Thus, it is very difficult to understand accurate HBV life cycle and we only test HBV amplification mechanism by transfecting cloned HBV genome into hepatocellular-originated cell lines such as HepG2, HuH6, HuH7 and so on. The limited transfection efficiency to these cell lines seems not to be appropriate how HBV affects the cellular gene expression profile. And there have been only two reports about cellular gene expression profiles under HBV producing and infecting models [2, 7].

In this report, we analyzed cellular gene expression profiles comparing HBV producing cells to its parental HBV non-producing cells and recognized that considerable numbers of gene expression level was altered by HBV amplification. We also picked up several characteristic gene expression level involved in cell cycle and interferon system.

MATERIALS AND METHODS

CELL. HuH6 and HB611 cells were grown in DMEM (low glucose; 1.0g/L) (Nakalai tesque®) supplemented with 10% fetal bovine serum, 10IU/ml penicillin G and 10μg/ml streptomycin (Nakalai tesque®). In case of HB611 culture, G418 (Nakalai tesque®) was added in the media at 0.5mg/ml.

RNA PURIFICATION. HuH6 and HB611 cells were grown on the 10 cm cell culture dish (Iwaki®) at almost confluent condition whose cell number was about 2~3 x 10^6/dish. The cells were washed with phosphate buffered saline (PBS) twice and then lysed directly in 3ml Trizol® (Invtrogen-Life Technologies). Each lysed solution was transferred into a 15ml centrifugation tube (BD™), respectively and total RNA was purified according to the manufacturer's protocol. The RNA was finally solved in TE solution (10mM Tris-HCl pH7.6, 1mM EDTA) and the concentration was measured with a UV Spectrophotometer (DU800™, Beckman)

DNA MICROARRAY ANALYSES. Each total RNA (250ng) was labeled with GeneChip® 3'IVT Express Kit aRNA amplification prodedure according to the manufacturer's protocol (GeneChip® 3'IVT Kit User Manual, P/N 702646 Rev.1; Chapter 2 aRNA Amplification Protocol and Chapter 3 Evaluation and Fragmentation of aRNA). The probe was hybridized on a DNA microarray, Human Genome U133 Plus2.0® (Affymetrix™)

with a hybridization oven (Hybridization Oven 640 110V®; Affymetrix 800138) and washed with Fluidics Station 450® (Affymetrix 00-0079). The microarray was scanned with GeneChip Scanner 3000® (Affymetrix 00-0074) and analyzed with GeneChip Operating Software ver1.4® (Affymetrix 690036). The analysis protocol was GeneChip Expression Analysis Data Fundamentals® (Chapter 4 First-Order Data Analysis and Data Quality Assesment and Chapter 5 Statistical Alogorithms Reference) with GeneChip Operating Software ver1.4®.

RESUTS

HB611. This cell was established by transfecting three tandemly arranged HBV genomes (adr4) in a plasmid containing the G418 resistance gene into HuH6 cells (Fig. 1) [19, 21]. HB611 cells maintains one copy of three tandemly arranged HBV genomes and is supposed to reflect all pathways of the HBV life cycle except the attachment and the entry process and the covalently closed circular DNA (cccDNA) formation (Fig.1. and Fig.2). The integrated HBV genome functions as cccDNA and produces HBV-related transcripts. The difference between HB611 and HuH6 cells is that the HBV genome is integrated into a host chromosome with transcription competence and there is no other system affecting cell physiology such as an immune system and therefore it could be possible to compare gene expression profiles purely by HBV production.

Global differences of gene expression profiles between HB611 and HuH6. This time, we utilized the Affymetrix Human Genome U133 Plus2.0™. This microarray loaded more than 50,000 probes. The scatter plot shows that 95% genes are expressed at the same level within eight folds difference (Fig. 3A, and 3B). Ten to twenty genes are drastically changed for their expression (arrows in the Fig. 3, Table 1, Table 2). Typical ones were picked up and drawn on the graph. Interestingly, some genes such as steroid sulfatace (microsomal) isozyme S, microsomal triglyceride transfere protein and fatty acid binding protein involved in fatty acids metabolism were highly expressed in HB611. And cell-growth related genes such as *CD24* (gi; 180167), platelet-derived growth factor receptor α (*PDGFRα*) and *myc* (*N-myc* related) were also highly expressed in HB611 (Table 2., Fig. 7).

Not a few genes seem to be changed by HBV production for their expression. 1014 genes (~2.0%)

were highly expressed in the HB611 cells more than eight times and 2063 genes (~4%) were highly expressed in the HuH6 cells more than eight times. These data suggest that HBV production should affect cellular gene expression program.

Cell cycle control genes. It is interesting how HBV production affects cell cycle since HBV is a major cause of liver cancer in the world. We mined the data related to cell cycle control; cyclins, cyclin dependent kinases (*CDKs*) and cyclin dependent kinase inhibitors (*CKIs*). In these cells, cyclin D1(*CYCD1*, gi; 12652656), cyclin A2 (*CYCA2*. gi; 4502612), cyclin B1 (*CYCB1*, gi; 1443518) and cyclin B2 (*CYCB2*, gi; 1093801) were modestly expressed, but the expression level did not show much difference (Fig.4). Among *CDKs*, these cells expressed *CDK4* that functions in G0-G1 phase. *CDK1*, *CDK2* and anther G0-G1 cyclin, *CDK6* were modestly expressed and their expression level was not different between HB611 cells and HuH6 cells (Fig. 5). As for *CKIs*, $p21^{Cip1}$ and $p27^{Kip1}$ were mainly expressed in these cells and suggest that they should probably work as major cell cycle controllers (Fig. 5). We have to take into consideration that 80 ~90 % cells were in the G1 in the ordinary culture condition. In this term, it is interesting that S phase and M phase cyclins were relatively highly expressed and *CDK4* was fairly highly expressed in these cells. This might represent that these cells were transformed cell lines.

