[Frontiers in Bioscience E2, 829-840, June 1, 2010]

Expression profile reveals novel prognostic biomarkers in hepatocellular carcinoma

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1. ABSTRACT

The purpose of this study was to identify and validate novel prognostic biomarkers in human hepatocellular carcinoma (HCC). We analyzed gene expression profiles not only between 33 HCCs and their corresponding noncancerous liver tissues, but also between 25 HCCs and pooled normal liver tissues using cDNA microarrays containing 12800 genes. Functional analysis of differentially expressed genes involved in HCC carcinogenesis and tumor progression revealed that up-regulated and down-regulated genes are mainly associated with cell cycle and immune response, respectively. We detected two regions of cytogenetic changes only in poorly-differentiated HCCs using the expression data. We identified a 9-gene expression signature, which was able to predict differentiation degree and survival of HCC samples. Among the 9 most discriminatory genes, minichromosome maintenance protein 2 (MCM2), a significantly up-regulated gene involved in cell cycle pathway, was selected for further analysis. Overexpression of MCM2 protein related to poor-differentiation in HCC was validated using tissue microarray-based immunohistochemistry containing 96 HCCs. Our studies show that the 9-gene expression signature may serve as promising prognostic biomarkers involved in hepatocarcinogenesis and tumor progression.

2. INTRODUCTION

Human hepatocellular carcinoma (HCC) is one of the most common cancers and a leading cause of death worldwide. Currently, there is no effective therapy for most HCC patients. It is critical to identify and validate novel anticancer targets in HCC. Multiple factors have been reported to be involved in hepatocarcinogenesis (1-3), including exposure to aflatoxin B1 (AFB1), chronic viral hepatitis and cirrhosis. Progress in basic scientific research has led to a better understanding of molecular mechanism responsible for HCC (1-4). For example, genomic alterations (1, 2) (5, 6) and many deregulated genes such as *HBx*, *TP53*, *IGF2*, *CDKN2A* ($p16^{INK4A}$), *RB1*, *PTEN*, *DLC1*, *MMP*, *APC*, *CTNNB1*, and *AXIN1* (1-4, 7) may play roles in development of HCC. Biological pathways such as MAPK/ERK, ras/raf/MAPK, NF_{κ}-B, ERBB2/NEU, JAK/STAT, and Wnt/β-catenin signal transduction pathways (1) have been found to be altered in HCC. However, these genetic changes do not precisely reflect biological nature of cancer.

It is generally accepted that DNA microarrays and tissue microarray (TMA) are useful tools for identification and validation of biomarkers in disease research. In recent years, DNA microarrays have been used

to identify genes involved in various diseases including HCC (8-32). To apply microarray data to clinical use, it is necessary to identify a small set of genes which can be used as clinical biomarkers. It is useful to combine DNA microarrays with TMA for cancer profiling. TMA has gained increased popularity in identifying proteins involved in disease diagnosis and therapy (13, 16, 22, 33-42).

In this study, we systematically combined cDNA microarrays and TMA analyses to identify and validate novel prognostic biomarkers involved in HCC carcinogenesis and tumor progression. Our findings may help further discover genetic mechanism of HCC, and provide clues for identifying novel prognostic, diagnostic and therapeutic targets.

3. MATERIALS AND METHODS

3.1. Tissue samples and RNA isolation

All the 58 primary HCC samples and noncancerous liver tissues used in cDNA microarray analysis were obtained with informed consent from patients who underwent curative resection at different Chinese hospitals in Guangxi and Shanghai, with full institutional review board approval. They are predominantly male and hepatitis B surface antigen (HBsAg)-positive. All liver tissues were verified by pathological examination. HCC samples were histopathologically diagnosed following Edmonson's classification (43). Normal liver tissues were obtained from 5 healthy individuals who died from accidents. Total RNA was extracted from each sample using TRIzol (GibcoBRL, Grand Island, NY) following manufacturer's instructions.

3.2. cDNA microarrays

Fabrication of cDNA microarray containing 12800 genes, probe preparation, microarray hybridization, image detection and data normalization were carried out as previously described (10, 11).

