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Original Article

Gene expression profiling of hepatitis B- and hepatitis C-related hepatocellular carcinoma using graphical Gaussian modeling

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Conflict of interest: The authors declare no conflict of interest.

Abbreviations: CH-B, chronic hepatitis B; CH-C, chronic hepatitis C; CLL, cells in liver lobules; CPA, cells in the portal area; EF, early fibrosis; EGR1, early growth response protein 1; ESR1, estrogen receptor 1; GGM, graphical Gaussian modeling; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; hTERT, human telomerase reverse transcriptase; LCM, laser capture microdissection; LF, late fibrosis; PCCM, partial correlation coefficient matrix; PTEN, phosphatase and tensin homolog; SD, standard deviation; SHC, src homology 2 domain containing; STAT1, signal

Erratum

Erratum to Gene expression profiling of hepatitis B- and hepatitis C-related hepatocellular carcinoma using graphical Gaussian modeling
[Genomics 101/4 (2013) 238–248]

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The publisher regrets that the on page 246, in 4.2.Microarray analysis, Accession NO. GSE41804 was a wrong number. The correct number should be GSE44074.

The publisher would like to apologise for any inconvenience caused.

DOI of original article: < http://dx.doi.org/10.1016/j.ygeno.2013.02.007 >
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transducer and activator of transcription 1; TCA, tricarboxylic acid cycle; VEGF, vascular endothelial growth factor.

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Abstract word count: 249

Total word count: 6,750

Number of figures: 4

Number of tables: 2

Abstract

Background & Aims

Gene expression profiling of hepatocellular carcinoma (HCC) and background liver has been studied extensively; however, the relationship between the gene expression profiles of different lesions has not been assessed.

Methods

We examined the expression profiles of 34 HCC specimens (17 hepatitis B virus [HBV]-related and 17 hepatitis C virus [HCV]-related) and 71 non-tumor liver specimens (36 chronic hepatitis B [CH-B] and 35 chronic hepatitis C [CH-C]) using an in-house cDNA microarray consisting of liver-predominant genes. Graphical Gaussian modeling (GGM) was applied to elucidate the interactions of gene clusters among the HCC and non-tumor lesions.

Results

In CH-B-related HCC, the expression of vascular endothelial growth factor-family signaling and regulation of T cell differentiation, apoptosis, and survival, as well as development-related genes was up-regulated. In CH-C-related HCC, the expression of ectodermal development and cell proliferation, wnt receptor signaling, cell adhesion, and defense response genes was also up-regulated. Many of the metabolism-related genes were down-regulated in both CH-B- and CH-C-related HCC. GGM analysis of the HCC and non-tumor lesions revealed that DNA damage response genes were associated with AP1 signaling in non-tumor lesions, which mediates the expression of many genes in CH-B-related HCC. In contrast, signal transducer and activator of transcription 1 and phosphatase and tensin homolog were associated with early growth response protein 1 signaling in non-tumor lesions, which potentially promotes angiogenesis, fibrogenesis, and tumorigenesis in CH-C-related HCC.

Conclusions

Gene expression profiling of HCC and non-tumor lesions revealed the predisposing changes of gene expression in HCC. This approach has potential for the early diagnosis and possible prevention of HCC.

Keywords: Hepatitis B virus, Hepatitis C virus, Hepatocellular carcinoma, Gene expression

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide with a particularly poor patient outcome [1]. It often develops as a result of chronic liver disease associated with hepatitis B (HBV) or hepatitis C virus (HCV) infection or with other etiologies such as long-term alcohol abuse, autoimmunity, and hemochromatosis [2]. HBV and HCV infection are the leading cause of HCC in the world [3]. In Japan, approximately 85% of patients with HCC are positive for the HBV surface antigen or anti-HCV antibody. Approximately 7% of patients with HCV-related liver cirrhosis develop HCC [4] and 3% of patients with HBV-related liver cirrhosis develop HCC [5].

