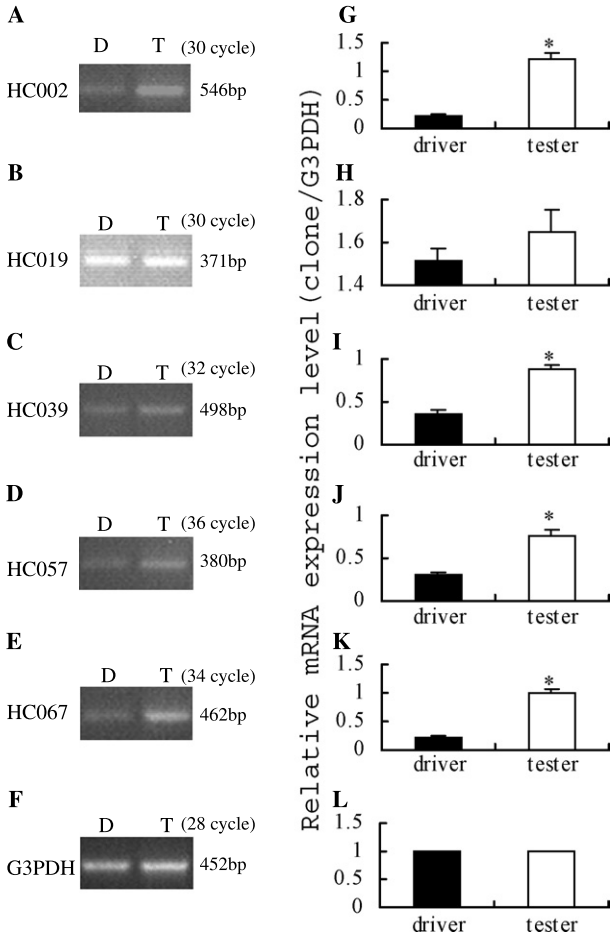

Fig. 3. Comparison of five differentially expressed genes between driver (D) and tester (T) using semi-quantitative RT-PCR. (A) Clone HC002; (B) clone HC019; (C) HC039; (D) HC057; (E) HC067; and (F) G3PDH control. Relative mRNA expression level was presented by dividing the intensity of gene with G3PDH. (G) clone HC002; (H) clone HC019; (I) HC039; (J) HC057; (K) HC067; and (L) G3PDH control. Values are means ± SD of three independent experiments. *$P < 0.05$ in comparison with driver.

driver. Interestingly, the expression of HC002, HC039, HC057 and HC067 in the tester was 5.4, 2.4, 2.4, and 4.4-fold higher ($P < 0.05$) than that in the driver, respectively. Among all the 11 clones that had no significant homology with any known genes in the GenBank database, a 567-bp cDNA clone (clone HC002) showed the most marked induction by HCS (Fig. 3). Therefore, we focused on this clone for further analysis.

The full-length cDNA of HCR2

The full-length cDNA of HCR2 obtained and the deduced amino acid sequence are shown in Fig. 4. The HCR2 cDNA is 1275 bp in length and contains a 237-bp open reading frame that extends from nucleotide positions 306–542 and encodes a protein of 78 amino acids. Comparison of the HCR2 cDNA sequence with GenBank database using the BLAST program revealed that there was no significant homology with any known genes. The HCR2 cDNA sequence was submitted to GenBank as a novel gene with Accession No. AY234417.

Bioinformatic analysis

The genomic structure of HCR2 gene was obtained from a 4689 270 bp sequence submission (GenBank Accession No. NW_043724.1) on chromosome 4q11, which includes the entire HCR2 gene sequence. The result suggested that HCR2 is a gene with single exon and mapped to rat chromosome 4q11. The predicted protein encoded by HCR2 gene contains 78 amino acids with a theoretical molecular weight of 8841.7 and isoelectric point of 8.59. This protein is probably a nuclear protein and no signal peptide and transmembrane sequence are predicted. It has one N-glycosylation site (amino acid positions 63–66, NGSE), four protein kinase C phosphorylation sites (amino acid positions 23–25, SPR; 29–31, SSR; 37–39, SAR; and 51–53, TSK) and two casein kinase II phosphorylation sites (amino acid positions 51–54, TSKD; 65–68, SEID). The sequence shared no significant homology with any known protein in the GenBank database.