For convenience of comparison, ratios of Cy5 (tumor) to Cy3 (nontumor) were $\log_2$-transformed and then converted back to fold change. Differentially expressed (DE) genes in HCC were selected according to criteria of $P<0.05$ by one-way analysis of variance (ANOVA) test (44), false discovery rate (FDR)≤5% (45), and fold change≥1.5. Among these genes, we identified discriminatory genes in poorly-differentiated HCCs (HCCs of grade III and IV) relative to well-differentiated HCCs (HCCs of grade I and II) according to criteria of $P<0.05$ by one-way ANOVA F test and FDR≤5% (44-47), which might be used to discriminate poorly-differentiated HCCs from well-differentiated HCCs. To perform Fisher discriminant analysis (FDA) classification (46-48), we selected the most discriminatory genes which were significantly up-regulated in poorly-differentiated HCCs relative to well-differentiated HCCs, which were selected based on criteria of $P<0.01$ by one-way ANOVA F test, FDR≤1%, fold change≥1.75, and misclassification rate by leave-one-out cross-validation (LOOCV) (44-48). We used permutation test (10000 permutations) to assess significance of our LOOCV misclassification rate, and $P<0.05$ was considered significant (49, 50). Further details of selection of most discriminatory genes based on ANOVA and LOOCV, and FDA classification were described in previous reports (44-50). Hierarchical clustering analysis was performed using Cluster and TreeView softwares (51).

We used EASE software (available at http://david.abcc.ncifcrf.gov/ease/ease.jsp) to assign DE genes to "Gene Ontology (GO) Biological Process" categories and test statistically (EASE Score, modified Fisher's exact test) for significant overrepresentation of identified genes within each category (52, 53). EASE Score<0.05 was considered significant.

Pathway analysis was performed using GenMAPP 2.0 software (available at http://www.genmapp.org) (54). $P<0.05$ calculated by MAPPFinder was considered significant.

To identify regions of frequent cytogenetic aberrations in HCCs using gene-expression microarray data, locally un-weighted smoothing cytogenetic aberrations prediction (LS-CAP) analysis was performed according to our previous report (55). Statistically significant standard was set up as $Z=1.96$ ($P=0.05$), and regions with $Z\geq1.96$ are identified as regional gene expression biases.

Full cDNA microarray data followed MIAME guidelines and will be available in NCBI's Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/. Accession number: GSE4108).

3.3. Tissue microarray-based immunohistochemistry

MCM2 protein expression in HCC was analyzed using TMA from Cybrdi (Gaithersburg, MD) containing 96 HCCs, 23 cirrhosis liver tissues and 24 normal liver tissues, which were spotted in two separate arrays. All liver tissues were verified by pathological examination. Immunohistochemistry analysis was performed using the Elivision plus two-step System (Dako, Carpinteria, CA) following manufacturer's instructions. Slides were incubated with primary anti-human MCM2 mouse monoclonal antibody (1:25, Abcam, Cambridge, UK). Diaminobenzidine and hematoxylin were used as chromogen and counterstain, respectively. Dark brown granules in nuclei were taken as positive reaction.

3.4. Statistical analysis

In cDNA microarray, Kaplan-Meier survival analysis was used to compare patient survival. Statistical P value was generated by log-rank test. Survival time was constructed from diagnosis date until death date or last follow-up. Patients known to be alive at their last follow-up were censored.

In TMA, chi-square test was used to assess significance of differences of MCM2 protein expression among HCCs with different clinicopathologic

characteristics, and among HCCs, cirrhosis liver tissues, and normal liver tissues.

All analyses were performed using SPSS 12.0 software (Chicago, IL). *Ps*<0.05 were considered significant.

4. RESULTS

4.1. Reproducibility of cDNA microarray

To evaluate reproducibility of cDNA microarray, duplicate microarray experiments were conducted. Correlation coefficient of Cy5/Cy3 ratios between two replicates was 0.94, indicating that our experiments are highly consistent as proved by our previous reports (11, 56).