Gene expression analysis of HCC has revealed from previous work, the activation of the wnt/β-catenin, pRb, p53, transforming growth factor-β, mitogen-activated protein kinase, and Janus kinase/signal transducer and activator of transcription pathways, stress response signaling, and epidermal growth factor receptor [6-8]. In addition, we have previously reported that the gene expression profiles in the livers of patients with chronic hepatitis B (CH-B) and chronic hepatitis C (CH-C) were different. Pro-apoptotic and DNA repair responses were predominant in CH-B, while inflammatory and anti-apoptotic phenotypes were predominant in CH-C [9, 10]. Furthermore, we optimized the laser capture microdissection (LCM) method to isolate cells in liver lobules (CLL) and cells in the portal area (CPA) for detailed gene expression analysis [10, 11]. However, it is still unknown how cancer signaling pathways are activated in HCC. As HCC frequently develops from a cirrhotic liver, analyzing the relationship of signaling pathways between HCC and non-cancerous liver tissue might be a useful approach for revealing the mechanism that ultimately leads to the development of HCC.

Graphical Gaussian modeling (GGM) is utilized widely to infer or test the relationships between multiple variables [12-14]. Previously, we developed a method that combines cluster analysis with GGM to infer a genetic network on the basis of expression profile data. Analysis of the expression profile of *Saccharomyces cerevisiae* revealed a model of its genetic network, and the accuracy of the inferred network was confirmed by its agreement with the cumulative results of experimental studies [15]. Therefore, GGM has the potential to be a useful analytical tool to identify the relationship between the gene expression profiles of HCC and non-cancerous liver tissue.

In the present study, we extended the analysis of gene expression in HCC and applied GGM analysis [15, 16]. Indeed, our procedure inferred the relationships between gene groups defined by clustering, and its application enabled us to elucidate the framework of the gene clusters in relation to the hepatocellular carcinogenesis of CH-B and CH-C.

2. Results

2.1 Expressed genes in CH-B-related HCC

The gene expression profiles of whole liver biopsy specimens and surgically resected liver were obtained from 36 patients with CH-B, 17 with CH-B-related HCC, 35 with CH-C, and 17 with CH-C-related HCC. The clinical characteristics of the patients are shown in Supplemental Tables A and B. We categorized the F1 and F2 fibrosis stages as early fibrosis (EF; n = 13 for CH-B and n = 12 for CH-C) and the F3 and F4 fibrosis stages as late fibrosis (LF; n = 22 for CH-B and n = 23 for CH-C).

The 783 differentially expressed genes in CH-B-related HCC were identified across 20 clusters, of which 4 (No. 8, 9, 11, and 20) were up-regulated and 12 (No. 1–7, 12–14, 16, and 17) were down-regulated (Figure 1 and Supplemental Table C). The

up-regulated clusters were comprised angiogenesis, cell cycle, apoptosis, and survival-related genes. Placental growth factor, vascular endothelial growth factor (VEGF)-related protein, SUMO-activating enzyme subunit 2, cyclin E1, and baculoviral IAP repeat-containing 5 were up-regulated (No. 8, 9, and 11). In addition, oncogene-related proteins, such as v-myc myelocytomatosis viral-related oncogene (No. 9), telomerase-associated protein 1, and stathmin 1/oncoprotein 18 (No. 8), tumor marker genes, such glypican 3, and growth factors, such as midkine (No. 9), were also up-regulated. In cluster No. 20, the proliferation and invasiveness-related gene and protein tyrosine kinase 2 were up-regulated.

Down-regulation was prominent in many metabolism-related genes including ornithine aminotransferase, insulin receptor substrate 1, glutamate dehydrogenase 2, acyl-coenzyme A oxidase 2, and acetyl-coenzyme A acyltransferase 2, as well as many cytochrome P450 family genes, suggesting impaired xenobiotic, amino acid, and lipid metabolism (No. 6, 7, 12, 13, 16, and 17). The characteristic genes expressed in CH-B-related HCC are shown in Table 1.