Liver specific genes. The analysis was based on the hepatocellular carcinoma (HCC) originated cells with or without HBV production and therefore liver specific gene expression could be maintained modestly but fairly reduced. As expected, some liver specific transcription factors such as hepatocyte nuclear factor 1A (*HNF1A*, gi; 184264), 1B (*HNF1B*, gi; 4507396), 4A (*HNF4A*, gi; 3250320) and 4G (*HNF4G*, gi; 5636455) were at low expression (Fig. 6). Albumin (*ALB*, gi; 7959790) is one of the typical liver specific genes [9]and its expression was a kind of repressed in these cell lines, which suggests that these HCC originated cell lines or otherwise, HCC itself were dedifferentiated in the course of transformation (Fig. 6). Interestingly, alphafetoprotein (*AFP*, gi; 4501988) was extremely highly expressed in HB611 cells. This might suggest that HBV production should drastically changed the expression program of this gene. As shown in Table 2, a couple of genes were much highly expressed in the HB611 cells. It seems to be interesting how the gene expression was controlled, in a

similar way or in a different way?

Other interesting genes. While we were mining data, we found interesting expression profiles of some genes. Sodium taurocholate co-transporting polypeptide (*NTCP* or *NTCP1*, gi; 4506970) was reported to be an HBV receptor last year [25]. The report pointed out that HCC originated cultured cell line such as HepG2 and HuH7 did not express this gene and correction of this gene expression by transfection endowed these cell lines with HBV infectivity and viral amplification. We checked the gene expression in this study. Beyond my expectations, HB611 cells expressed moderately NTCP (Fig. 7), which suggests that HBV produced from the HB611 cells could reinfect the cells. But so far, cccDNA was not observed in the HB611 [19, 21], which suggests that there was some obstruction to HBV infection in this system. HBV membrane protein, large S (LS), middle S (MS) and small S (SS or simply HBs) produced from the cells might interfere the HBV reinfection. On the other hand, another similar gene, *NTCP2* (gi; 456972) was at no expression level.

We checked about hundreds interferon related genes including INFs, IFN receptors and IFN regulatory factors (IRFs) but there was almost no difference between HB611 cells and HuH6 cell, expressed or not expressed (data not shown). Likewise, Janus kinase (JAK)/ signal transduction and transcription factors) STATs related genes were not changed (data not shown), although it was reported that HBV polymerase (HBVpol) inhibited the activities of the STAT proteins [24].

DISCUSSION

In this report, we analyzed how HBV production affected cellular gene expression profiles using an artificially HBV producing system based on an HCC-originated cultured cell line. HBV never infects cultured cell lines and experimental animals. Needless to say, this kind of analysis should be done in natural infection system. HBV, however, has strict species specificity and there is no very convenient and useful infection system for HBV. HepaRG, a HCC derived cell line, and PHH separated from SCID-hu hepatocyte are commercially available. Even though PHH shows high competency for HBV infection, both cell lines are very expensive and inconvenient for daily HBV study. Therefore, more convenient and useful systems for HBV infection

must be explored and currently we can utilize an HBV production system designed artificially in HCC derived cultured cell lines [18, 19]. Nevertheless, it was clear that such analysis was very informative and HBV production affected remarkably the cellular gene expression profiles and expression level of some genes appeared to be drastically changed by HBV production.

Considerable numbers of gene seemed to be affected for their gene expression. Since it was reported that hepatitis C virus infection affected lipid metabolism [15], it is very interesting that the expression level of some genes such as fatty acid binding protein (gi; 4557584) involved in lipid metabolism was remarkably changed in the presence of HBV related products in the cells. Change in lipid metabolism might lead to cellular phenotypic change.

Some genes related to cell growth such as PDGFR α and myc (N-myc related in this case, gi; 33877057) were extremely overexpressed in the HB611 cells. N-myc overexpression was observed in woodchuck hepatitis virus (WHV) involved hepatocarcinogenesis [8, 22]. WHV genome was found near or in the N-myc locus, which resulted in overexpression of N-myc gene [22]. In our case or human HCC, there has been no evidence that the HBV genome integrated in or near the N-myc locus. Therefore, N-myc overexpression in our system is not the case and maybe there could be different mechanism. HBV infection might increase the gene expression and facilitate hepatocyte growth.

Cell cycle engine is accelerated by cyclins (*CYCs*) and cyclin dependent kinase (*CDKs*) which are checked and controlled by CKI. Although *CYCs* and *CDKs* should be activated in immortalized and transformed cells, *CYCD1* (gi; 12652656) as a G1 phase cyclin, *CYCA2* (gi; 4502612) as a S phase cyclin and *CYCB2* (gi; 1093801) and less *CYCB1* (gi; 1443518) as an M phase cyclin were relatively activated in both HB611 and HuH6 cells. *CDK4* rather than *CDK6* was favored in these cell lines. $p57^{Kip2}$ (gi; 854949) is thought to be a CKI unique for stem cells and $p21^{Cip1}$ (gi; 11386202) and $p27^{Kip1}$ (gi; 12805034) were main CKIs in this case as expected.

Liver specific gene expression could be important to show how much such cell lines maintained well-differentiated hepatocyte characters. Although normal liver gene expression profiles were not investi-

gated, liver specific transcription factors such as *HNF1A, 1B, 4A* and *4G* were expressed at low level but not absent. *ALB* (gi; 7959790) is one of the characteristic liver specific genes and was at low expression level. In contrast, *AFP* (gi; 4501988) was upregulated in HB611 cells, the mechanism of which could not be assessed in this report but HBV production might cause blastic change in hepatic cells. *NTCP* (gi; 4506970) is a notable factor as an HBV receptor [25]. Considering that cultured HCC derived cell lines never permit HBV infection, NTCP expression had not been expected. HB611 cells, however, showed moderate expression of *NTCP*. This fact let us expect that HB611 cells should competent for HBV infection and reinfection circuit could go around. So far, we have not observed cccDNA formation in the HB611 cells and this is not the case perhaps because S related membrane proteins secreted from HB611 cells should block the receptor and the ligand interaction.

We found a big difference of three genes influencing cell growth. EGF as well as hepatocyte growth factor (HGF) is a hepatocyte growth factor [12, 14] and therefore its receptor, *EGFR* (gi; 6228471) expression should be important for tumor growth dependent on EGF. HBV production, however, could change drastically growth factor usage to platelet derived growth factor (PDGF) by increasing PDGFR α (gi; 5453869).

It is fascinating how gene expression profiles are altered by HBV production. Core protein, polymerase and X protein could be good candidates that change the gene expression and these phenomenon have to be assessed in a natural HBV infection system using PHH. Furthermore, we should pay attention to non-coding short RNA (ncsRNA) expression status in presence or absence of HBV. ncsRNA including miRNA basically have a negative effect on the target gene [3]. Such RNA molecules should have remarkable effects on cellular and viral gene expression program and might be expressed from HBV genome as well as host genome (Fig. 8), though ten or more miRNAs have been reported to regulate HBV gene expression [11, 26].

