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Construction of a hepatic stellate cells subtracted cDNA library of differentially expressed genes in normal mice and mice with Schistosomiasis japonica*

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Abstract: To construct a hepatic stellate cells (HSCs) subtracted cDNA library to find differentially expressed genes in normal mice and mice infected with *Schistosoma japonicum* (*S. japonicum*). Suppression subtractive hybridization (SSH) was used. The cDNA fragments of normal mouse were compared to those of *schistosoma*-infected mice to find differentially expressed genes. Then differentially expressed cDNA fragments were directly inserted into T/A cloning vector to set up the subtractive library. Amplification of the library was carried out with transformation of DH5α. The amplified library contained more than 400 positive bacterial clones, which were then hybridized with forward and backward subtracted probes for differential screening. One hundred positive bacterial clones were randomly selected for sequencing and BLAST analysis. Finally, virtual Northern Blot confirmed such differential expression. The subtracted cDNA library of differentially expressed genes of HSCs was constructed successfully, the library is efficient and lays foundation for screening and cloning new and specific genes of schistosomiasis.

Key words: Schistosomiasis japonica, Suppression subtractive hybridization, Differential expression
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INTRODUCTION

Differentially expressed genes are often important in disease pathogenesis. A comparative study of the gene expression profile in different developmental stages of *Schistosoma mansoni* (*S. mansoni*) was initiated based on the expressed sequence tag (EST) approach. A total of 1401 ESTs were generated from seven different cDNA libraries constructed from four distinct stages of the parasite life cycle (Franco *et al*., 1997). Sorensen *et al*.(1999) reported that the absence/presence of the RsaI site in the NDI provides a useful marker for the delineation of cohorts of *S. japonicum*. Drew and Brindley (1995) isolated and characterized two female-specific sequences by representational difference analysis (RDA) indicating that this procedure should also find use in the definition of traits and sequences that differ among other groups of schistosomes. Reis *et al*.(1989) investigated the mechanisms involved in sex-specific gene expression in *S. mansoni*, and isolated a cDNA (fs800) that hybridized to an 800 nucleotide mRNA present in high levels only in mature female worms. Data shown above indicated that differentially expressed genes are involved in the process of pathogenesis, but further investigation of the mechanisms is required.

The construction of a differential expression library of Schistosomiasis japonica (S. japonica) in an animal model, isolation and identification of genes

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related to this disease is of great importance for characterizing pathogenesis. Liver fibrosis is an important pathological change due to S. japonica. Hepatic stellate cells (HSCs), also known as Ito cells, fat-storing cells, or lipocytes, seem to be the primary target cells for mitogenic and fibrogenic stimuli (Pinzani, 1995). HSCs are perisinusoidally located, stellate-shaped cells that contain a remarkable number of intermediate filament subtypes (Niki *et al*., 1999; Levy *et al*., 1999). During liver fibrogenesis HSCs proliferate, transform from the quiescent state into myofibroblast-like cells ("activated" HSC), a process characterized by the induction of alpha-smooth muscle actin (α-SMA) and a switch in the expression profile and synthesis of large amounts of connective tissue components (Friedman, 1993).

Identification of molecular markers for various diseases has become an important aim for diagnostics and disease therapy. Many of the currently available technologies such as microarrays, differential display and subtractive hybridization can be used for identification of disease-specific markers. However, proving statistical significance of data can be very challenging. In addition, it is often difficult to collect the required amount of RNA from small clinical samples. As a result, there is an increasing need for methods that amplify limiting amounts of RNA while maintaining the original RNA representation. A new and highly effective method, termed suppression subtractive hybridization (SSH) (Diatchenko *et al*., 1996; Von Stein *et al*., 1997), had been developed for the generation of subtracted cDNA libraries. SSH is a genome-wide approach that enriches differentially expressed mRNA transcripts. We aimed to make novel observations of differential gene expression in hepatic fibrosis in mice with S. japonica using SSH combined with virtual Northern Blot.

