Differential gene expression analysis of \textit{in vitro} duck hepatitis B virus infected primary duck hepatocyte cultures

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Abstract

\textbf{Background:} The human hepatitis B virus (HBV), a member of the hepadnaviridae, causes acute or chronic hepatitis B, and hepatocellular carcinoma (HCC). The duck hepatitis B virus (DHBV) infection, a dependable and reproducible model for hepadnaviral studies, does not result in HCC unlike chronic HBV infection. Information on differential gene expression in DHBV infection might help to compare corresponding changes during HBV infection, and to delineate the reasons for this difference.

\textbf{Findings:} A subtractive hybridization cDNA library screening of \textit{in vitro} DHBV infected, cultured primary duck hepatocytes (PDH) identified cDNAs of 42 up-regulated and 36 down-regulated genes coding for proteins associated with signal transduction, cellular respiration, transcription, translation, ubiquitin/proteasome pathway, apoptosis, and membrane and cytoskeletal organization. Those coding for both novel as well as previously reported proteins in HBV/DHBV infection were present in the library. An inverse modulation of the cDNAs of ten proteins, reported to play role in human HCC, such as that of Y-box binding protein1, Platelet-activating factor acetylhydrolase isoform 1B, ribosomal protein L35a, Ferritin, \(\alpha\)-enolase, Acid \(\alpha\)-glucosidase and Caspase 3, copper-zinc superoxide dismutase (CuZnSOD), Filamin and Pyruvate dehydrogenase, was also observed in this \textit{in vitro} study.

\textbf{Conclusions:} The present study identified cDNAs of a number of genes that are differentially modulated in \textit{in vitro} DHBV infection of primary duck hepatocytes. Further correlation of this differential gene expression in \textit{in vivo} infection models would be valuable to understand the little known aspects of the hepadnavirus biology.

Introduction

The human hepatitis B virus (HBV) and the duck hepatitis B virus (DHBV), which are members of the same virus family, hepadnaviridae, share several features in common [1]. Unavailability of primary animal models susceptible to HBV infection, and inefficiency and unreliability of the infection process in \textit{in vitro} systems [2] are major limitations in HBV research which restrain the study of this major human pathogen. But the establishment of the animal model with domestic duck employing the DHBV has helped greatly to overcome the shortcomings in HBV research [1,3]. However, this model has its own limitations as revealed by the differences in the clinical manifestations of the disease in humans and birds infected by these viruses. This mainly pertains to the chronicity in DHBV infection without liver injury/hepatocellular carcinoma (HCC)/cirrhosis; spontaneous elimination of infection in adult ducks; and at the molecular level, the expression of only a cryptic X-protein [4]. A major lacuna in HBV biology is the lack of sufficient information on the molecular mechanisms involved in the development of HCC in chronic HBV patients, which has become a major medical challenge [5].

A few studies have been performed comparing the gene expression in HBV positive HCC and non-cancerous liver [6] and viral and non-viral HCC [7] in patient samples. However, no study has focused to identify the differential gene expression in infection with DHBV either \textit{in vivo} or \textit{in vitro} to facilitate a comparative analysis. A recent \textit{in vitro} study has addressed the proteomic changes during DHBV infection, which has brought to light a number of genes that are involved in the infection process [8]. However, a purely proteome based approach might not reveal
changes in the expression levels of many of the low abundant proteins due to technical limitations, which needs to be complemented by mRNA/cDNA differential expression based approaches. In this context, we carried out a subtractive hybridization cDNA library construction and screening to identify the differential gene expression during DHBV infection in primary duck hepatocytes (PDH) in culture. The protocol we followed identified 42 up-regulated and 36 down-regulated genes in DHBV infected PDH in culture.

Methods

Primary duck hepatocytes (PDH) were isolated from 27-day old embryonated, un-hatched, duck eggs free of duck hepatitis B virus (DHBV) infection as previously described [9] and maintained at 5 × 10^6 cells/ml in DMEM+F12 medium and this concentrated virus was used to infect PDH at an MOI of 10^3 genome equivalents per hepatocyte, as previously described [11] in presence of 1% DMSO (Sigma). DHBV infection was confirmed by PCR on the DNA obtained from the culture supernatant using DHBV specific primers P1F and P1R (Additional File 1, Table 1). DHBV stock was concentrated from LMH-D2 cell culture supernatant, a chicken hepatoma cell line that constitutively replicate DHBV, (a kind gift from Dr. William S Mason, Fox Chase Cancer Centre, California), by precipitation with 10% polyethylene glycol 8000 (USB, USA) [10]. The pellet was re-suspended in DMEM+F12 medium and this concentrated virus was used to infect PDH at an MOI of 10^3 genome equivalents per hepatocyte, as previously described [11] in presence of 1% DMSO (Sigma). DHBV infection was confirmed by PCR on the DNA obtained from the culture supernatant using DHBV specific primers P1F and P1R (Additional File 1, Table 1).