4.2. Identification and validation of differentially expressed genes in HCC

To minimize bias from different reference controls, two sets of patients were analyzed using cDNA microarrays. Set one: we compared gene expression profiles between 33 HCCs and their corresponding noncancerous liver tissues; set two: we compared gene expression profiles between 25 HCCs and pooled normal liver tissues. We identified 642 and 1848 DE genes (P<0.05 by ANOVA; FDR≤5%; ≥1.5-fold) in two sets of patients, respectively, and intersection between two sets was 416 DE genes in HCC, in which 145 genes were up-regulated and 271 genes were down-regulated. Among 416 DE genes, 226 genes have not been reported in HCC before.

To confirm microarray result, we randomly selected two up-regulated genes (*SPP1* and *DAP3*) and two down-regulated genes (*ALDH2* and *ADH4*) to examine their expression levels using slot blot with 24 pairs of HCCs among 33 HCCs used for microarray analysis. Results of slot blot showed changes in gene expression consistent with microarray data.

4.3. Identification of a gene expression signature predicting differentiation degree and survival of HCC samples

Hierarchical clustering was performed using 416 DE genes to evaluate relationships of gene expression patterns and clinical phenotypes, such as sex, age, hepatitis virus infection, tumor stage, tumor size, tumor number, venous invasion, encapsulation, metastasis and differentiation degree. Except for differentiation degree, patients dendrograms obtained by this evaluation showed no placing related to any of the other clinical phenotypes.

To discriminate poorly-differentiated HCCs from well-differentiated HCCs, we identified 231 and 248 discriminatory genes in poorly-differentiated HCCs relative to well-differentiated HCCs in 33 HCCs and 25 HCCs sets, respectively (P<0.05 by one-way ANOVA F test; FDR≤5%), and 160 intersection genes were found in both sets, in which 65 genes were up-regulated and 95 genes were down-regulated. Among 160 discriminatory genes, 149 genes have not been reported in poorly-differentiated HCC before.

To decrease complexity, we selected 9 most discriminatory genes (*MCM2, CCNB1, SPP1, CDC7, SMC4L1, BIRC5, ASNS, CCT6A,* and *KNTC1*) which were significantly up-regulated in poorly-differentiated HCCs relative to well-differentiated HCCs (≥1.75-fold; P<0.01 by one-way ANOVA F test; FDR≤1%; misclassification rate calculated by LOOCV) to perform FDA. Gene expression signature based on less than the 9 discriminatory genes decreased discriminatory accuracy, and addition of more genes to the 9-gene signature provided no additional discriminated value. HCC samples were found to be divided into two distinct groups related to differentiation degree using hierarchical clustering analysis with 160 discriminatory genes (Figure 1A) and 9 most discriminatory genes (Figure 1B), respectively.

LOOCV was used to comfirm and evaluate effectiveness of the 9-gene expression signature, and class prediction accuracy resulted in 88% and 94% correct assignments in 25 HCCs and 33 HCCs sets, respectively (P<0.0005 and P<0.01 as measured by permutation testing in 25 HCCs and 33 HCCs sets, respectively). To further validate predictive performance, we used FDA classification with the 9-gene expression signature to reciprocally predict differentiation degree of 33 HCCs and 25 HCCs, in which poorly-differentiated HCCs and well-differentiated HCCs were separated in the FDA projection defined discriminant axes of the 9 most discriminatory genes (Figure 1C and D). Using the 9-gene expression signature in 25 HCCs to predict 33 HCCs, 12 of 17 patients were correctly classified as poorly-differentiated HCCs (71%), and 16 of 16 patients were correctly classified as well-differentiated HCCs (100%), with overall accuracy of 85% (Figure 1C). Using the 9-gene expression signature in 33 HCCs to predict 25 HCCs, 14 of 15 patients were correctly classified as poorly-differentiated HCCs (93%) and 8 of 10 patients were correctly classified as well-differentiated HCCs (80%), with overall accuracy of 88% (Figure 1D). These results indicated that the 9-gene expression signature was able to predict differentiation degree of HCC samples.