MATERIALS AND METHODS

Animal and animal models

Four weeks old male BABL/C mice (provided by Zhejiang University Animal Study Center) were challenged by cercariae of *S. japonicum* (25 cercariae per mouse). Simultaneously, mice from the same center were used as normal control in order to suppress background that could otherwise be induced by inter-individual genetic differences. The mice were maintained under 12 h light/dark cycles and allowed free access to food and water.

Isolation of mouse HSCs

Sixteen weeks after challenge, liver fibrosis was established as shown by histopathological examination. Primary HSCs were isolated from male BALB/C mice by the pronase-collagenase method followed by density centrifugation on a Nycodenz gradient as described previously (Cassiman *et al*., 2001). HSCs from mice with fibrosis will be referred to as "Tester" sample, and HSCs from control mice as "Driver" sample.

SMART cDNA synthesis

Total RNA was prepared using Trizol reagent (Gibco BRL, USA). RNA integrity was checked on 1% formaldehyde agarose gel. Poly(A)+RNA was then purified using PolyATract mRNA cDNA system kit (Promega, USA) as per manufacturer's instructions. mRNAs (0.5 μg) from each sample were reversely transcribed using MuLV SuperScript II reverse transcriptase (Gibco BRL, USA), SMART II oligonucleotides and CDS primers from SMART cDNA synthesis kit (Clontech, USA). The first strand cDNA mixtures were amplified by LD PCR. Before amplification, the number of PCR cycles was optimized (19 for Driver and 17 for Tester) to ensure that cDNA products remained in the exponential phase of amplification to reduce nonspecific amplification.

SSH

cDNAs produced were purified by passing through Chroma spin-400 columns (Clontech, USA). Each purified tester cDNA was digested with RsaI to give an average insert length approximately longer than that of 600 base pairs, then were phenol chloroform-extracted, ethanol-precipitated, and dissolved in 6 μl ddH_2O. The tester cDNAs were subdivided into two equal groups and then ligated to adaptors 1R and 2R in separate ligation reactions. Ligation efficiency was tested. Subtractive hybridization was performed by annealing an excess of driver cDNAs with each sample of adaptor-ligated tester cDNAs. The cDNAs were heat-denatured and incubated at 68 °C for 8 h. After the first hybridization, the two samples were mixed together and hybridized again with freshly

heat-denatured driver cDNAs for 20 h at 68 °C. Two rounds of hybridization generated a normalized population of tester-specific cDNAs with different adaptors on each end.

After filling in the ends, two rounds of PCR amplification were performed to enrich desired cDNAs containing both adaptors by exponential amplification of these products. The optimized cycles for the first and second PCR were 26 and 12 respectively to increase representation and reduce redundancy of subtracted cDNA libraries. Secondary PCR products were used as templates for PCR amplification of human G3PDH at 18, 23, 28 and 33 cycles to enssure subtraction efficiency. PCR products were run on 1.8% agarose gels.

Both forward and backward SSH were performed. The difference between them was that the tester sample in forward SSH was used as driver sample in backward SSH.

Cloning and differential screening of subtracted cDNA libraries

Products from the secondary PCR were T-A cloned into pGEM-T Easy Vector using pGEM-T Easy Vector System kit (Promega, USA). The ligation products were transformed into DH5α competent cells. The transformed cells were plated on 2-YT agar plates containing ampicillin, X-Gal and IPTG, which allowed for color selection of colonies. Transformation efficiency was calculated and white clones were selected for further characterization.