Northern blot analysis

HCR2 mRNA expression in various rat tissues was investigated by Northern blot analysis using mRNA of each tissue. Results showed that, unlike the positive signal in aorta tissue (Figs. 2 and 3), transcript of HCR2 in rat heart, brain, spleen, lung, liver, skeletal muscle, kidney, and testis was undetectable (Fig. 5).

Discussion

To identify novel genes relevant for hypercoagulable pathophysiology, we used the high carbohydrate diet-induced hypercoagulable rats as model animals with which to pursue our study. So far two other methods to establish hypercoagulable animal model were reported. One is to induce hypercoagulable state by injecting animals with steroid hormones, such as dexamethasone and methylprednisolone [17]. Steroid hormones can decrease the fibrinolytic activity of reticuloendothelial cells. This resulted in hypercoagulable state and hypofibrinolysis. Another was by injecting animals with steroid hormones after administrating with *Escherichia coli* endotoxin [18,19]. Bacterial endotoxin can activate endothelial cells, macrophages, and platelets to express procoagulant molecules, leading to hypercoagulable state and possible thrombus formation. *E. coli* endotoxin and steroid hormones might induce ischemic necrosis in synergy. However, the states in both the animal mod-

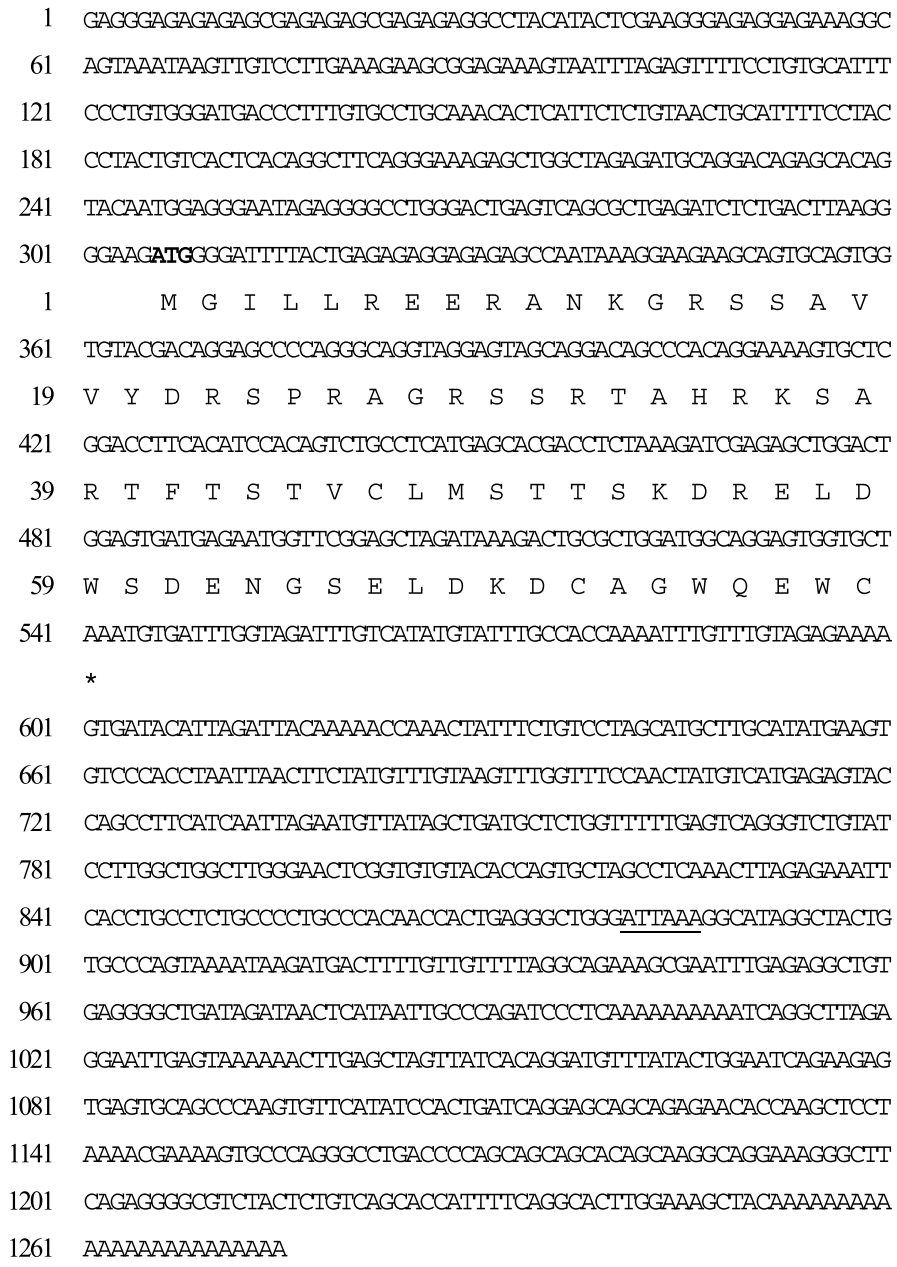

Fig. 4. Nucleotide sequence and deduced amino acid sequence of HCR2 gene. Polyadenylation signal is underlined; initiation codon is bolded; stop codon is indicated by asterisk (*).