Kaplan–Meier curves suggested significantly shorter survival among poorly-differentiated HCC patients than among well-differentiated HCC patients (Figure 1E-G). Notably, the difference could be more significant in subdivision based on the 9-gene expression signature (P<0.01, Figure 1F and G) than subdivision based on clinical and histopathological criteria (P=0.0205, Figure 1E).

4.4. Functional analysis of Gene Ontology biological process categories and pathways involved in HCC carcinogenesis and tumor progression

Using EASE analysis, we identified overrepresented GO biological process categories (EASE score<0.05) with 416 DE genes in HCCs relative to nontumor liver tissues and 160 discriminatory genes in poorly-differentiated HCCs relative to well-differentiated HCCs, respectively (Table 1). Among these themes, we identified some important categories which have not been reported before, such as category of response to external

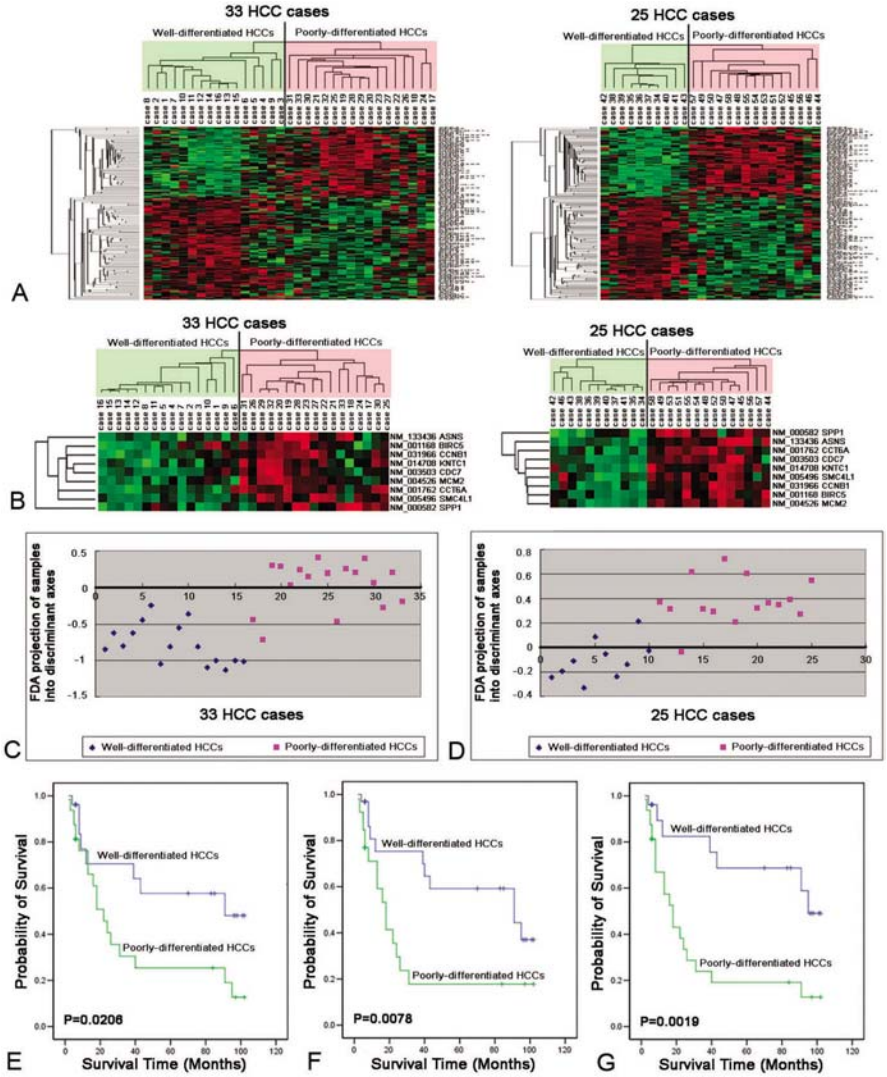

Figure 1. Identification of a gene expression signature predicting differentiation degree and survival of HCC samples. ***(A),*** hierachical clustering analysis using the common 160 discriminatory genes in poorly-differentiated HCCs relative to well-differentiated HCCs (P<0.05 with threshold (F0.05(1,31)=4.17 in 33 HCCs and F0.05(1,23)=4.28 in 25 HCCs, respectively) by one-way ANOVA F test; FDR≤5%). Columns represent tumor samples; rows represent genes. Changes in relative expression are presented in color patterns. Red, overexpression; green, underexpression; black, unchanged expression. ***(B)***, hierachical clustering analysis using the 9 most discriminatory genes which were significantly up-regulated in poorly-differentiated HCCs relative to well-differentiated HCCs (≥1.75-fold; P<0.01 with threshold ($F_{0.01(1,31)}$=7.56 in 33 HCCs and $F_{0.01(1,23)}$=7.88 in 25 HCCs, respectively) by one-way ANOVA F test; FDR≤1%; misclassification rate of 6% and 12% calculated by leave-one-out cross-validation (LOOCV) in 33 HCCs and 25 HCCs sets, respectively (P<0.01 and P<0.0005 as measured by permutation testing in 33 HCCs and 25 HCCs sets, respectively)). Columns represent tumor samples; rows represent genes. Changes in relative expression are presented in color patterns. *Red*, overexpression; *green*, underexpression; *black*, unchanged expression. ***(C-D)***, validation of predictive performance using Fisher discriminant analysis (FDA) classification with the 9-gene expression signature to reciprocally predict the differentiation degree of 33 HCCs and 25 HCCs. Poorly-differentiated HCCs and well-differentiated HCCs were separated in the FDA projection defined discriminant axes of the 9 most discriminatory