2 μg of polyA RNA each from DHBV infected and uninfected PDH on zero and 4th day of infection was isolated using PolyATract mRNA isolation system-III (Promega, USA) and was used to construct forward and reverse subtracted cDNA libraries using Clontech PCR-Select cDNA subtraction kit (Clontech, USA), as per kit protocols. PCR amplification of a house-keeping gene GAPDH (Additional File 1, Table 1) from subtracted and un-subtracted samples was used for confirmation of the subtraction efficiency. The subtracted cDNAs were ligated with the pGEM-T (Easy) vector (Promega), competent JM109 Escherichia coli cells (Promega) were transformed and plasmids were isolated following standard molecular biology protocols to obtain 137 forward and 148 reverse subtracted clones.

Macroarrays of these plasmids were generated by vacuum transferring 100 ng each of the denatured clone plasmid in duplicate spots onto nylon membranes (Hybond-N+, Amersham Biosciences UK) using a dot-blot apparatus (Bio-Dot, Bio-Rad). The arrays were hybridized with α-32 P labelled forward and reverse subtracted cDNA mixtures as radioactive probes in a reverse-northern procedure. The probes were radio-labelled in a 50 μl PCR reaction using [α-32 P]-dCTP, dATP, dGTP, dTTP (0.2 mM each) and unlabelled dCTP (0.02 mM) using the nested PCR primers 1 and 2R (10 μM each) (Additional File 1, Table 1) and the Advantage 2 polymerase mix (Clontech). The adaptor regions common to both the probe and library clones were removed by digestion with RsaI restriction enzyme (NEB). The arrays were individually hybridized with both forward and reverse radio-labelled probes. Subsequent to a pre-hybridization of the membrane for 30 min in the hybridization solution(10% Polyethylene glycol, 1.5x SSPE and 7% sodium dodecyl sulphate), heat denatured probe solution containing 100 μl of RsaI digested radio-labelled probe, 250 μl of 10 mg/ml Herring sperm DNA(Promega) and 100 μl of 0.2N NaOH was added. The probe solution was removed after 16 hrs of hybridization at 65°C and the membrane was washed twice in 2× SSC and 0.1%SDS for 10 min at room temperature followed by two high stringency washes using 0.2× SSC and 0.1%SDS at 65°C for 10 min, and exposure to a phosphor screen for 30 min. The images were captured in Molecular Imager FX (Bio-Rad). The hybridization intensity was measured in the captured images by densitometry analysis of the signal on individual clones using VisionWorksLS image acquisition and analysis software (UVP, USA). The relative abundance ratio of gene expression was calculated using the following formulas.

Abundance Ratio (Up − regulated clones) = Signal Intensity when hybridized with reverse subtracted library probe − Signal Intensity when hybridized with forward subtracted library probe

Abundance Ratio (Down − regulated clones) = Signal Intensity when hybridized with forward subtracted library probe − Signal Intensity when hybridized with reverse subtracted library probe

All genes with an abundance ratio of more than one, a cut-off fixed arbitrarily, were then short-listed as the ones with true differential expression. These clones were subjected to automated DNA sequencing in an ABI Prism 310 sequencer (Applied Biosystems) with the Big Dye Terminator 3.0 kit (ABI Prism; Applied Biosystems) as per the manufacturer’s directions using the primers TvecF and TvecR (Additional File 1, Table 1). The sequences thus obtained were analyzed using the BLAST online software (NCBI).