Randomly selected individual clones were grown for 8 h in 96-well culture plates and then used for clone PCR amplification. The primers used were nested Primers 1R (NP1R) and 2R (NP2R) of adaptors. After 27 cycles amplification, the PCR products were subjected to a differential screening process. Briefly, 3 μl of amplified products of each candidate gene was mixed with DNA dilution buffer (50 μg/ml herring sperm DNA, 10 mmol/L Tris·Cl, pH 8.0, 1 mmol/L EDTA), spotted onto duplicate Hybond N+ Membranes (Roch, Germany) and UV cross-linked for 1 min. The forward and backward subtracted cDNAs were used as probes. These cDNAs were DIG-labelled by PCR amplification using PCR DIG probe synthesis kit (Roch, Germany), digested with RsaI, EagI and SmalI to remove the adaptors and purified with column. After prehybridization, the duplicate membranes were hybridized with forward and backward subtracted cDNA libraries probes respectively at 42 °C for 16~20 h in hybridization solution with 50% deionized formamide, 0.1% (w/v)-Lauroylsarcosine, 2% blocking reagent, 0.02% (w/v) Sodium Dedecyl Sulfate (SDS), 2 μg/ml NP1R, NP2R and CDS primer sequences. The membranes were then washed for 2×5 min with 2×SSC, 0.1% SDS at room temperature and for 2×15 min in 0.5×SSC, 0.1% SDS at 68 °C. Then they were blocked by gentle agitation in blocking solution at 37 °C for 1 h, incubated in buffer I with Anti-DIG (1:4000) at 37 °C for 30 min, washed in buffer I for 2×15 min and equilibrated in buffer III for 5 min. Finally, the membranes were incubated in a mixture of NBT and BCIP for about 15 min. The clones giving at least 5-fold higher hybridization signals with the forward probes were selected for further analysis.

Sequencing and BLAST homology search

Candidate positive clones from the subtracted cDNA library were selected for sequencing. Cycle sequencing reactions were conducted with Thermo Sequenase cycle sequencing core kit (Amersham Life Science, USA). Each clone was sequenced in both directions. Each sequence of the isolated clones was used for homology searches in the public databases such as GenBank, SWISS-PROT, and PIR by Blastn program.

Virtual Northern blot

High yields of full-length cDNAs were generated from 1 μg total RNA of normal and fibrosis HSCs using SMART PCR cDNA synthesis kit (Clontech, USA). cDNAs (0.2 μg) were electrophosed on a 1.0% agarose gel, transferred onto Hybond N+ Membrane (Roch, Germany) and hybridized with DIG-labelled clone probes. These probes were generated by PCR amplification of the relevant inserts, adaptor-cutting and column-purification. Hybridization and washing conditions were the same as described above in screening of cDNA libraries. DIG-labelled G3PDH probes were used as control.

RESULTS

Subtracted cDNA library construction by SSH

In this research, SSH was done to identify dif-

ferentially expressed genes among cDNAs of HSC from normal and diseased mice. The subtracted cDNA library was constructed. Fig.1 shows the subtracted cDNA library after secondary PCR amplification which usually looked like smears with or without several discrete bands. However, the patterns among them were different.

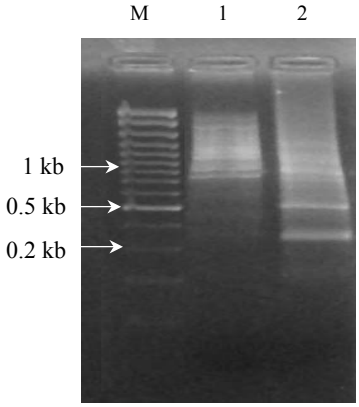

Fig.1 Analysis of differentially expressed cDNA with nest PCR
Lane 1 indicated unsubtracted sample; Lane 2 indicated subtracted sample; Lane M: 100 bp Marker

Subtraction efficiency analysis showed the effectively reduced abundance of non-differentially expressed genes. In nonsubtracted cDNA libraries, housekeeping gene G3PDH PCR products were visible after 18 cycles of amplification and became saturated after 23–28 cycles. However, subtracted libraries required a higher number of amplification cycles for G3PDH to be detected, suggesting that the library subtractions were complete (Fig.2).

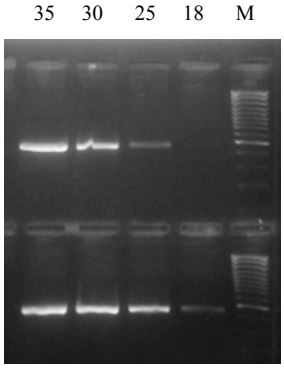

Fig.2 PCR analysis of subtraction efficiency
Lanes up indicated the subtracted sample was amplified by PCR with different cycles: 18, 25, 30, 35. Lanes down indicated the unsubtracted sample was amplified by PCR with different cycles: 18, 25, 30, 35. Lane M: 100 bp Marker

Differential screening of subtracted cDNA libraries

About 3×102 clones were respectively identified in subtracted cDNA library with average insert size of 0.2–1 kb (Fig.3). One-hundred randomly selected clones were screened by hybridization with both forward and backward subtracted cDNA library probes. Clones corresponding to truly differentially expressed genes would mainly hybridize with the forward subtracted cDNA probe, but not, or only faintly, with the backward subtracted cDNA probe, as shown by signal comparison between two blots.