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gene	gi No.	HuH6	HB611
hox transcript antisense RNA	10939596	235.7	0.7
EST*	5863388	1128.8	1.4
plastin 3	7549808	1398.5	2.1
transmembrane channel like 5	13417048	3166.5	4.8
kelch-like	9511250	1970.7	9.2
Wntless homolog	2835015	10967.0	55.9

Table 1. Genes extremely highly expressed in HuH6.

* is a transcript identified as an expression sequence tag (EST). Gene name and its gi number are shown on the left two columns. The values represent signal intensity detected in this analysis.

gene	gi No.	HuH6	HB611
CD24	180167	77.5	7083.8
gremlin-1	10863087	2.0	1136.2
steroid sulfatase (microsomal) isozyme S	3538520	3.7	4229.7
steroid sulfatase (microsomal) isozyme S*	13162281	7.7	1179.1
microsomal triglyceride transfer protein	4648246	6.3	1855.5
fatty acid binding protein	4557584	41.4	22460.8
lysyl oxidase	4505008	7.3	1696.3
PDGFR-α	5453869	7.7	1297.9
myc (N-myc related)	12803748	6.8	1495.5

Table 2. Genes extremely highly expressed in HB611.

* is another gene of steroid sulfatase (microsomal) isozyme S. Gene name and its gi number are shown on the left two columns. The values represent signal intensity detected in this analysis.

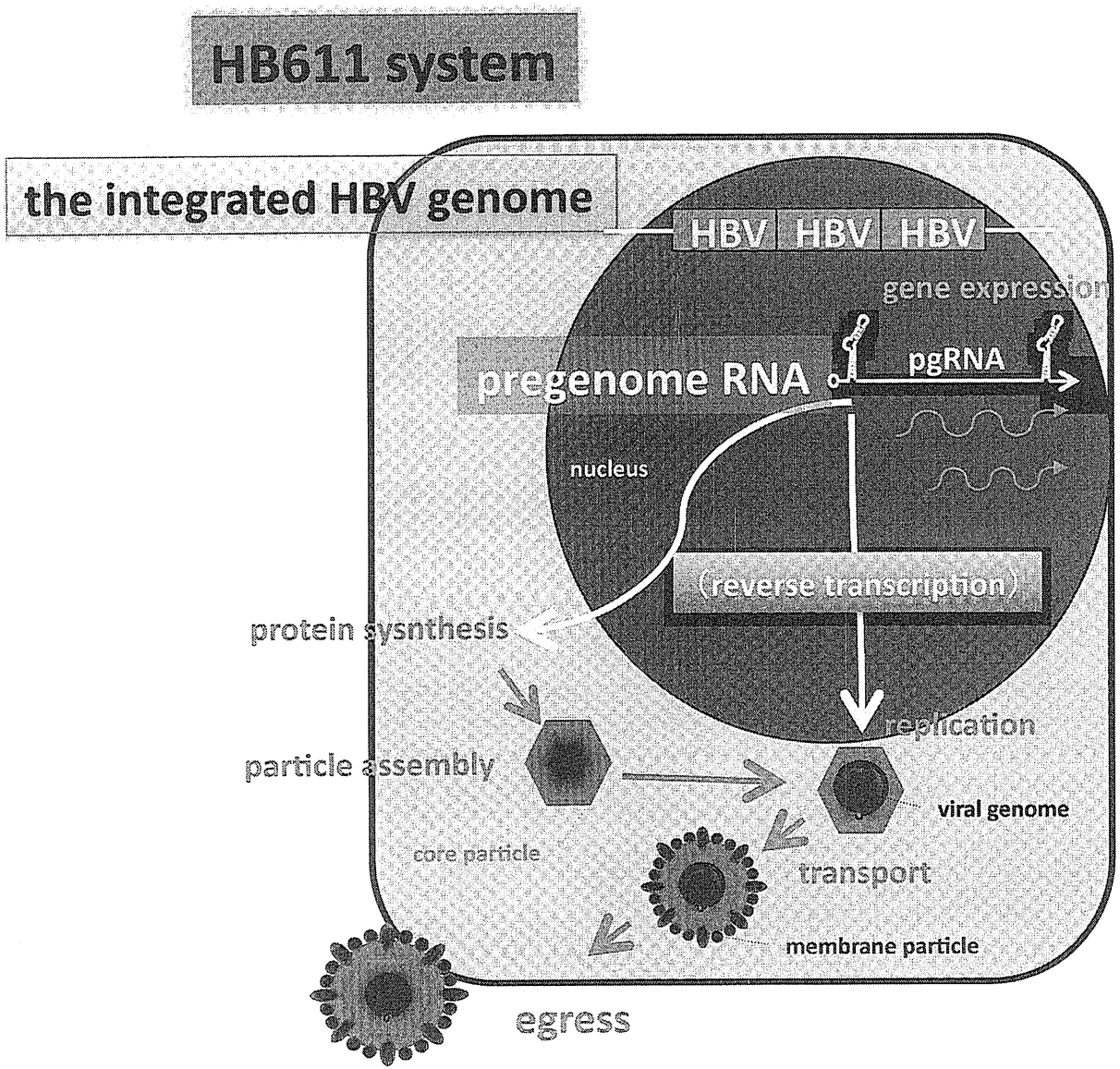

Fig. 1. HB611 system. In HB611 cells three tandemly arranged HBV genomes are integrated in the host chromosome. Viral genes including pregenome RNA (pgRNA) are transcribed from the genome. The viral genome is synthesized through reverse transcription pathway while packaging into core particle.