genes. Each point represents a HCC sample. Poorly-differentiated samples yielded positive values; well-differentiated samples yielded negative values. ***(C),*** prediction of 33 HCCs using the 9-gene expression signature in 25 HCCs. 12 of 17 poorly-differentiated HCCs (71%) and 16 of 16 well-differentiated HCCs (100%) were classified correctly. ***(D)***, prediction of 25 HCCs using the 9-gene expression signature in 33 HCCs. 14 of 15 poorly-differentiated HCCs (93%) and 8 of 10 well-differentiated HCCs (80%) were classified correctly. ***(E-G),*** Kaplan-Meier survival curves in all the 58 HCC patients after subdivision into two subgroups (well-differentiated HCCs and poorly-differentiated HCCs) based on clinical and histopathological criteria(*E*), the 9-gene expression signature in 25 HCCs(*F*), and the 9-gene expression signature in 33 HCCs(*G*), respectively. *P* values were calculated by log-rank test and the differences between groups were significant (P<0.05). +, time of censorship.

Table 1. GO biological process categories overrepresented by differentially expressed genes involved in HCC carcinogenesis and tumor progression (EASE score<0.05)

Gene category	EASE score	No. of up-regulated genes	Gene category	EASE score	No. of down-regulated genes
In HCCs relative to nontumor liver tissues					
cell cycle	0.0000	19	complement activation	0.0000	10
cell proliferation	0.0002	22	blood coagulation	0.0000	12
DNA replication and chromosome cycle	0.0002	9	cell ion homeostasis	0.0011	8
cell growth and death	0.0033	8	immune response	0.0019	26
DNA metabolism	0.0067	12	response to external stimulus	0.0020	42
cell growth and/or maintenance	0.0073	45	regulation of blood pressure	0.0022	5
cell-matrix adhesion	0.0160	3	metabolism	0.0044	150
pathogenic Invasion	0.0309	4	signal transduction	0.0350	6
protein metabolism	0.0351	30			
In poorly-differentiated HCCs relative to well-differentiated HCCs					
cell cycle	0.0000	14	complement activation	0.0000	6
cell proliferation	0.0000	16	blood coagulation	0.0000	7
cell growth and/or maintenance	0.0016	25	immune response	0.0003	15
DNA replication and chromosome cycle	0.0054	5	response to external stimulus	0.0046	19
protein metabolism	0.0136	17	metabolism	0.0377	56
DNA metabolism	0.0154	7			
cell growth and death	0.0160	5			

stimulus involved in HCC development, and categories of DNA replication and chromosome cycle, complement activation, blood coagulation, and response to external stimulus involved in poorly-differentiated HCC progression. The categories in poorly-differentiated HCCs relative to well-differentiated HCCs were of particular interest because they reflect groups of genes correlated with tumor progression and poor prognosis.