Three genes, randomly selected from the top five genes in Table 1 and 2 (with high abundance); one gene from the bottom (with lower abundance) of the table; and one gene, which was not short-listed, were used for real-time PCR analysis for validation of the short-listing procedure. Specific primers for these 10 genes (five from each of the up-regulated and down-regulated library) and primers for the house keeping gene GAPDH were designed (Additional File 1, Table 1) and used in the real-time PCR. cDNA was synthesized using
| No. | Name of the clone | Abundance Ratio | BLAST Result | Amplicon Size (bp) | e-value | GenBank Accession No. |
|-----|-------------------|-----------------|--------------|--------------------|---------|----------------------|
| 1   | F22               | 2.41            | BLAST        | 462                | 0       | JG662697             |
| 2   | F125              | 2.23            | BLAST        | 268                | 2.00E-85| JG662698             |
| 3   | F106              | 2.08            | tBLASTx      | 371                | 3.00E-04| JG662699             |
| 4   | F8                | 2.04            | tBLASTx      | 678                | 4.00E-07| JG662700             |
| 5   | F71               | 2               | BLAST        | 593                | 0       | JG662701             |
| 6   | F21               | 1.87            | BLAST        | 681                | 0       | JG662702             |
| 7   | F13               | 1.77            | BLAST        | 767                | 0       | JG662703             |
| 8   | F76               | 1.76            | tBLASTx      | 218                | 1.00E-14| JG662704             |
| 9   | F19               | 1.58            | BLAST        | 680                | 0       | JG662705             |
| 10  | F70               | 1.55            | BLAST        | 544                | 1.00E-150| JG662706        |
| 11  | F46               | 1.51            | BLAST        | 655                | 1.00E-140| JG662707       |
| 12  | F77               | 1.45            | tBLASTx      | 527                | 5.00E-07| JG662708             |
| 13  | F131              | 1.44            | BLAST        | 448                | 0       | JG662709             |
| 14  | F45               | 1.42            | BLAST        | 755                | 0       | JG662710             |
| 15  | F74               | 1.42            | BLAST        | 209                | 2.00E-49| JG662711             |
| 16  | F6                | 1.4             | BLAST        | 541                | 0       | JG662712             |
| 17  | F16               | 1.35            | BLAST        | 346                | 8.00E-31| JG662713             |
| 18  | F43               | 1.33            | tBLASTx      | 680                | 9.00E-143| JG662714           |
| 19  | F26               | 1.32            | tBLASTx      | 308                | 0.002   | JG662715             |
| 20  | F42               | 1.31            | BLAST        | 748                | 0       | JG662716             |
| 21  | F127              | 1.31            | tBLASTx      | 234                | 5.00E-16| JG662717             |
| 22  | F17               | 1.29            | BLAST        | 678                | 0       | JG662718             |
| 23  | F44               | 1.29            | tBLASTx      | 721                | 5.00E-21| JG662719             |

Table 1 List of cDNAs up-regulated during PDH infection with DHBV

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total RNA from fresh sets of primary duck hepatocyte cultures either infected with DHBV or uninfected, as described above, using Avian Myeloblastosis Virus (AMV) reverse transcription system (Promega). Real-time PCR was carried out as previously described [12]. The experiments were repeated thrice, each in duplicates, and average fold change in gene expression was calculated for individual genes.

The threshold cycle (Ct) values obtained in the real-time PCR analysis were normalized with the expression of the house-keeping gene GAPDH, and the relative expression of individual genes in infected and uninfected cells were calculated by Pfaffl method [13] for Day 0 and Day 4 of infection using the equation:

\[
\text{Ratio} = \frac{(E_{\text{target}})^{\Delta C_{T,\text{target}}(\text{calibrator-test})}}{(E_{\text{ref}})^{\Delta C_{T,\text{ref}}(\text{calibrator-test})}}
\]

The ratios for day 0 and day 4 infected samples were compared and analysed statistically by paired Student’s t-test to validate the significance of gene expression changes. P-values < 0.05 were considered significant.