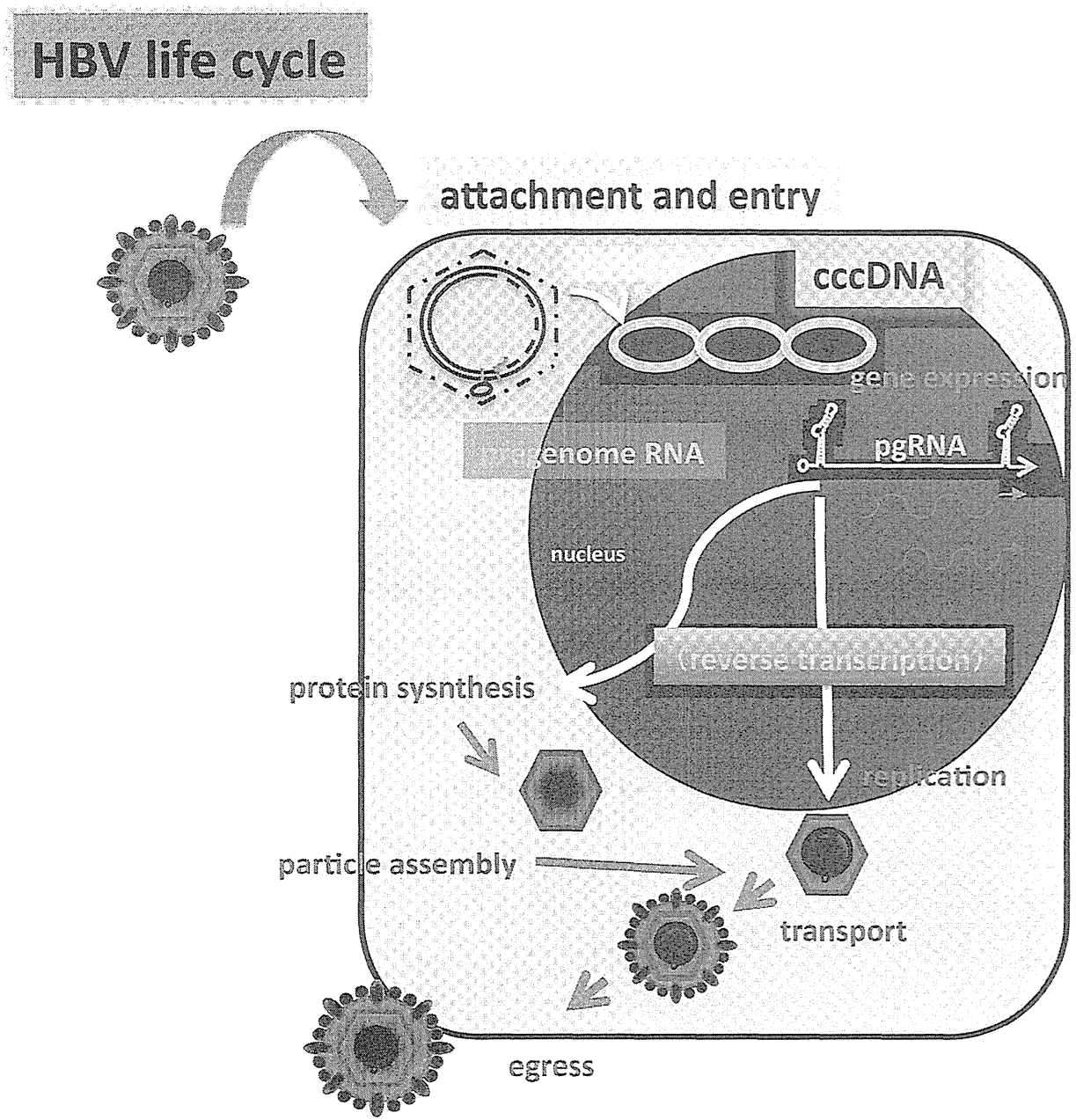

Fig. 2. Natural HBV life cycle. In the natural infection course of HBV, the viral genome is not integrated into the host genome. The partially double stranded DNA genome is converted to covalently closed circular DNA, which is maintained in the infected cell nucleus and produces viral related transcripts including pgRNA. The pathway after this is the same as shown in Fig. 1.