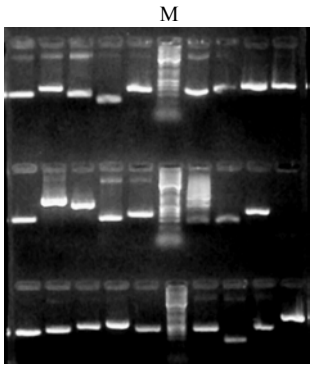

Fig.3 Clone PCR amplification with an average insert size of 0.2–1 kb. M: 100 bp Marker

Sequencing and homology search

Automated sequencing proved that 76 ESTs were obtained by sequencing in the substructed cDNA library, and that most clones were isolated no more than two times. BLASTN homology search revealed that 70 clones contained substantial sequence homologies with known gene fragments and 6 possibly novel genes, showing no significant homologies with any known sequences in the GenBank. The results of homology search are shown in Table 1. Clone name, insert size, sequence identity and redundancy are shown.

Virtual Northern Blot

The cDNAs of selected clones were mainly expressed in tester tissue but not, or to a lesser extent, in driver tissue. Virtual Northern Blot showed such differential expression in most clones. These results were also confirmed by RNA Dot blot.

DISCUSSION

The novel technique called SSH is an ideal sub-

Table 1 Characteristion of some randomly selected differentially expressed clones

Clone	Insert	Homologous gene	Score	*E*-value	Match	Change*
9-5	501 bp	Cytochrome b, mitochondrial	1191	0.0	99%	↓
2-12D	441 bp	Rps2 Ribosomal protein S2	809	0.0	98%	↓
49	449 bp	Anxa1 annexin A1	2288	1.0e–95	99%	↑
7-1	565 bp	Immunoglobulin kappa chain variable 28	938	0.0	96%	↑
10D	487 bp	Cathepsin K	908	0.0	96%	↑
2-4A	502 bp	Chitinase 3-like 1, (Chi3l1)	916	0.0	98%	↑
4C	530 bp	1810057I13Rik RIKEN cDNA gene	940	0.0	98%	↑
7A	405 bp	Myadm Myeloid-associated differentiation marker	664	0.0	93%	↑
9A	794 bp	Cathelin related antimicrobial peptide precursor	688	0.0	93%	↑
9D	718 bp	5' similar to gb: J00544 Mouse Ig active J chain, partial mRNA	874	0.0	96%	↑
12H	316 bp	Mus musculus Rho, GDP dissociation inhibitor (GDI) beta (Arhgdib)	615	e–173	99%	↑

*Change means gene expression level compared to normal control. ↑ means up-regulate; ↓ means down-regulate

tractive system that combines high subtraction efficiency with normalized representation of differentially expressed genes, and is based on suppression PCR that permits exponential amplification of genes differing in abundance, at the same time that amplification of sequences of identically abundant genes in two populations is suppressed. Therefore, differentially expressed genes of low abundance can be cloned, while differentially expressed genes of high abundance are not excessively isolated (Von Stein *et al*., 1997; Kuang *et al*., 1998). SSH is based primarily on a recently described technique called suppression PCR and combines normalization and subtraction in a single procedure (Diatchenko *et al*., 1996). The normalization step equalizes the abundance of cDNAs within the target population and the subtraction step excludes the common sequences between the target and driver populations. It was reported that SSH achieved more than 1000-fold enrichment of low-abundance genes but only required 2 μg mRNA instead of the 10–20 μg required in other methods. This method has been successfully used to isolate significant genes in many researches. These results suggest that the SSH technique is applicable to many molecular genetic and positional cloning studies for the identification of disease, developmental, tissue-specific, or other differentially expressed genes (Lee *et al*., 1996; Pitzer *et al*., 1999; Zhang *et al*., 2000). Combining elements of SSH with high throughput differential screening permits the efficient and rapid cloning of rarely transcribed differentially expressed genes (Von Stein *et al*., 1997). Here we report the efficiency of SSH technique in identifying differentially expressed genes in Schistosomiasis japonica-infected mice with liver fibrosis.