|   |   |   |   |   |   |   |
|---|---|---|---|---|---|---|
| 24 | F135 | 1.27 | Leucine-rich repeats and calponin homology (CH) domain containing 4-Orctolagus cuniculus | tBLASTx | 288 | 0.4 | JG662720 |
| 25 | F14 | 1.24 | Clathrin, light chain A (CLTA)-Gallus gallus | BLAST | 673 | 0 | JG662721 |
| 26 | F83 | 1.2 | Sequestosoma 1-Gallus gallus | BLAST | 562 | 0 | JG662722 |
| 27 | F10 | 1.18 | RAB 32, member of Ras oncogene-Gallus gallus | BLAST | 743 | 0 | JG662723 |
| 28 | F30 | 1.16 | Ribosomal protein L6 (RPL6)-Gallus gallus | BLAST | 591 | 0 | JG662724 |
| 29 | F64 | 1.16 | Holocytochrome c synthase (cytochrome c heme-lyase)-Gallus gallus | tBLASTx | 421 | 8.00E-52 | JG662725 |
| 30 | F32 | 1.16 | Lysosomal-associated membrane protein 1-Taeniopygia guttata | tBLASTx | 591 | 4.00E-93 | JG662726 |
| 31 | F7 | 1.14 | Serine protease 23-Gallus gallus | BLAST | 740 | 3.00E-168 | JG662727 |
| 32 | F18 | 1.09 | Beta-catenin isolate 3-Anas platyrhynchos | BLAST | 710 | 0 | JG662728 |
| 33 | F52 | 1.09 | Zebratfish DNA sequence from clone CH211-276C22 in linkage group 6 | tBLASTx | 218 | 2.2 | JG662729 |
| 34 | F25 | 1.08 | Gallus gallus finished cDNA, clone ChEST457d18 | tBLASTx | 696 | 2.00E-27 | JG662730 |
| 35 | F59 | 1.07 | Leucine proline-enriched proteoglycan (leprecan)1/1,prolyl 3-hydroxylase 1 (P3H1)-Gallus gallus | BLAST | 581 | 0 | JG662731 |
| 36 | F12 | 1.07 | Ribophorin 1-Gallus gallus | BLAST | 796 | 0 | JG662732 |
| 37 | F87 | 1.06 | Gallus gallus finished cDNA, clone ChEST855m19 | BLAST | 505 | 3.00E-91 | JG662733 |
| 38 | F95 | 1.06 | Spastic paraplegia 3A (autosomal dominant)-Gallus gallus | BLAST | 316 | 3.00E-54 | JG662734 |
| 39 | F107 | 1.06 | High-mobility group box 3-Taeniopygia guttata | BLAST | 276 | 2.00E-136 | JG662735 |
| 40 | F1 | 1.05 | ATP synthase, H+-transporting, mitochondrial Fo complex, subunit F2 (ATP52)-Gallus gallus | tBLASTx | 199 | 9.00E-18 | JG662736 |
| 41 | F78 | 1.03 | Ubiquitin specific peptidase 47 (USP47)-Gallus gallus | BLAST | 695 | 0 | JG662737 |
| 42 | F88 | 1.03 | No significant similarity found | tBLASTx | 360 | - | JG662738 |
| No. | Name of the clone | Abundance Ratio | BLAST Result | tBLASTx Result | Amplicon Size (bp) | e-value | GenBank Accession No. |
|-----|------------------|-----------------|--------------|----------------|-------------------|---------|----------------------|
| 1   | R73              | 1.52            | BLAST        |                | 342               | 4.00E-143 | JG662661             |
| 2   | R90              | 1.41            | tBLASTx      |                | 328               | 1.00E-14  | JG662662             |
| 3   | R130             | 1.39            | BLAST        |                | 400               | 6.00E-172 | JG662663             |
| 4   | R108             | 1.39            | BLAST        |                | 486               | 0        | JG662664             |
| 5   | R96              | 1.34            | BLAST        |                | 646               | 1.00E-17  | JG662665             |
| 6   | R97              | 1.33            | BLAST        |                | 743               | 0        | JG662666             |
| 7   | R111             | 1.31            | BLAST        |                | 646               | 0        | JG662667             |
| 8   | R134             | 1.3             | tBLASTx      |                | 490               | -        | JG662668             |
| 9   | R123             | 1.25            | BLAST        |                | 462               | 0        | JG662669             |
| 10  | R133             | 1.24            | BLAST        |                | 257               | 3.00E-98  | JG662670             |
| 11  | R103             | 1.22            | BLAST        |                | 508               | 4.00E-14  | JG662671             |
| 12  | R16              | 1.18            | BLAST        |                | 593               | 0        | JG662672             |
| 13  | R100             | 1.17            | BLAST        |                | 631               | 0        | JG662673             |
| 14  | R84              | 1.16            | tBLASTx      |                | 756               | 3.8      | JG662674             |
| 15  | R15              | 1.15            | BLAST        |                | 438               | 5.00E-168 | JG662675             |
| 16  | R126             | 1.15            | tBLASTx      |                | 395               | 1.00E-25  | JG662676             |
| 17  | R135             | 1.14            | tBLASTx      |                | 239               | 0.048    | JG662677             |
| 18  | R143             | 1.13            | BLAST        |                | 546               | 0        | JG662678             |
| 19  | R45              | 1.13            | tBLASTx      |                | 476               | 1.00E-145 | JG662679             |
| 20  | R141             | 1.11            | BLAST        |                | 336               | 1.00E-152 | JG662680             |
| 21  | R10              | 1.11            | BLAST        |                | 735               | 0        | JG662681             |
| 22  | R129             | 1.11            | BLAST        |                | 664               | 0        | JG662682             |
| 23  | R106             | 1.1             | BLAST        |                | 381               | 0        | JG662683             |
| 24  | R93              | 1.1             | tBLASTx      |                | 488               | -        | JG662684             |
| 25  | R139             | 1.1             | BLAST        |                | 279               | 7.00E-100 | JG662685             |
Results & Discussion