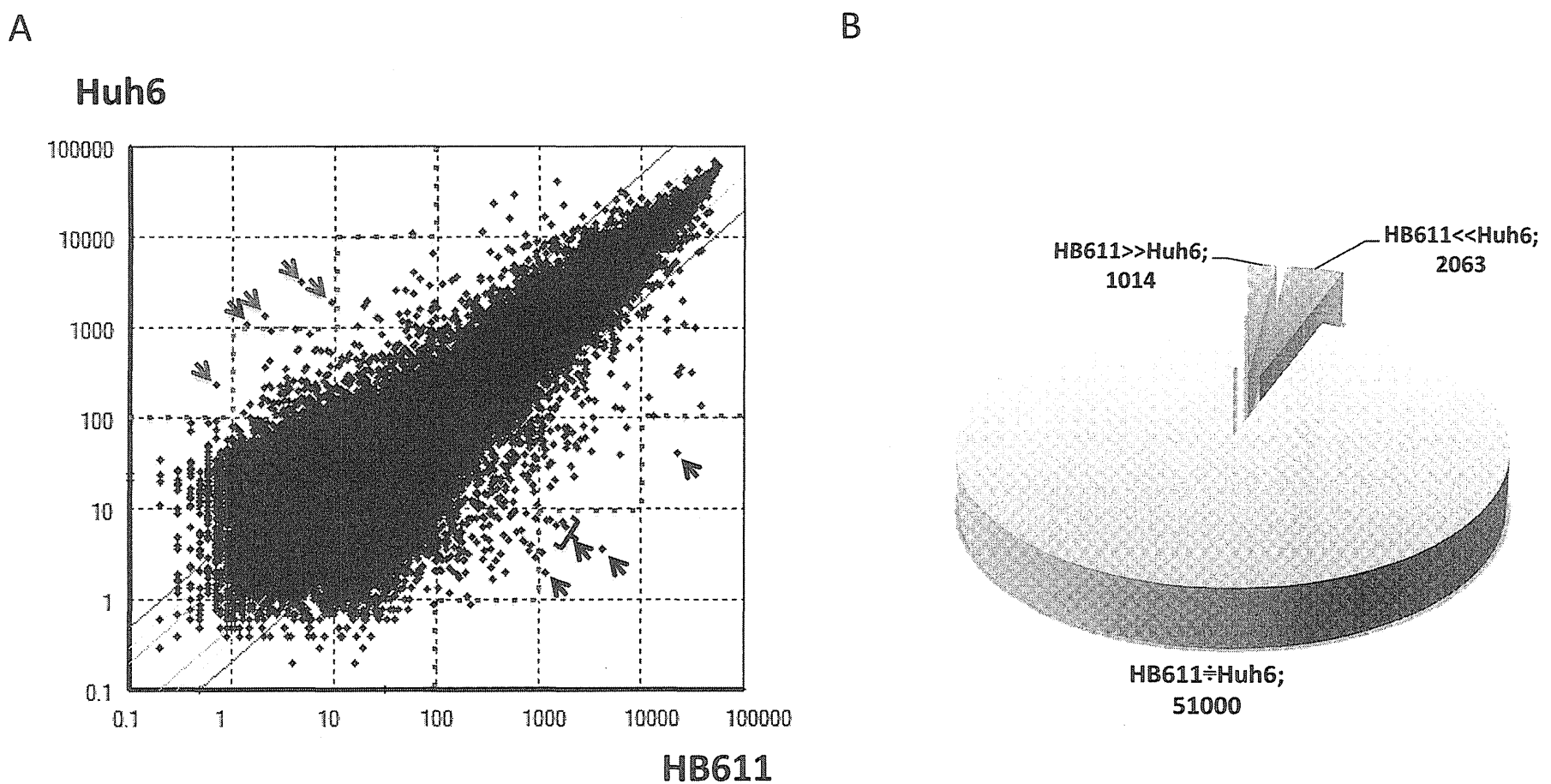

Fig. 3. Gene expression profile of HB611 and its parental HuH6 cells. A. Scatter plot analysis of expressed genes in HB611 and HuH6 cells. Orange dotted lines shows the boundary of extreme difference in expression level more than one hundred. Arrows represent picked-up genes that show extreme difference in expression. B. Pie chart of the gene expression profile. The light blue zone represents highly expressed genes in HB611 more than eight times and light red section represents highly expressed genes in HuH6 more than eight times.

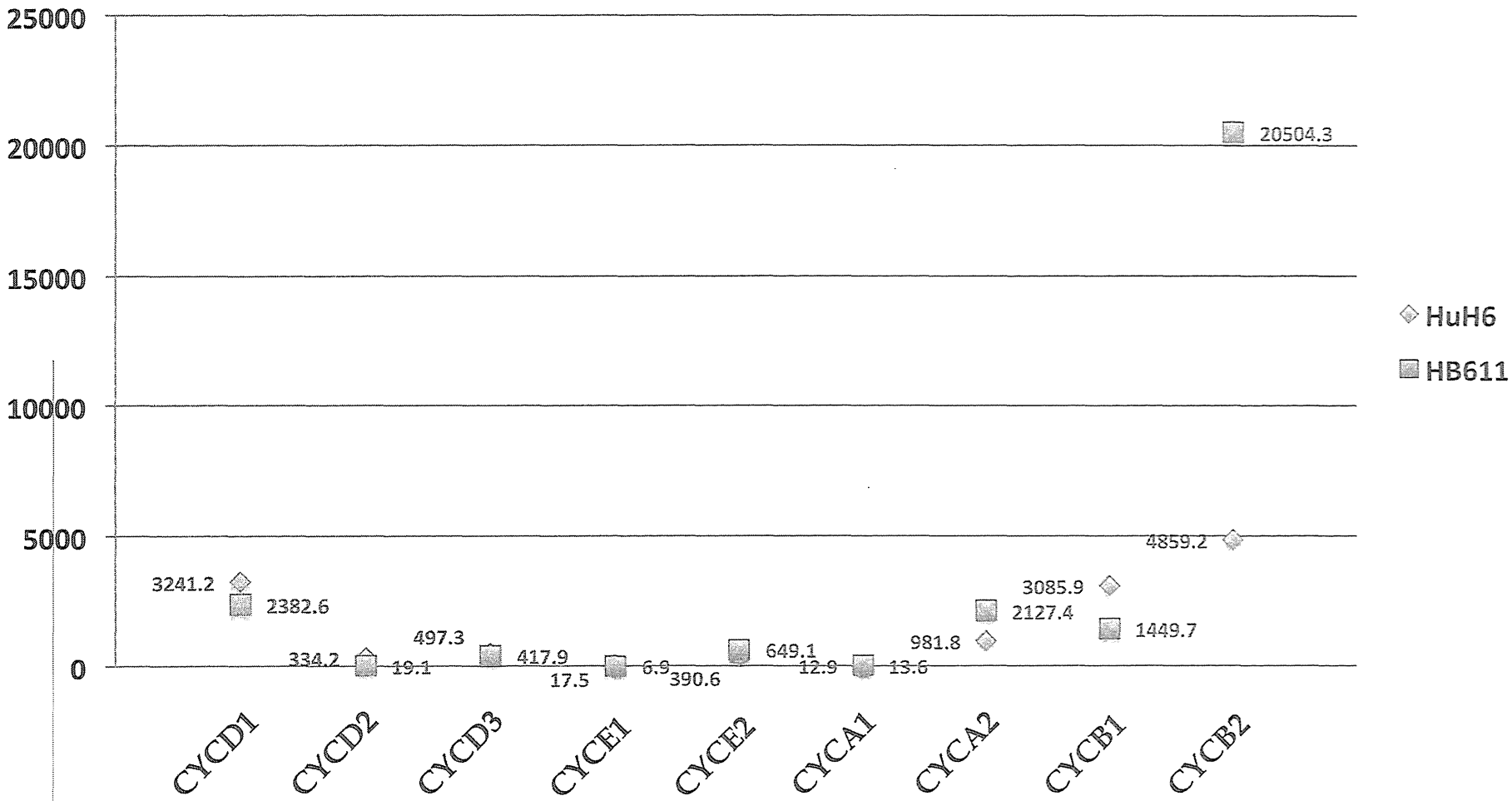

Fig. 4. Expression level of cell cycle control genes. The values represent signal strength in the DNA microarray analysis. Orange figures shown on the right of the marker represent the value for HB611 cells and the blue ones on the left for HuH6.