2.2 Expressed genes in CH-C-related HCC

The 668 differentially expressed genes in CH-C-related HCC were identified across 18 genetic clusters, of which 5 (No. 10, 12, 14, 15, and 18) were up-regulated and 11 (No. 1–7, 11, 13, 16, and 17) were down-regulated (Figure 2 and Supplemental Table D). Cluster No. 12 comprised immune defense response genes, such as chemokine (the C-C motif) ligand 19, natural killer cell transcript 4, major histocompatibility complex class I B, major histocompatibility complex class II DQ beta 1, and ubiquitin-specific protease 8. Cluster No. 14 comprised cytoskeleton-associated, cell cycle, mitosis-related, and MAPKKK cascade-related genes, such as tubulin, src homology 2 domain containing (SHC) transforming protein 1, sterile alpha motif domain

containing 9, S100 calcium binding protein A10, annexin A2, cyclin B1, platelet-activating factor acetylhydrolase, isoform Ib, and vimentin. In cluster No. 15, glypican 3, aldo-keto reductase family 1, member B10, ATP citrate lyase, farnesyl diphosphate synthase, serine protease inhibitor, and Kazal type 1 were up-regulated. Cluster No. 15 included many candidate tumor markers of HCC. Interestingly, LCM analysis revealed that many of the up-regulated genes in clusters No. 12, 14, and 15 were preferentially expressed in CPA. Cluster No. 18 comprised regulation of G1/S checkpoint, signal transduction, and ectoderm development-related genes, such as bone morphogenetic protein 4, cyclin-dependent kinase inhibitor 2A, fibroblast growth factor 9, and ornithine decarboxylase 1. Similar to CH-B-related HCC, many of the metabolism-related genes, including glucose, lipid, and amino acid genes, were down-regulated. The unique feature of lipid metabolism in CH-C-related HCC was the up-regulation of cholesterol and fatty acid synthesis genes and down-regulation of cholesterol metabolism and β oxidation genes. It was characterized by the up-regulation of stearoyl-CoA desaturase, farnesyl diphosphate synthase (No. 14), and ATP citrate lyase (No. 15), and down-regulation of acetyl-coenzyme A acetyltransferase 1. The characteristic genes expressed in CH-C-related HCC are shown in Table 2. Representative gene expression levels confirmed by TaqMan PCR are shown in Supplemental Figure C1.

Pathway analysis of the combined up- and down-regulated clusters is shown in Supplemental Figure D and Supplemental Table E. In CH-C-related HCC, immune response- and cytoskeleton-related genes, such as actin, tubulin, and vimentin, were up-regulated, while in CH-B-related HCC, cell matrix interaction genes, such as collagen IV and matrix metalloproteinase, were up-regulated. Immune-related genes were shown to be down-regulated in both CH-C- and CH-B-related HCC by MetaCore™ database analysis (Thomson Reuters, New York, NY) (Supplemental Figure D). Gene

annotation by DAVID Bioinformatics Resources 6.7 (http://david.abcc.ncifcrf.gov/) [17] showed that oxidative phosphorylation and ATP synthesis coupled electron transport were up-regulated more in CH-C-related HCC than in CH-B-related HCC (Supplemental Table E).

2.3 Expressed genes in CH-B and CH-C

Differentially expressed genes in CH-B or CH-C were identified by backward selection, which did not include genes that were differentially expressed in CH-B- or CH-C-related HCC. As HCC frequently develops in the LF stage of liver disease, gene expression was evaluated in this stage. A total of 352 genes were differentially expressed in the LF stage of CH-B and classified into 21 clusters, of which 7 (No. 2, 3, 9, 10, 15, 16, and 18) were up-regulated and 11 (No. 5–7, 8, 11–14, 17, 20, and 21) were down-regulated (Supplemental Figure B and Supplemental Table F).

In the CH-B fibrotic liver, genes involved in apoptosis, survival, and response to stress, as well as chemokine- and cytokine-related genes and wnt beta-catenin and angiogenesis-related genes, were up-regulated. Interestingly, these genes were already up-regulated in the EF stage of CH-B. In contrast, metabolism-related genes, such as those for pyruvate, cholesterol, and retinol metabolism and the mitochondrial tricarboxylic acid (TCA) cycle, were down-regulated.