The infection of PDH with DHBV did not produce any visible changes on the cell monolayer (Figure 1A). The virus infection was confirmed by PCR detection of a 300 bp DHBV glycoprotein 1 (gp1) gene fragment in the DNA isolated from infected PDH culture supernatant (Figure 1B) and by sequence analysis. The establishment of a productive infection was indicated by the increasing PCR amplification intensity of the gene fragment with every successive day of culture for the total culture period of eight days. For RNA isolation for subtraction library construction, we selected an early time point of 4 days as described in previous studies [14]. Two libraries were generated- the forward subtracted or up-regulated genes and the reverse subtracted or down-regulated genes. The efficiency of subtraction procedure was indicated by a decrease in the intensity and appearance of discrete banding patterns in the lanes with subtracted products (Figure 1C) and was confirmed by PCR detection of the house-keeping gene GAPDH, the amplicons of which appeared at an earlier time point (25 cycles) in un-subtracted samples compared to a later time point (30 cycles) in both forward and reverse subtracted libraries (Figure 1D). Hybridization of macroarrays blotted with 137 up-regulated and 148 down-regulated clones (Figure 1E) and short-listing only the ones with an abundance ratio of more than 1, we obtained 42 non-redundant up-regulated clones and 36 non-redundant down-regulated clones (Tables 1 and 2). Real-time PCR done using the representative sets of short-listed clones gave results confirming the reliability of the short-listing procedure. Genes that topped the differential expression among the up-regulated genes (F22, F8, F71) showed a significant (P < 0.05) increase at 4-day compared to the 0 day in infected PDH (Figure 2A), while the reverse was the case of the down-regulated genes (R73, R90, R130) (Figure 2B), all of whose expression decreased significantly (P < 0.05) at 4-day DHBV infection. F88 and R86, which were selected from the bottom end of the up-regulated and down-regulated gene-tables, respectively, also showed the expected modulation albeit at a lower fold. F62 and R47, picked from the genes left-out did not show any significant difference in their expression pattern.

Functional classification of the short-listed clones using gene ontology based on BLAST results grouped them mainly into those belonging to cellular processes such as cellular respiration, signal transduction, transcription/translation, ubiquitin/proteasome pathway and apoptosis besides those coding for membrane and cytoskeletal proteins (Table 3). Among them, the category that was maximum up-regulated were the ones involved...
Figure 1 Subtractive hybridization cDNA library construction and screening