Seventy-six aligning ESTs were obtained by sequencing clones in the substructed cDNA library of schistosomiasis-infected mice. BLAST programme results showed 70 ESTs correlated with cell differentiation and protein binding to growth factors, suggesting that they are related to HSC activation and proliferation. Six ESTs were not found to be homologous to sequences in public databases, needing further investigation to determine their function. Although each clone may be involved in schistosomiasis liver fibrogenesis, selection of suitable clones to study first by bioinformatics is important. According to Choi's standard (Choi *et al*., 1995), ESTs may be classified into four groups: (1) previously known genes: BLASTN with >95% identity and >70 bp overlap length; (2) novel gene transcripts with significant homologies to known genes of the same organisms, with 60%–95% identity and >70 bp overlap length; (3) novel gene transcripts with significant homologies to known genes of other organisms, with >60% identity and >70 bp overlap length; (4) unknown genes: genes that have no statistically significant match to any known genes. Genes in groups (2) and (3) are often the first groups to be further studied.

1. Chitinase 3-like: In humans, the gene YKL-40, also known as human cartilage glycoprotein 39 (HC-gp39), is a member of a family 18 glycosylhydrolases. Its production by chondrocytes, synovial

cells, activated macrophages, neutrophils, and osteosarcoma cells (MG-63) has been reported (Hakala *et al*., 1993; Nyirkos and Golds 1990; Renkema *et al*., 1998; Volck *et al*., 1998; Johansen *et al*, 1992). Although the exact physiological function of YKL-40 is unknown at present, the pattern of its expression in normal and disease states suggests a role in inflammation, degradation and/or remodeling of the extracellular matrix (Cintin *et al*., 2001). Several papers reported that YKL-40 is increased in patient with alcoholic cirrhosis and patients with hepatitis C (Johansen *et al*., 2000; Nojgaard *et al*., 2003a; 2003b). Dose-dependent growth stimulation was observed when each of the fibroblastic cell lines was exposed to HC-gp39 in a concentration range from 0.1 to 2 nmol/L. HC-gp39 is involved in both the mitogen-activated protein kinase and the protein kinase B (AKT) signalling pathways mediating the mitosis. Thus HC-gp39 initiates a signalling cascade in connective-tissue cells which leads to increased cell proliferation, suggesting that this protein could play a major role in the pathological conditions leading to tissue fibrosis (Recklies *et al*., 2002). HSC is the target cell in liver fibrogenesis, platelet-derived growth factor (PDGF) is one of the most potent mitogens for cultured HSC isolated from rat, mouse, or human liver. PDGF-related mitogenesis and cell migration occur by pathways that are at least in part independent of ERK activation (Pinzani, 2002). So this gene may also be involved in HSC activation. Which cells express this gene in the liver is also unknown; the fact that we detected it in HSC substructed cDNA library may give a clue to its source in the liver.

2. Ribosomal protein S2 (RPS2): The ribosomal protein S2 (RPS2) is encoded by a gene from the highly conserved mammalian repetitive gene family LLRep3. It participates in aminoacyl-transfer RNA binding to the ribosome, potentially affecting the fidelity of mRNA translation. Kowalczyk *et al*.(2002) found that the levels of RPS2 protein and its corresponding mRNA were higher in mouse hepatocellular carcinoma, in mouse livers after one-third partial hepatectomy, and in serum-starved cultured hepatocytes following serum treatment. Their study shows that the increased expression of RPS2 correlates with increased cell proliferation. However, whether the altered expression of this protein reflects its involvement in cellular proliferation or represents associated phenomena is still a key question that needs to be explored. The decreased expression of RPS2 may provide negative regulation of HSC proliferation.