els were not considered to be very relevant to human hypercoagulable state. Therefore, we used hypercoagulable animals induced by high carbohydrate diet (79.2% of total calories) developed in our laboratory for our study [20]. High carbohydrate diet could increase the level of blood glucose leading to the elevated level of plasma insulin in human and animals. As a result, insulin promotes the synthesis and secretion of TG-rich VLDL in liver and results in a state of endogenous hypertriglyceridemia (HTG). Many investigations have demonstrated that the elevated plasma triglyceride can activate the blood coagulation, inhibit the fibrinolytic activity and promote the aggregation of platelet [21–23]. Kjalke et al. [24] reported that plasma lipoproteins, particularly TG-rich VLDL enhanced tissue factor-independent factor VII activation. Li et al. [25] and Eriksson et al. [26] found that TG-rich VLDL increased the expression of plasminogen activator inhibitor-1 (PAI-1) gene via VLDL response element in the PAI-1 promoter in cultured human endothelial cells, resulting in impaired fibrinolysis. Tabengwa et al. [27] reported that hypertriglyceridemic VLDL downregulated tissue plasminogen activator gene transcription through *cis*-repressive regions in the t-PA promoter in cultured human

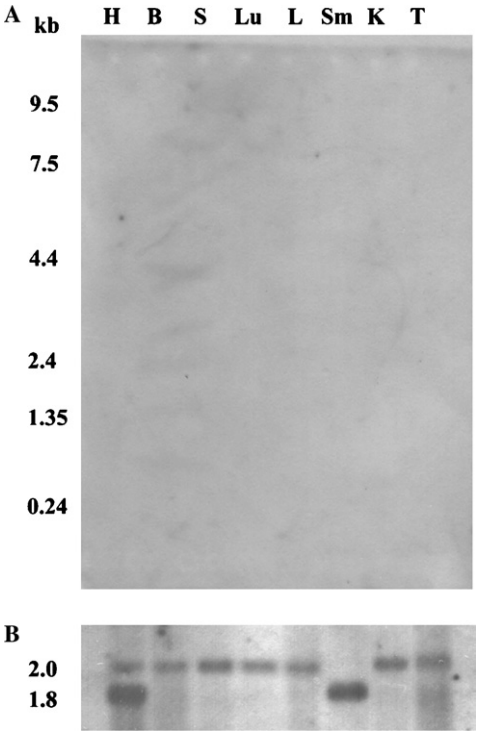

Fig. 5. Expression analysis of HCR2 mRNA transcription in various rat tissues by Northern blot. Expression of HCR2 gene (A) and β-actin control (B); H, heart; B, brain; S, spleen; Lu, lung; L, liver; Sm, skeletal muscle; K, kidney; and T, testis. Unlike the positive signal in the tissue isolated from the subtractive library of aorta on reverse dot-blot analysis, the transcript of HCR2 in these tissues was undetectable. In contract β-actin control in identical samples showed the significant signal.