In total, 214 genes were differentially expressed in the LF stage of CH-C and classified into 7 gene clusters, of which 1 was up-regulated (No. 1) and 3 were down-regulated (No. 3, 5, and 6) (Supplemental Figure B and Supplemental Table G). In CH-C, genes involved in the interferon signaling pathway, leukocyte chemotaxis, and immune response were preferentially up-regulated. These genes were expressed at a significantly higher level in CPA than in CLL in the liver (No. 1). Conversely, many metabolism and liver function-related genes were down-regulated (No. 3, 5, and 6).

These genes were expressed at significantly higher levels in CLL compared to CPA in the liver.

2.4 Framework of gene clusters in relation to hepatocarcinogenesis of CH-B using GGM

We used GGM to examine the relationship between non-cancerous and HCC gene clusters. The partial correlation coefficient matrix (PCCM) generated by GGM is shown in Supplemental Tables H and I. The frame networks of genetic clusters are shown in Figure 3. The blue lines indicate a negative partial correlation and the black lines indicate a positive partial correlation. Multiple correlations were observed within the non-cancerous and HCC clusters. In addition, some interesting correlations between non-cancerous and HCC clusters were noted. In CH-B (Figure 3A), non-cancerous cluster No. 3 was up-regulated and correlated with HCC clusters No. 8 and 18. Non-cancerous cluster No. 3 was composed of wnt signaling and oxidative stress-related genes, HCC cluster No. 8 was composed of VEGF family signaling-related genes, and HCC cluster No. 18 was composed of estrogen receptor 1 (ESR1) regulation of G1/S transition-related genes. Moreover, non-cancerous cluster No. 16 correlated positively with HCC cluster No. 11 and negatively with HCC cluster No. 18. Non-cancerous cluster No. 16 was composed of cytokine production and apoptosis-related genes, while HCC cluster No. 11 was composed of apoptosis and survival-related genes. The down-regulated non-cancerous cluster No. 13 in CH-B correlated negatively with HCC cluster No. 8. Non-cancerous cluster No. 13 was composed of hepatic functional genes, such as those related to cholesterol metabolism and the TCA cycle.

The correlations between these clusters were further confirmed by examining individual gene interactions with reference to the MetaCore database (Figure 4A). Eight

genes in non-cancerous clusters No. 3 and 16 were directly associated with AP1 in HCC cluster No. 18. These genes are related to development and the DNA damage response. In HCC cluster No. 18, many of the cell cycle, development, immune system, and metabolism-related genes were regulated by AP1 [18-20]. In addition, it is interesting to note that the HBV transcript clustered in HCC cluster No. 18 (Figure 1). The up-regulated HCC cluster No. 11 was associated with AP1 [21]. In addition, the down-regulated HCC cluster No. 13, which included many liver function-related genes, was also associated with AP1 [22, 23]. Thus, in CH-B, the DNA damage response might trigger the signaling pathway of HCC, while AP1 in HCC is likely the key regulator of HBV-related HCC.

2.5 Framework of genetic clusters in relation to hepatocarcinogenesis of CH-C using GGM

In CH-C (Figure 3B), the up-regulated non-cancerous cluster No. 1 correlated negatively with HCC cluster No. 9 and positively with HCC cluster No. 2. Non-cancerous cluster No. 1 was composed of interferon alpha/beta signaling pathway and leukocyte chemotaxis genes. HCC cluster No. 9 was composed of signal transduction and regulation of cell proliferation genes and associated directly with HCC cluster No. 18. HCC clusters No. 15 and 18 were composed of development process and wnt signaling pathway genes. HCC clusters No. 12 and 14 were composed of immune development, cell adhesion, and defense response genes. These clusters were directly and indirectly associated with HCC cluster No.9. HCC cluster No. 2 was composed of liver function genes, including those for lipid metabolism and iron ion transport. Non-cancerous cluster No. 7, which was composed of immune response, G-protein signaling, and regulation of lipid metabolism genes, correlated positively with HCC cluster No. 18.