(A) PDH infected with DHBV, 8 days post-infection. (B) PCR Confirmation of DHBV infection. Upper lane shows the increase in amplification of a DHBV specific gene from days 1 through 8, while the amplification is missing from uninfected controls. (C) Comparison of subtracted and unsubtracted cDNAs on a 2% agarose gel. Individual lanes are marked. Lane 5 is a 100 bp DNA ladder. Lane 8 is a positive control provided with the kit. (D) Analysis of subtraction efficiency using PCR for GAPDH. (E) Macroarray screening by dot-blot hybridization. Each clone is spotted in duplicates. Membranes were hybridized with radio-labelled probes as indicated. The average densitometric intensities of each duplicate clone pair was read for relative abundance calculation.
Figure 2 Real-time PCR of representative genes in DHBV infected PDH, 0-day and 4-days post-infection. (A) Significant up-regulation of cDNAs (F22, F8, and F71) selected from the top of short-listed clones in the up-regulated gene table (Table-1). (B) Significant down-regulation of cDNAs (R73, R90, and R130) selected from the top of short-listed clones in the down-regulated gene table (Table-2). The Y-axis represents relative gene expression values obtained from the Pfaffl analysis (see Methods). Significant P-values (< 0.05) are indicated. The values in parenthesis indicate fold-change in expression.
| Category                        | FORWARD                                                                                                           | REVERSE                                                                                       |
|--------------------------------|------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------|
| **Membrane proteins**          | Cadherin 11                                                                                                      | Transmembrane protein 30A                                                                    |
|                                | Lysosomal-associated membrane protein 1                                                                         |                                               |
|                                | CD9 protein                                                                                                      |                                               |
|                                | Leucine-rich repeats and calponin homology (CH) domain containing 4                                             |                                               |
| **Cellular Respiration**       | Pyruvate dehydrogenase E1-beta subunit                                                                       | Alpha enolase                                                                                 |
|                                | Succinate-CoA ligase, GDP-forming, alpha subunit(SUCLG1)                                                        | Hydroxacyl glutathione hydrolase-like                                                        |
|                                | ATP synthase, H+ transporting, mitochondrial F0 complex, subunit F2(ATPSJ2)                                       | ATPase8, ATPase6 genes for F0-ATP synthase subunit 8, F0-ATP synthase subunit 6               |
|                                | Cytochrome oxidase subunit 1 (COI)                                                                             |                                               |
| **Cytoskeletal**               | Filamin B, beta                                                                                                  | Beta-actin                                                                                  |
|                                | T-complex 1                                                                                                      |                                               |
|                                | Microtubule-associated protein RP/EB family, member 1                                                           |                                               |
|                                | Similar to AnkyrinB                                                   |                                               |
| **Signal Transduction**        | Beta-catenin isolate 3                                                                                           | TRAF interacting protein (TRAIIP)                                                             |
|                                | Quaking homolog, KH4 domain                                                                                       |                                               |
| **Transcription and Translation** | High mobility group AT-hook 2 (HMGA2)                                                                           | Splicing factor, arginine/serine-rich 18 (SFRS18)                                              |
|                                | High-mobility group box 3                                                                                         | MYST/Esa1-associated factor 6                                                                 |
|                                | Heat shock transcription factor 2 (H5F2)                                                                         | Y box binding protein 1                                                                       |
|                                | CWC22 spliceosome-associated protein homolog                                                                   | Ribosomal protein L35a                                                                       |
|                                | Ubiquitin-like, containing PHD and RING finger domains, 1 (UHRF1)                                               | Eukaryotic translation initiation factor 5 (EIF5)                                              |
|                                | Nuclear protein Matrin 3 (MATR3)                                                                                  |                                               |
|                                | Ribosomal protein L6 (RPL6)                                                                                      |                                               |
|                                | Ribophorin 1                                                                                                     |                                               |
| **Ubiquitin-proteasome**       | Ubiquitin specific peptidase 47 (USP47)                                                                          | Proteasome (prosome, macropain) 26S subunit, ATPase1 (PSMC1)                                 |
|                                | Sequestosome 1                                                                                                   |                                               |
| **Apoptosis**                  | Tumor necrosis factor receptor superfamily, member 6b, decoy (TNFRSF6B)                                       | Caspase 3, apoptosis-related cysteine peptidase (CASP3)                                       |
| **Others**                     | Anas platyrhynchos female-specific sequence                                                                      | No significant similarity found                                                              |
|                                | Junco hyemalis 164 gene, partial sequence                                                                       | UPF0308 protein                                                                              |
|                                | Gallus gallus finished cDNA, clone CHEST4573d18                                                                 | No significant similarity found                                                              |
|                                | No significant similarity found                                                                                   | Gallus gallus finished cDNA, clone CHEST7573h13                                              |
|                                | Gallus gallus finished cDNA, clone CHEST855m19                                                                   | Gallus gallus finished cDNA, clone CHEST1915                                                |
|                                | Zebrafish DNA sequence from clone CH211-276C22 in linkage group 6                                              | Gallus gallus BAC clone CH261-189F16 from chromosome z                                       |
|                                | Similar to SH3 domain containing 19                                                                              | Gallus gallus hypothetical protein                                                             |
|                                | Zinc finger CCCH-type containing 13 (ZC3H13)                                                                     | Gallus gallus similar to MGC53471 protein                                                     |
|                                | E1aC homolog 2 (E. coli) (ELAC2)                                                                                 | Ferritin, heavy polypeptide 1 (FTH1)                                                         |
|                                | Similar to KIAA2019 protein/AHNAK nucleoprotein 2                                                               | Zinc finger CCCH-type, antiviral 1 (ZC3HAV1)                                                 |
|                                | CLE7                                                               | Similar to RGD-CAP                                                                            |
|                                | Cu/Zn superoxide dismutase (SOD1)                                                                               | Versican                                                                                     |
|                                | Exonuclease NEF-sp                                                                                               | Platelet-activating factor acetylhydrolase isoenform 1b, alpha subunit 49kDa (PAFAH1B1)      |
|                                | Component of oligomeric golgi complex 3 (COG3)                                                                    | Catechol-O-methyltransferase                                                                 |
|                                | Clathrin, light chain A (CLTA)                                                                                   | Chromosome 15 hypothetical ATG/GTP binding protein                                            |
|                                | RAB 32, member of Ras oncogene                                                                                    | Ankyrin repeat domain 17 (ANKRD17)                                                          |
|                                | Holocytchrome c synthase (cytochrome c heme-lysase)                                                             | Similar to KIAA1824 protein/WD repeat domain                                                 |
|                                | Serine protease 23                                                                                               | Ral guanine nucleotide dissociation stimulator-like 1 (RGL1)                                 |
|                                | Leucine proline-enriched proteoglycan (leprecan)/prolyl 3-hydroxylase 1 (P3H1)                                  | Acid alpha-glucosidase                                                                       |
|                                | Spastic paraplegia 3A (autosomal dominant)                                                                       |                                               |
|                                | Alanine-glyoxylate aminotransferase 2                                                                           |                                               |
in transcription/translation (19%), whereas the ones maximum down regulated (11%) belonged to cytoskeletal proteins. The former included the HMG Box proteins and Y-box binding proteins. Previous studies have implicated the Y-box binding protein1, Platelet-activating factor acetylhydrolase isofrom 1B (PAFAH1B1), Ribosomal Protein L35a, Ferritin, α-enolase, Caspase 3, CuZn Superoxide Dismutase (CuZnSOD), Filamin B, Pyruvate dehydrogenase 1-β, β-catenin, prolyl-3-hydroxylase 1, β-actin, acid α-glucosidase, and clathrin, the cDNAs of which were identified to be up-regulated, with chronic HBV infections and HCC development [6,15-26]. In comparison to the earlier report based on proteome analysis in DHBV infected PDH [8], except for β-actin and α-enolase, all the cDNAs identified in the present study represented new genes. The difference could be due to multiple reasons, and importantly it might include the selective enrichment/removal of some of the cDNAs during the process of RT-PCR amplification and cloning as part of the subtraction library construction. Nevertheless, our data provides a new set of candidate genes worthy further investigation in hepabnaviral infection.