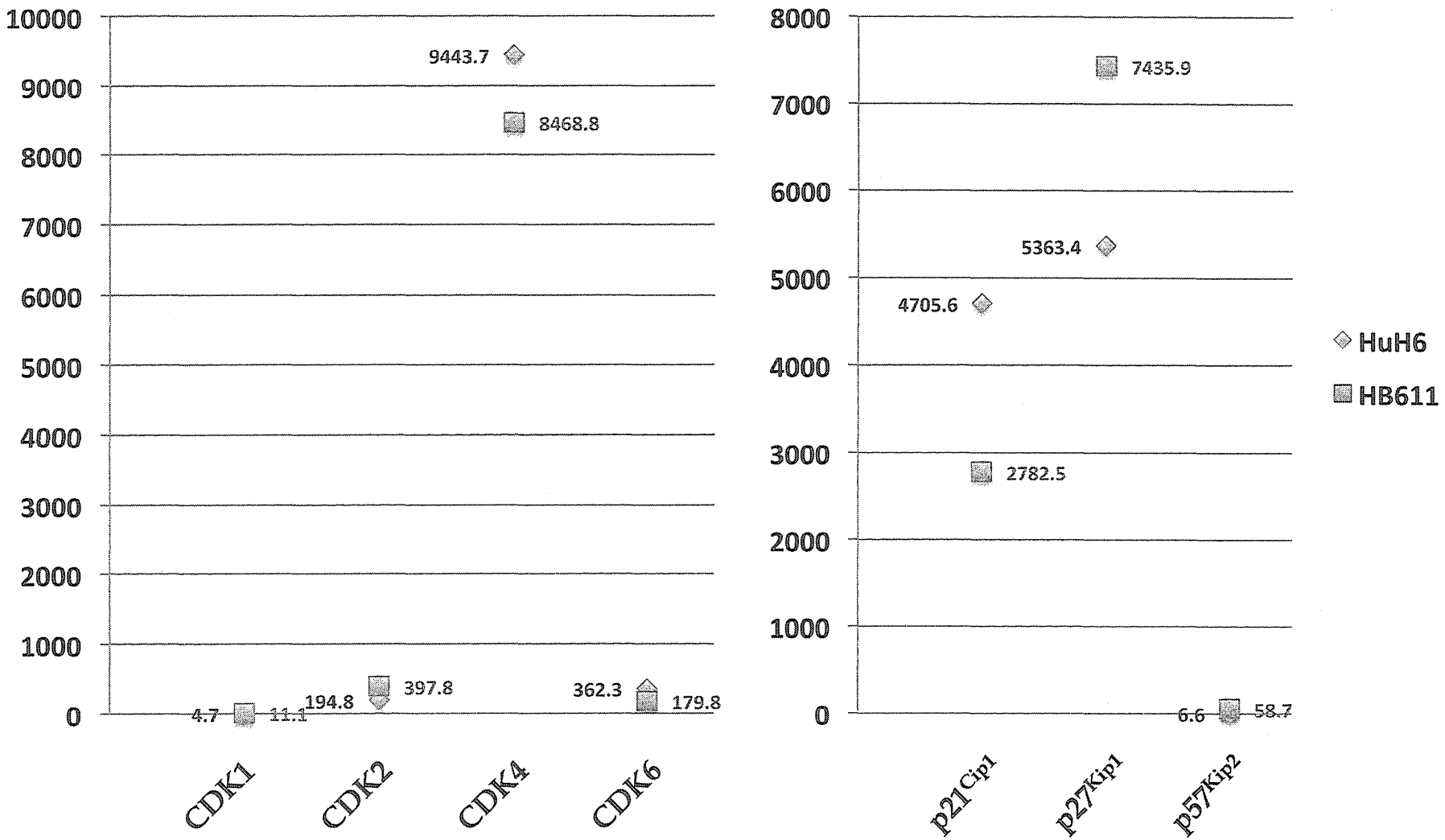

Fig. 5. Expression level of cyclin dependent kinases (CDKs) (left) and cyclin dependent kinase inhibitors (CKIs) (right). Orange figures shown on the right of the marker represent the value for HB611 cells and the blue ones on the left for HuH6.