Analysis of the individual gene interactions (Figure 4B) showed that a key regulator gene of non-cancerous cluster No. 1, signal transducer and activator of transcription 1 (STAT1), negatively regulated early growth response protein 1 (EGR1) in HCC cluster No. 9 [24]. EGR1 was a key regulator of angiogenesis and fibrogenesis-inducing genes, such as PAI-1 (No. 9), COL1A1, and FAK1 (No. 18) [25-27]. In addition, EGR1 negatively regulated a key regulator of gluconeogenesis, PEPCK (No. 2) [28]. Thus, EGR1 regulated the tissue repair response as well as the metabolic process. In addition to STAT1, phosphatase and tensin homolog (PTEN), in non-cancerous cluster No. 7, negatively regulated FAK1 in HCC cluster No. 18 [29]. FAK1 regulated oncogene SHC (No. 14) and might be involved in the cancer signaling pathway [30, 31]. Interestingly, PTEN was associated with Oct-3/4, a regulator of liver differentiation through its target gene C/EBP alpha (No. 3); C/EBP alpha regulated CYP27A1 and CYP3A5 (No. 5). Thus, in CH-C, two antitumor genes, STAT1 and PTEN, were associated with the expression of EGR1 and FAK1, which promote angiogenesis, fibrogenesis, and tumorigenesis in cancerous lesions. Interestingly, the expression of PTEN was related to the metabolic process of CH-C.

2.6 Serial gene expression in non-cancerous gene clusters and the occurrence of HCC

Analysis of the framework of gene clusters in relation to hepatocarcinogenesis by GGM and individual gene interactions revealed several key genes that were associated with hepatocarcinogenesis in non-cancerous clusters. We focused on STAT1 and PTEN in non-cancerous clusters in CH-C and evaluated serial changes of their expression at 2 time points (tumor free and tumor present) in additional 11 patients. The clinical characteristics of these patients at both time points are shown in Supplemental Table J. The expression of STAT1 and its related genes significantly

decreased at the time of HCC development compared with the tumor-free time. Similarly, the expression of PTEN significantly decreased when HCC developed compared with the tumor-free time (Supplemental Figure C2, 3).

3. Discussion

HCC frequently develops in the advanced stage of liver fibrosis. Although gene expression profiling of HCC and the background liver has been studied extensively [32-35], the relationship between the gene expression profiles of different lesions has not been elucidated. In the present study, we utilized GGM [15, 16] to analyze the relationship between gene expression in HCC and non-cancerous liver. GGM is widely utilized to study gene association networks [12-14].

We first performed gene expression profiling in CH-B- and CH-C-related HCC. The up- and down-regulated genes were identified by a comparison with a single reference sample of normal liver. There may be some variations in gene expression among normal livers; however, the identified genes were characteristic of HCC and were consistent with previous reports [33, 34]. Differences in the signaling pathways between CH-B- and CH-C-related HCC are clearly shown in Figures 1 and 2 and Supplemental Figure D. In CH-C-related HCC, immune response- and cytoskeleton-related genes, such as actin, tubulin, and vimentin, were up-regulated, while in CH-B-related HCC, cell matrix interaction genes, such as collagen IV and matrix metalloproteinase, were up-regulated. HBV-X protein reportedly promotes HCC metastasis by the up-regulation of matrix metalloproteinases [36]. The differences in the gene expression profiles between CH-C- and CH-B-related HCC were concordant with those reported previously [34, 37].

In the present study, GGM analysis also revealed the interactions of each cluster within HCC as well as within non-cancerous lesions. GGM analysis in

CH-B-related HCC showed that 3 up-regulated clusters and 6 down-regulated clusters were associated with each other. In CH-C-related HCC, 4 up-regulated gene clusters and 5 down-regulated gene clusters were associated with each other (Figure 3). Interestingly, the up-regulated gene clusters were preferentially expressed in CPA in the liver. This prompted us to consider the origin of the HCC cells. Recent reports of immunohistochemical staining of liver tissue using stem cell markers, such as EpCAM and CD133, have suggested the presence of hepatic stem cells in the periportal area [38]. In contrast, many of the down-regulated genes were liver function and metabolism-related genes that were preferentially expressed in CLL in the liver.