3. GDP dissociation inhibitor (GDI): GDI belongs to the Rho-GDIs family or GDP dissociation inhibitor for the Ras-related Rho family. GDI includes RhoGDI-1, D4-GDI/RhoGDI-2, and RhoGDI-3. D4-GDI is a GDP dissociation inhibitor for the Ras-related Rho family. It inhibits GDP dissociation from Rho protein, which is needed in active Ras-related Rho family GTPase. The Rho small G protein family members regulate various actin cytoskeleton-dependent cell functions. And the signaling pathways of the Rho family members regulated by Rho. GDIalpha play important roles in maintaining cell structure and physiological function (Aikawa *et al*., 2000). For example, D4-GDI is a substrate for the caspase-3, D4-GDI may be regulated during apoptosis through the caspase-3 mediated cleavage of GDI protein. D4-GDI cleavage promotes cytoskeletal changes during apoptosis (Kwon *et al*., 2002). Cytoskeletal reorganization plays an important role in the regulation of different cell functions, such as proliferation and migration. Since platelet-derived growth factor (PDGF) stimulates both proliferation and chemotaxis of HSC. PDGF-induced cytoskeletal reorganization in HSC is dependent on PKC and Rho, thus suggesting that these two pathways may play an important role in the response of liver to injury (Di Sario *et al*., 2002). So Rho signaling pathways are involved in the activation of HSC in vitro. Tada *et al*.(2001) reported that inhibitors of the Rho-ROCK pathway might be useful therapeutically in hepatic fibrosis.

4. Cathepsin K: Cathepsin K is a member of the papain superfamily of cysteine proteases and has been proposed to play a pivotal role in osteoclast-mediated bone resorption. Dodds *et al*.(2001) developed a sensitive cytochemical assay to localize and quantify osteoclast cathepsin K activity in sections of osteoclastoma and human bone. In tissue sections, osteoclasts that are distant from bone express high levels of cathepsin K messenger RNA (mRNA) and protein. However, the majority of the cathepsin K in these cells is in an inactive zymogen form, as assessed using both cytochemical assay and specific immu-

nostaining. In contrast, osteoclasts that are closer to bone contain high levels of immunoreactive mature cathepsin K that codistributes with enzyme activity in a polarized fashion toward the bone surface. Polarization of active enzyme was clearly evident in osteoclasts in the vicinity of bone. The osteoclasts apposed to the bone surface were almost exclusively expressing the mature form of cathepsin K. These cells showed intense enzyme activity, which was polarized at the ruffled border. These results suggest that the in vivo activation of cathepsin K occurs intracellularly, before secretion into the resorption lacunae and the onset of bone resorption.

Diaz *et al*.(2000) provided evidence for the association of cathepsin K with the granulomatous reaction. In the granulomatous response to the hydatid cyst, cathepsin K is expressed by epithelioid and giant multinucleated cells. It is proposed that, by analogy with bone resorption, cathepsin K is secreted by the host in an attempt to digest the persistent foreign body. Both processes, bone resorption and granulomatous reactions, therefore tackle persistent extracellular material (the bone matrix or the foreign body), and utilize specialized cells of the monocytic lineage (osteoclasts or epithelioid/giant cells) secreting cathepsin K as an effector. As we found it in the subtracted library of HSC, it suggested that cathepsin K is probably secreted by the cell in an attempt to digest the persistent foreign body and ECM during fibrogenesis.

We also detected MYADM (Myeloid-associated differentiation marker), a new marker gene for hematopoietic differentiation (Pettersson *et al*., 2000), whether it is involved in HSC differentiation is worth investigating.

Other researchers have reported on differentially expressed genes related to cirrhosis caused by HCV (Shackel *et al*., 2003), but this is the investigation by SSH of differentially expressed genes in liver fibrosis caused by *S. japonicum* SSH. This study provided a new method to identify genes related to schistosomiasis hepatic fibrosis. The constructed library also provides the basis for new diagnostics and therapy for this disease.

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