endothelial cells. Our data showed that HTG could affect some markers of haemostasis, such as prothrombin time (PT), activated partial thromboplastin time (APTT), and plasminogen activator inhibitor-1 (PAI-1) (as shown in Table 2). A shortened PT and APTT could indicate an enhanced activity of extrinsic and intrinsic coagulation pathway, respectively. PAI-1 is a potent inhibitor of fibrinolytic pathway and its activity was increased in this study. The changes of these parameters indicated that the balance between the coagulation and the fibrinolytic system shifted to a prothrombotic state. High carbohydrate diet-induced hypercoagulable rats were model animals which were well compatible with the characteristics of human dietary regimen high in complex carbohydrates and low in fat (especially in oriental populations), therefore they represented a physiological state model for human HCS and were used as ideal animals for screening novel genes related to HCS.

It is known that a large number of the coagulation factors are synthesized in liver and most of the genes have been identified. Unlike liver, aorta tissues contain components of smooth muscle cells, macrophages, and endothelial cells, which can synthesize and secrete many active factors, such as thrombinmodulin (TM), antithrombin III (AT-III), tissue factor (TF), TF pathway inhibitor (TFPI), coagulation factor V, tPA, tPA receptor, thrombin activatable fibrinolysis inhibitor (TAFI), and PAI-1 [28]. This suggested that aorta is a particularly important site for the activity of coagulation and fibrinolysis. It is also an initial lesion site for atherosclerosis. Therefore, we chose aorta as the candidate tissue to search for the novel genes which might be in high level of differential expression in hypercoagulable state.

In the present study, we showed an isolation and identification of a novel gene, HCR2, in aorta tissue of hypercoagulable rats by a suppression subtractive hybridization technique. This gene encoded as a putative protein of 78 amino acids. Bioinformatic analysis revealed that HCR2 was encoded by a single exon and mapped to rat chromosome 4q11. The protein is characterized as having no signal peptide and transmembrane sequence, and most likely a nuclear protein. Amino acid sequence analysis showed that HCR2 protein has the Asn_{63}-Gly_{64}-Ser_{65} sequence, which is necessary for the glycosylation of Asn. Therefore, this is a potential target for protein glycosylation via N-glycosidic linkage. In addition, HCR2 protein contains six potential phosphorylation sites. Consequently, this molecule is a potential target for protein phosphorylation via protein kinase C and casein kinase II. Phosphorylation and dephosphorylation of proteins is the most common model of regulation and plays important roles in many physiological and pathological processes, such as cell growth and development, proliferation and differentiation, immune response, inflammatory reaction, and carcinomatous change. Once the protein kinase C is activated, the hydroxyl groups of Ser_{23}, Ser_{29}, Ser_{37}, and Ser_{51} could be phosphorylated. This suggests that HCR2 protein may play a role in the inositol triphosphate/diacylglycerol (IP_3/DAG) and Ca^{2+} dependent signal pathway (IP_3/DAG-Ca^{2+} signal pathway).

The multiple tissue Northern (MTN) blot membrane was available to detect tissue mRNA of rat heart, brain, spleen, lung, liver, skeletal muscle, kidney, and testis, not the mRNA of aorta. The results showed that, unlike the positive signal in the tissue isolated from the subtractive library of aorta on reverse dot-blot analysis (Fig. 2), the transcript of HCR2 in these tissues was undetectable. In addition, RT-PCR analysis has also shown that HCR2 is also over-expressed in aorta of hypercoagulable rats (Fig. 3). Therefore, we speculated that the expression of HCR2 is unique in rat aorta. The specific expression and significance of HCR2 in HCS requires further investigation.