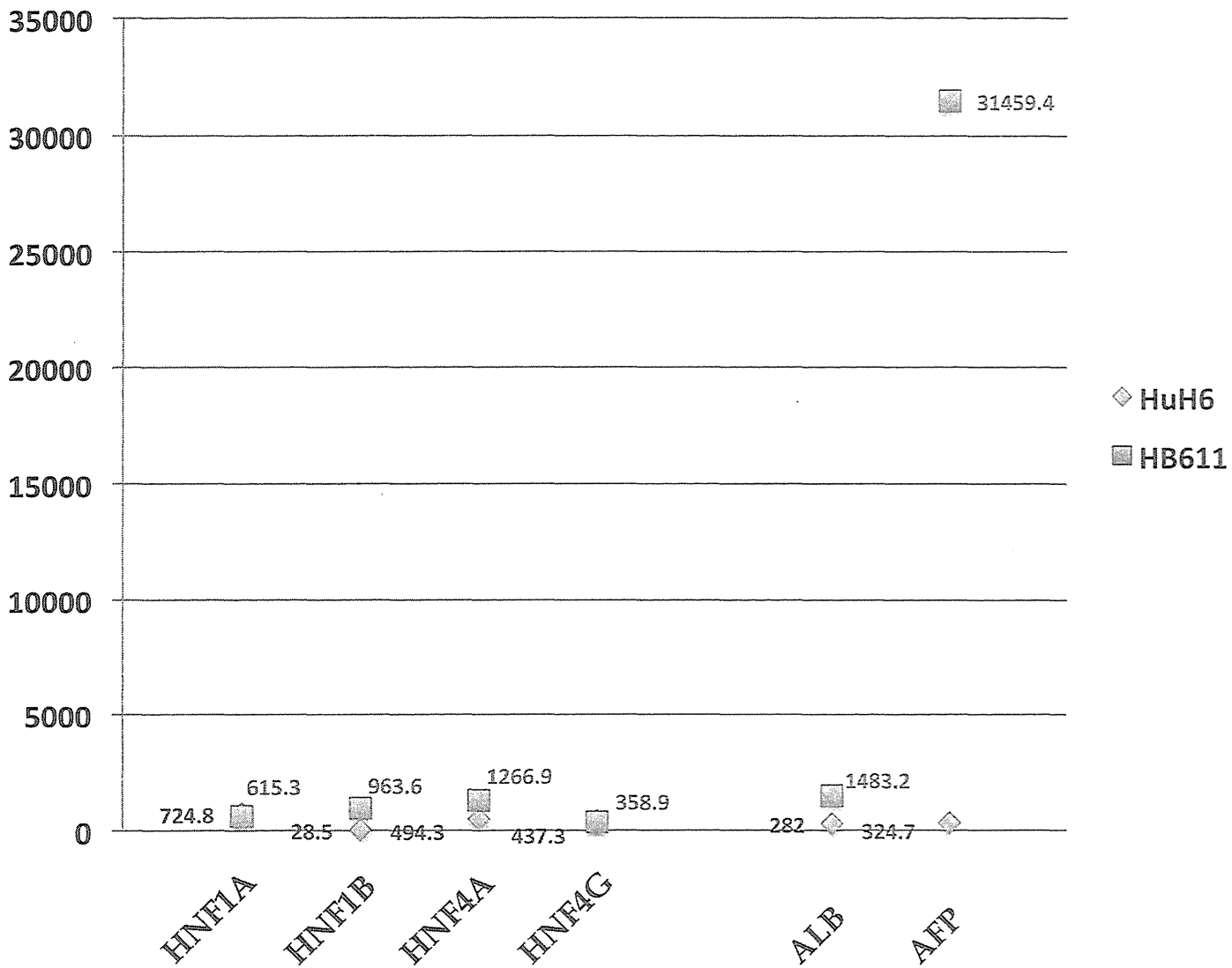

Fig. 6. Expression level of liver specific genes. Orange figures shown on the right of the marker represent the value for HB611 cells and the blue ones on the left for HuH6.

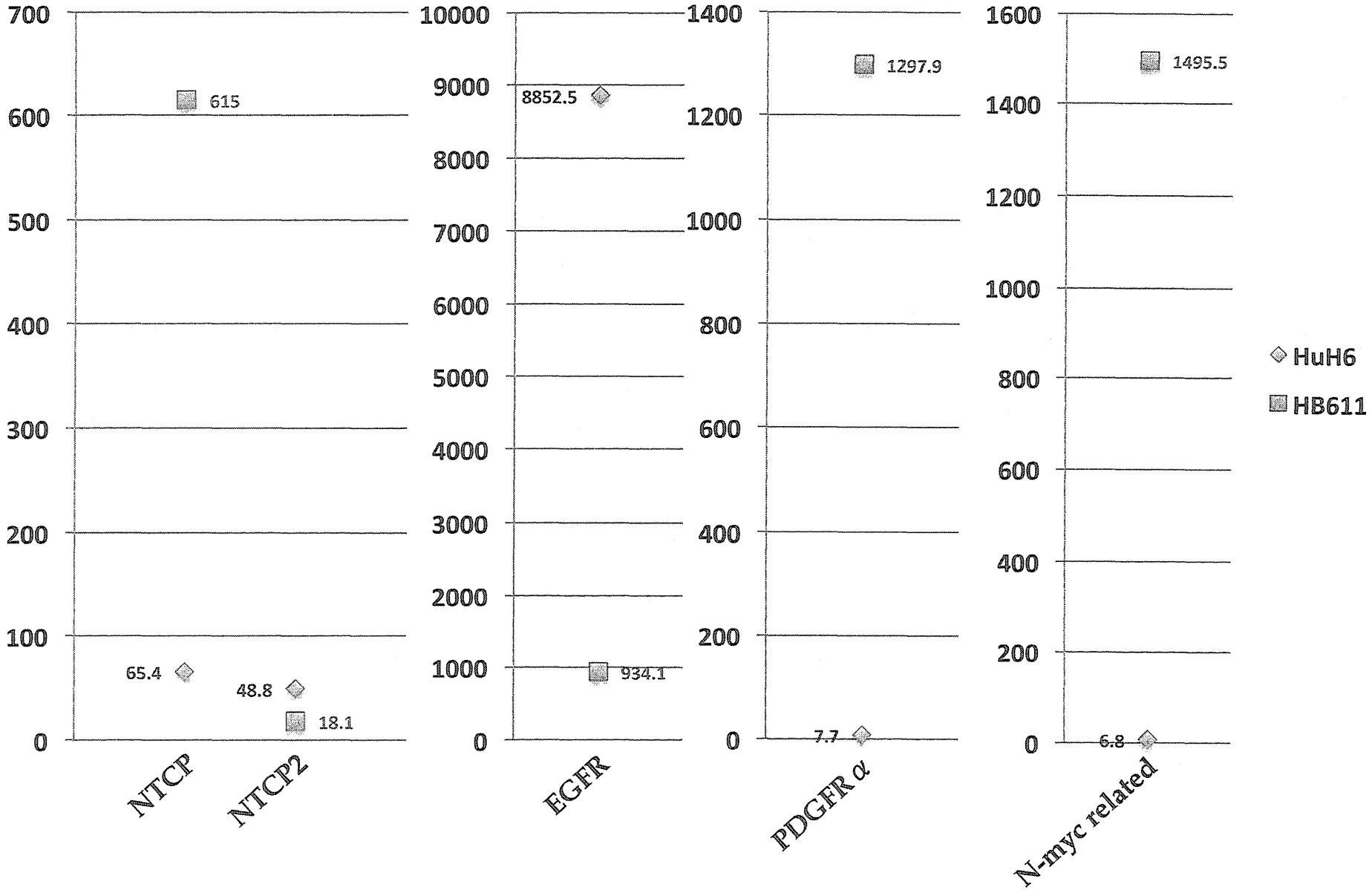

Fig. 7. Expression level of NTCPs and typical growth related genes. Orange figures shown on the right of the marker represent the value for HB611 cells and the blue ones on the left for HuH6.