GGM analysis between the HCC and non-cancerous liver revealed the unique interactions of 2 lesions in this study. In CH-B, up-regulated clusters No. 3 and 16, development and DNA damage response gene clusters, regulated HCC clusters No. 8, 11, and 18, VEGF-family signaling, apoptosis and survival-related, and ESR1 regulation of G1/S transition-related gene clusters. Down-regulated cluster No. 13, a metabolism-related gene cluster, negatively regulated the up-regulated HCC cluster No. 8. These results suggest that the metabolic status of non-cancerous liver influences the gene expression of HCC. Individual gene interactions with reference to the MetaCore database showed that 8 genes in non-cancerous clusters No. 3 and 16 were directly associated with AP1 in HCC cluster No. 18, which regulated the expression of many HCC genes (Figure 4) [18-23]. Interestingly, the HBV transcript was clustered in HCC cluster No. 18. It has been reported that the HBV transcript enhances AP-1 activation [39, 40]. The results suggest a role for the HBV transcript in CH-B-related HCC. Recently, a next generation sequencing approach revealed the frequent integration of HBV in HCC (86.4%), where the putative cancer-related human telomerase reverse transcriptase (hTERT), mixed-lineage leukemia 4, and cyclin E1 genes were located [41]. Although, we could not find the up-regulation of these genes in CH-B-related HCC,

HBV genome integration should have important roles for HBV-related hepatocarcinogenesis. A previous report demonstrated that HBx retained the ability to overcome active oncogene RAS-induced senescence by using hTERT, which was introduced into human immortalized primary cells [42].

In CH-C, STAT1 and PTEN signaling in clusters No. 1 and 7, respectively, were associated with HCC clusters No. 9, 18, and 2, EGR1 signaling, ectodermal development and cell proliferation, lipid metabolism, and iron transport gene clusters. Individual gene interactions with reference to the MetaCore database showed that EGR1 regulates multiple genes in HCC cluster No. 9 as well as genes in up-regulated HCC cluster No. 18 and down-regulated HCC cluster No. 2 (Figure 4). STAT1 and PTEN in non-cancerous clusters No. 1 and 7 exhibited an anti-tumor effect.
STAT1 negatively regulated EGR1 [24] and, interestingly, the expression of PTEN was associated with metabolic-related genes in non-cancerous clusters No. 3 and 5 (Figure 4). PTEN reportedly promotes oxidative phosphorylation, decreases glycolysis, and prevents the metabolic reprogramming of cancer cells [43].

The reduced expression of these antitumor genes in CH-C might increase the expression of EGR1 and FAK1, which promote angiogenesis, fibrogenesis, and tumorigenesis in HCC (Figure 4). EGR-1 promotes hepatocellular mitotic progression [44], while p53 and PTEN are downstream targets of EGR1. EGR1 might be involved in a negative feedback mechanism of cell cycle progression by inducing p53 and PTEN [45]. Recent reports described the tumorigenic role of EGR1 in the presence of p53 and PTEN mutations [46, 47]. Thus, interferon signaling evoked by an innate immune response and the PTEN expression-associated metabolic process (No. 3 and 5) will likely regulate the gene expression profile of HCC through EGR1.

It is reported that HBV X protein represses the expression of PTEN by inhibiting the function of p53 [48] and c-Jun promotes cellular survival by suppression of PTEN

[49]. In this study, the expression of PTEN was repressed in CH-B (Supplemental Figure B, CH-B, cluster 21). Possible involvement of HBx, AP-1 and PTEN signaling in HBV-related hepatocarcinogenesis should be explored furthermore.

Recently, Hoshida et al. reported that gene expression profiling of the background liver of patients with HCC predicts their outcome [35]. In their report, gene sets, which correlated with good survival, included many metabolic process genes, such as those of fatty acid, amino acid, and glucose metabolism. In accordance with their results, our findings showed that the possible involvement of metabolic process genes in the background liver might influence gene expression in HCC. In addition, our study revealed the predisposing changes of gene expression in non-cancerous liver that precede the changes of gene expression in HCC. Interestingly, we found that the expression of the anti-tumor genes STAT1 and PTEN was decreased significantly at the onset of HCC compared with the tumor-free time. Therefore, serial analysis of the expression of these genes might be useful for predicting the development of HCC. Several reports have shown that the decreased expression of some chemokines, such as CXCL10, CCL2, and CCL5, is associated with the poor prognosis of resectable HCC [50, 51]. In this study, the expression of CXCL10, CXCL6, CXCL9, and macrophage migration inhibitory factor was decreased at the onset of HCC compared with the tumor-free time (Supplemental Figure C). It would be worthwhile to examine the expression of these genes in serum samples to predict the development of HCC.