An interesting observation in this study was the inverse pattern of differential expression of ten of these genes in in vitro DHBV infected cells as against the reports on HCC clinical samples [6,15-20]. The mRNAs for the Y-box binding protein 1, PAFAH1B1, Ribosomal Protein L35a, Ferritin, α-enolase, acid alpha-glucosidase and Caspase 3 were shown to be down-regulated during in vitro DHBV infection, whereas those of CuZnSOD, Filamin B and Pyruvate dehydrogenase were shown to be up-regulated, where as the reverse was the trend in human HCC. This observation may be purely coincidental owing to the fact that the experimental method we used was an in vitro system, and the changes in primary hepatocytes during culture itself, such as de-differentiation, might have led to these alterations in gene expression.

Conclusions
In summary, the present study identified cDNAs of a number of genes that are differentially modulated in cultured PDH, invito infected with DHBV. cDNAs of both novel as well as already reported genes/proteins associated with HBV/DHBV infection or HCC were identified in the library. The genes short-listed here could be valuable leads for further studies in animal models, which might help to understand the pathology of chronic HBV infections and pathogenesis of HCC.

Additional material

Additional file 1: Primers used in the study

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Authors’ contributions
SN, DSA and AI carried out the experiments. SN drafted the manuscript. ES conceived the study, edited and completed the final version of the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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