Although the function of HCR2 in hypercoagulable state is still elusive, the identification of the gene will provide important clues for further cloning HCS-associated novel genes and be beneficial to the elucidation of the mechanism underlying the hypercoagulable state in diseases such as coronary heart disease and stroke.

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References

[1] Z.L. Liu, S.L. He, J.Z. Li, Diagnosis and Therapy of Thromboembolism, second ed., People Health Press, Beijing, 2000.
[2] E. Verdy, N. Berkane, A. Magdelaine, F. Soubrier, S. Uzan, Prevalence of factor V Leiden, hyperhomocysteinemia, prothrombin G20210A, and methylene tetrahydrofolate reductase C677T mutations in obstetrical complications, Ann. Biol. Clin. 57 (1999) 539–544.
[3] H. Weiler, V. Lindner, B. Kerlin, B.H. Isermann, S.B. Hendrickson, B.C. Cooley, D.A. Meh, M.W. Mosesson, N.W. Shworak, M.J. Post, E.M. Conway, L.H. Ulfman, U.H. von Andrian, J.I. Weitz, Characterization of a mouse model for thrombomodulin deficiency, Arterioscler. Thromb. Vasc. Biol. 21 (2001) 1531–1537.
[4] B. Zoller, F.P. Garcia de, A. Hillarp, B. Dahlback, Thrombophilia as a multigenic disease, Haematologica 84 (1999) 59–70.
[5] K. Yamamoto, K. Takeshita, T. Shimokawa, H. Yi, K. Isobe, D.J. Loskutoff, H. Saito, Plasminogen activator inhibitor-1 is a major stress-regulated gene: implications for stress-induced thrombosis in aged individuals, Proc. Natl. Acad. Sci. USA 99 (2002) 890–895.
[6] T.K. Makris, G.A. Stavroulakis, U.G. Dafni, A.E. Gialeraki, P.G. Krespi, A.N. Hatzizacharias, C.G. Tsoukala, J.S. Vythoulkas, M.K. Kyriakidis, ACE/DD genotype is associated with hemostasis balance disturbances reflecting hypercoagulability and endothelial dysfunction in patients with untreated hypertension, Am. Heart J. 140 (2000) 760–765.
[7] P. Liang, A.B. Pardee, Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction, Science 257 (1992) 967–971.
[8] N. Lisitsyn, N. Lisitsyn, M. Wigler, Cloning the differences between two complex genomes, Science 259 (1993) 946–951.
[9] M. Schena, D. Shalon, R.W. Davis, P.O. Brown, Quantitative monitoring of gene expression patterns with a complementary DNA microarray, Science 270 (1995) 467–470.
[10] V.E. Velculescu, L. Zhang, B. Vogelstein, K.W. Kinzler, Serial analysis of gene expression, Science 270 (1995) 484–487.
[11] L. Diatchenko, Y.F. Lau, A.P. Campbell, A. Chenchik, F. Moqadam, B. Huang, S. Lukyanov, K. Lukyanov, N. Gurskaya, E.D. Sverdlov, P.D. Siebert, Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries, Proc. Natl. Acad. Sci. USA 93 (1996) 6025–6030.
[12] S.Z. Guo, Z.Y. Zhang, T.J. Tong, Cloning and characterization of cellular senescence-associated genes in human fibroblasts by suppression subtractive hybridization, Exp. Cell Res. 298 (2004) 465–472.
[13] S.A. Fox, S. Loh, A.L. Thean, M.J. Garlepp, Identification of differentially expressed genes in murine mesothelioma cell lines of differing tumorigenicity using suppression subtractive hybridization, Biochim. Biophys. Acta 1688 (2004) 237–244.