In summary, using a bioinformatics approach, we performed gene expression profiling of HCC and non-cancerous liver, which revealed the predisposing changes of gene expression in HCC. This approach will be useful for the early diagnosis of HCC. Further studies with a larger sample population are needed to confirm our data and to determine possible means for preventing the development of HCC.

4. Materials and methods

4.1 Patients and tissue samples

HCC and non-cancerous liver specimens were obtained from 17 patients with HCV-related HCC and 17 with HBV-related HCC who underwent surgical resection of the liver (Supplemental Tables A and B). For the control normal liver, a surgically obtained tissue sample from a patient who showed no clinical signs of hepatitis was used as described previously [9, 10]. The liver tissue was histologically normal, and the patient tested negative for all hepatitis virus markers and had normal levels of serum aminotransferase. HCC and non-cancerous liver tissues were enucleated from resected specimens and frozen immediately in liquid nitrogen for RNA isolation [10]. In a previous study, expression profiling of the liver of 19 patients with CH-B and 18 with CH-C was performed (Table 2) [10]. The other experimental procedures are described in the Supplemental Materials and Methods.

4.2 Microarray analysis

cDNA microarray slides (Liver chip 10k) were used as described previously [10]. For the selection of genes, we utilized data from the cDNA microarray and hepatic SAGE libraries derived from normal liver, CH-C, CH-C-related HCC, CH-B, and CH-B-related HCC, including 52,149 unique tags. We selected 9614 non-redundant genes that are expressed in diseased and normal liver. The detailed procedures for the preparation of the cDNA microarray slides are described in the Supplemental Material and Methods. RNA isolation, amplification of antisense RNA, labeling, and hybridization were performed according to the protocols described previously [10]. Quantitative assessment of the signals on the slides was performed by scanning on a ScanArray 5000 (General Scanning, Watertown, MA) followed by image analysis using GenePix

Pro 4.1 (Axon Instruments, Union City, CA) as described previously [10]. The microarray data have been submitted to the Gene Expression Omnibus (GEO) public database at NCBI (Accession No. GSE41804). The details are also described in the Supplemental Material and Methods.

4.3 Graphical Gaussian modeling data processing

GGM [15, 16] enabled us to reveal the gene cluster framework in relation to hepatocellular carcinogenesis of CH-B and CH-C. The procedure included: 1) gene clustering; 2) construction of the PCCM by GGM algorithms; and 3) visualization of the cluster pathway (Supplemental Figure A).

4.4 Gene selection

To utilize a variety of tissue samples, we first calculated the ratio of gene expression in non-cancerous tissue (36 with CH-B and 35 with CH-C) to that in normal tissue and the ratio of gene expression in HCC tissue (17 with CH-B related HCC and 17 with CH-C related HCC) to that in normal tissue. Then, the expression ratios of non-cancerous and HCC tissues in individual samples were standardized in the two tissues, respectively, by transformation to the Z score (each value was subtracted by the average value and divided by the standard deviation (SD)) such that the mean expression value was 0 and the SD was 1. A gene was regarded as differentially expressed if the Z score was >1 or <-1 ($1 > |AV \pm SD|$). Although the criterion for a differentially expressed gene is usually $|AV \pm 2SD|$, the selection procedure described above is simply designed to gather as many differentially expressed genes as possible, and is suitable for determining the macroscopic relationships between gene systems estimated by cluster analysis. Gene selection from non-HCC samples was performed similarly by avoiding the selected genes in HCC (backward selection). Therefore, a

correlation between HCC and non-HCC genes could be obtained as there was no overlap between the genes.