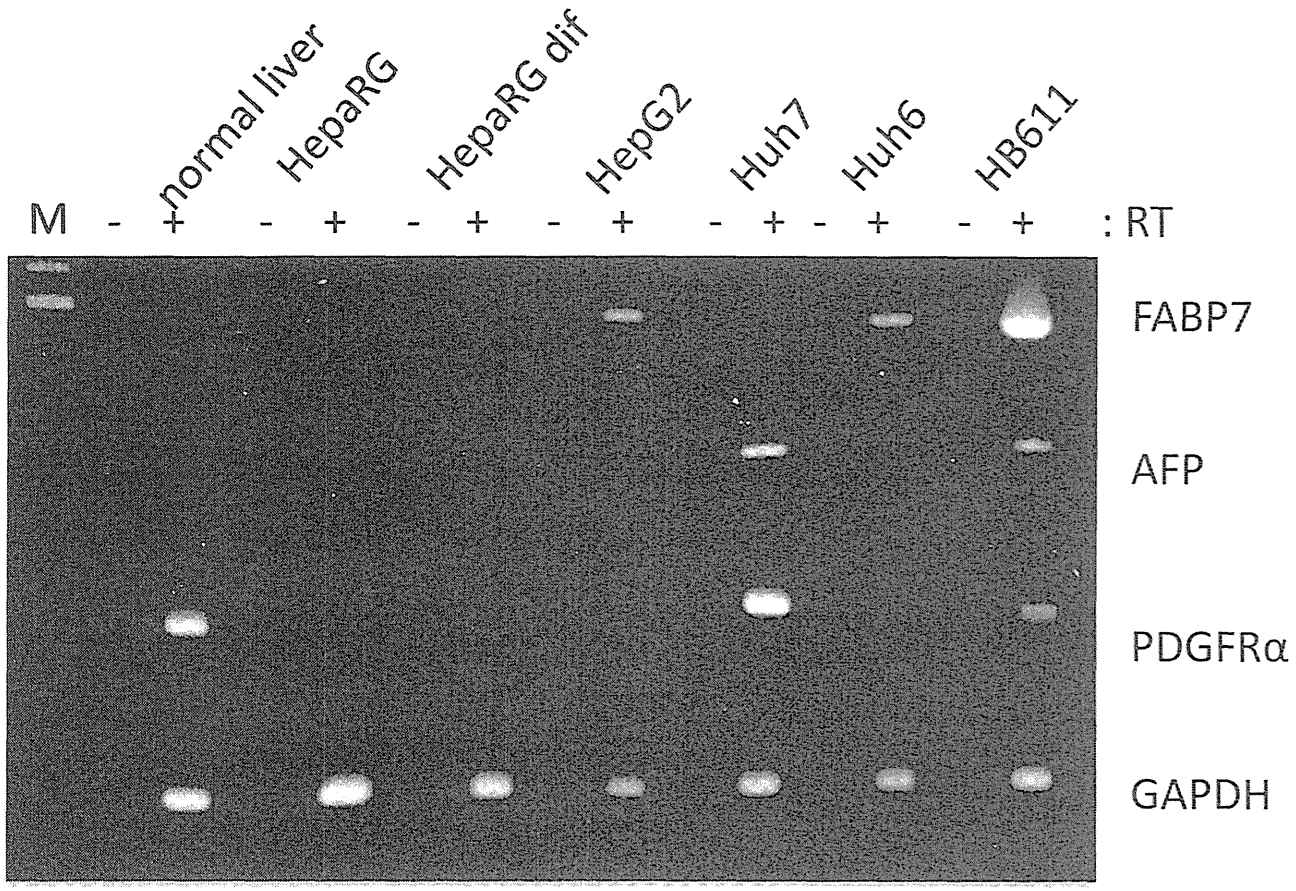

Fig. 8. RT-PCR of RNA extracted from several hepatocellular carcinoma cells. Typical genes which showed much difference in the expression level were tested with RT-PCR, including GAPDH as a control for RNA preparation and expression.

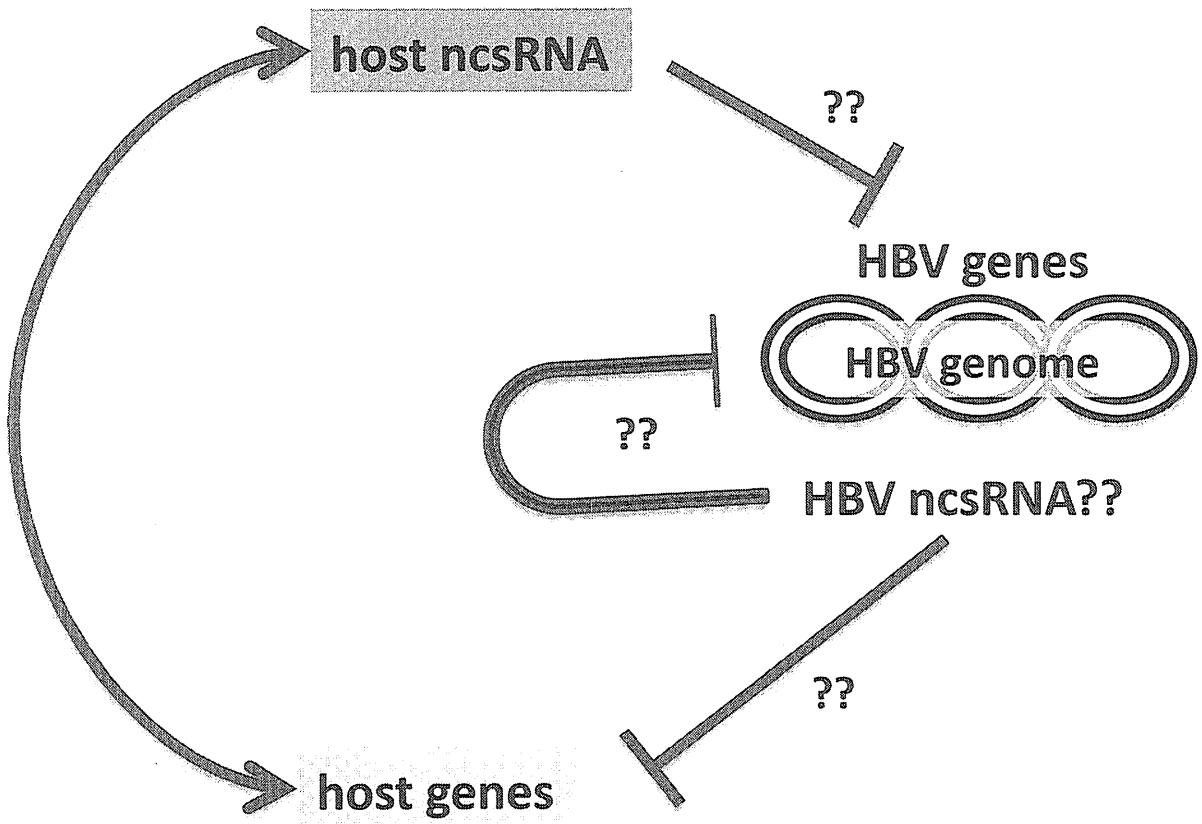

Fig. 9. Non-coding viral RNA could be expressed from the HBV genome and affects cellular gene expression program as well as the viral one.