[14] M.H. Qin, Z.H. Zeng, J. Zheng, P.K. Shah, S.M. Schwartz, L.D. Adams, B.G. Sharifi, Suppression subtractive hybridization identifies distinctive expression markers for coronary and internal mammary arteries, Arterioscler. Thromb. Vasc. Biol. 23 (2003) 425–433.
[15] T. Shen, H. Bai, B.W. Liu, Z.H. Zhang, Changes of blood coagulation and fibrinogen of experimental hypertriglyceridemia rats, Chin. J. Arterioscler. 10 (2002) 199–202.
[16] M. Uchijima, E. Raz, D.A. Carson, T. Nagata, Y. Koide, Identification of immunostimulatory DNA-induced genes by suppression subtractive hybridization, Biochem. Biophys. Res. Commun. 286 (2001) 688–691.
[17] L.C. Song, K.Z. Chen, J.Y. Zhu, Influence of coptis root on lipid peroxide, hyperlipemia and thrombosis in vitro, Shan Xi Chin. Med. 17 (1996) 137–138.
[18] W.L. Liu, W.T. Guo, W.Q. Li, S.Z. Wen, H.J. Huo, X.J. Yang, Y.Y. Liu, R.D. Wang, H.K. Sun, Q.L. Sun, Induction of animal model of ischemic necrosis in femoral head with steroids treatment, Acta Acad. Med. Nei Mongol 20 (1998) 71–73.
[19] M. Matsui, S. Saito, K. Ohzono, N. Sugano, M. Saito, K. Takaoka, K. Ono, Experimental steroid-induced osteonecrosis in adult rabbits with hypersensitivity vasculitis, Clin. Orthop. Relat. Res. 277 (1992) 61–72.
[20] L.H. Zhang, B.W. Liu, Z.F. Wu, T.H. Lan, Effects of high-carbohydrate diet on plasma insulin and cAMP metabolism in rat, Chin. Biochem. J. 2 (1986) 19–25.
[21] B.W. Liu, Plasma triglyceride and arteriosclerosis, Adv. Cardiovas. Dis. 20 (1999) 3–6.
[22] T. Shen, H. Bai, B.W. Liu, Y. Liu, Z.H. Zhang, Z.Y. Deng, Plasma haemostatic and fibrinolytic activities and their relationship to levels of serum lipids and apolipoproteins in endogenous hypertriglyceridemic patients, Sichuan Da Xue Xue Bao Yi Xue Ban 34 (2003) 405–408.
[23] H.C. Simpson, J.I. Mann, T.W. Meade, R. Chakrabarti, Y. Stirling, L. Woolf, Hypertriglyceridaemia and hypercoagulability, Lancet 1 (1983) 786–790.
[24] M, Kjalke, A. Silveira, A. Hamsten, U. Hedner, M. Ezban, Plasma lipoproteins enhance tissue factor-independent factor VII activation, Arterioscler. Thromb. Vasc. Biol. 20 (2000) 1835–1841.
[25] X.N. Li, H.E. Grenett, R.L. Benza, S. Demissie, S.L. Brown, E.M. Tabengwa, S.H. Gianturco, W.A. Bradley, G.M. Fless, F.M. Booyse, Genotype-specific transcriptional regulation of PAI-1 expression by hypertriglyceridemic VLDL and Lp (a) in cultured human endothelial cells, Arterioscler. Thromb. Vasc. Biol. 17 (1997) 3215–3223.
[26] P. Eriksson, L. Nilsson, F. Karpe, A. Hamsten, Very-low-density lipoprotein response element in the promoter region of the human plasminogen activator inhibitor-1 gene implicated in the impaired fibrinolysis of hypertriglyceridemia, Arterioscler. Thromb. Vasc. Biol. 18 (1998) 20–26.
[27] E.M. Tabengwa, R.L. Benza, H.E. Grenett, F.M. Booyse, Hypertriglyceridemic VLDL downregulates tissue plasminogen activator gene transcription through *cis*-repressive region(s) in the tissue plasminogen activator promoter in cultured human endothelial cells, Arterioscler. Thromb. Vasc. Biol. 20 (2000) 1675–1681.
[28] Y.H. Zhao, B.W. Liu, Mutation and unusual expression of clotting system-related genes and hypercoagulability, Sheng Li Ke Xue Jin Zhan 34 (2003) 324–328.