4.5 Clustering with automatic determination of cluster number

In gene profile clustering, the Euclidian distance between Pearson's correlation coefficient of profiles and the unweighted pair group method using the arithmetic average (UPGMA or group average method) were adopted as the metric and the technique, respectively, with reference to previous GGM analysis [15, 16]. Note that the present metric and technique were selected to estimate robustly the clusters against the noise of gene expression measurements [15]. In cluster number estimation, the variance inflation factor was adopted as a stopping rule for the hierarchical clustering of expression profiles [15], and the popular cutoff value of 10.0 [52] was adopted as the threshold.

4.6 Graphical Gaussian modeling

The average expression profiles were calculated for the members of each cluster, and the average correlation coefficient matrix between the clusters was calculated. The average correlation coefficient matrix between the clusters was then subjected to GGM as described previously [15, 16]. The correlation coefficient can return a false value in the presence of confounding factors. Partial correlation enables replacement of a false-positive correlation with the actual correlation. The PCCM was calculated using GGM (Supplemental Figure A). All calculations for clustering analysis and GGM were performed via the ASIAN web site (http://eureka.cbrc.jp/asian/index_j.html) [53] and "Auto Net Finder," a commercial desktop version of ASIAN (Infocom Corporation, Shibuya, Tokyo, Japan, http://www.infocom.co.jp/bio/download/).

4.7 Rearrangement of the inferred network

Since the magnitude of the partial correlation coefficient indicates the strength of the association between clusters, the intact network can be rearranged according to the partial correlation coefficient to interpret the association between clusters. The strength of the association can be assigned by a standard test for the partial correlation coefficient. In the present study, the significance level in the t-test was 1% (Supplemental Figure A).

4.8 Gene ontology of cluster members

Functional ontology enrichment analysis was performed to examine the gene ontology process distribution of each cluster gene using MetaCore™ (Thomson Reuters, New York, NY). Gene ontology was also confirmed by DAVID Bioinformatics Resources 6.7 (http://david.abcc.ncifcrf.gov/) [17].

Acknowledgements

The authors thank Nami Nishiyama for excellent technical assistance.

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Figure legends

Fig. 1. One way hierarchical clustering of 783 differentially expressed genes in CH-B-related HCC

A total of 783 genes were differentially expressed in CH-B-related HCC. Up-regulated genes are shown in red, down-regulated genes are shown in green, and unchanged genes are shown in white (Figure 1).

Fig. 2. One way hierarchical clustering of 668 differentially expressed genes in CH-B-related HCC

A total of 668 genes were differentially expressed in CH-C-related HCC. Up-regulated genes are shown in red, down-regulated genes are shown in green, and unchanged genes are shown in white (Figure 2).

Fig. 3. GGM analysis of each cluster in HCC and non-cancerous lesions

Each cluster in the HCC and non-cancerous lesions was connected according to partial correlation coefficient matrix (PCCM) by GGM algorithms (Supplemental Tables H and I). The blue lines indicate a negative partial correlation and the black lines indicate a positive partial correlation. The size of each cluster reflects the number of clustered genes. The red circles are up-regulated gene clusters, while the green circles are down-regulated gene clusters. Within each cluster, the blue area indicates the proportion of genes that are over-expressed in CPA, while the deep purple area indicates the proportion of genes that are over-expressed in CLL.

A; interactions of HBV related clusters

B; interactions of HCV related clusters

Fig. 4. Individual gene interactions between gene clusters in HCC and non-cancerous lesions

Direct interactions of individual genes among each cluster were confirmed by reference to the MetaCore database. The blue arrows indicate negative regulation, while the black arrows indicate positive regulation. Unspecified interactions are shown with black lines. The red squares are up-regulated gene clusters, while the green squares are down-regulated gene clusters.

A; direct interactions of genes in HBV related clusters

B; direct interactions of genes in HCV related clusters

Erratum

Erratum to Gene expression profiling of hepatitis B- and hepatitis C-related hepatocellular carcinoma using graphical Gaussian modeling
[Genomics 101/4 (2013) 238–248]

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The publisher regrets that the on page 246, in 4.2.Microarray analysis, Accession NO. GSE41804 was a wrong number. The correct number should be GSE44074.

The publisher would like to apologise for any inconvenience caused.

DOI of original article: < http://dx.doi.org/10.1016/j.ygeno.2013.02.007 >
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