GenMAPP 2.0 software was used to identify biological pathways with 416 DE genes and 160 discriminatory genes, respectively. Genes in three major pathways (Cell_cycle, Complement_and_Coagulation_Cascades, and Fatty_Acid_Degradation) were significantly altered in HCCs relative to nontumor liver tissues (P<0.05) (Figure 2A). Genes in two major pathways (Cell_cycle, and Complement_and_Coagulation_Cascades) were significantly altered in poorly-differentiated HCCs relative to well-differentiated HCCs (P<0.05) (Figure 2B). Notably, seven cell cycle-related genes up-regulated in HCC (*MCM2, CCNB1, CCNB2, CDC7, CDC25C, BUB1B,* and *MAD2L1*) were more significantly up-regulated in poorly-differentiated HCCs. They play important roles in regulating different phase progression of cell cycle -- MCM2 and CDC7 in S phase; CCNB1, CCNB2 and CDC25C in G2 phase; BUB1B and MAD2L1 in M phase. MCM2 is phosphorylated, and regulated by CDC7 (57). CDC25C directs dephosphorylation of CCNB-bound CDC2 and triggers entry into mitosis. BUB1B and MAD2L1 act cooperatively to prevent premature sister chromatids separation by directly inhibiting anaphase-promoting complex.

GO and pathway analysis of DE genes involved in both HCC carcinogenesis and tumor progression revealed that up-regulated genes are mainly associated with cell cycle and cell proliferation, while down-regulated genes are mainly associated with immune response.

4.5. Cytogenetic aberrations analysis

Cytogenetic aberrations, such as amplification and deletion, could be identified precisely using locally un-weighted smoothing cytogenetic aberrations prediction (LS-CAP) with gene-expression microarray data (55). With span being 250 genes and fold change being 1.5, 12 regions of frequent cytogenetic changes were identified in HCCs ($Z \geq 1.96$), including 7 gains (1q, 6p, 7p, 7q, 8q, 17q and 20q) and 5 losses (3p, 4q, 6p, 11p and 11q). The Z statistic for cytogenetic changes and precise localizations of cytogenetic aberrations are shown in Figure 3. Notably, gains of chromosome 7p11.1-p22.3 and 8q24.1-q24.3 were identified only in poorly-differentiated HCCs, but not in well-differentiated HCCs. In addition, cytogenetic aberrations and candidate genes were analyzed with the same dataset using LS-CAP.

4.6. Validation of MCM2 protein overexpression related to poor-differentiation in HCC

Among the 9 most discriminatory genes, two up-regulated genes involved in cell cycle pathway (*MCM2* and *CCNB1*) were significantly up-regulated in at least 50% HCCs in cDNA microarray. Validation of CCNB1 protein overexpression in HCC using TMA has been reported (33), whereas analysis of MCM2 protein in HCC has not been demonstrated before. Here, we analyzed MCM2 protein expression in HCC with TMA-based immunohistochemistry for further investigation (Figure 4A). MCM2 protein expression levels were significantly higher in HCCs (57% positive, 55 of 96 HCCs, P<0.001) (Figure 4B) than in cirrhosis liver tissues (35% positive, 8 of 23 tissues) and normal liver tissues (13% positive, 3 of 24 tissues) (Figure 4C). Moreover, MCM2 protein expression levels, which showed a significant positive association with histopathological grade, were significantly higher in poorly-differentiated HCCs (HCCs of grade III) (68% positive, 32 of 47 cases, P=0.036) than in well-differentiated HCCs (HCCs of grade I and II) (47% positive, 23 of 49 cases). These results indicated that overexpression of MCM2 protein related to poor-

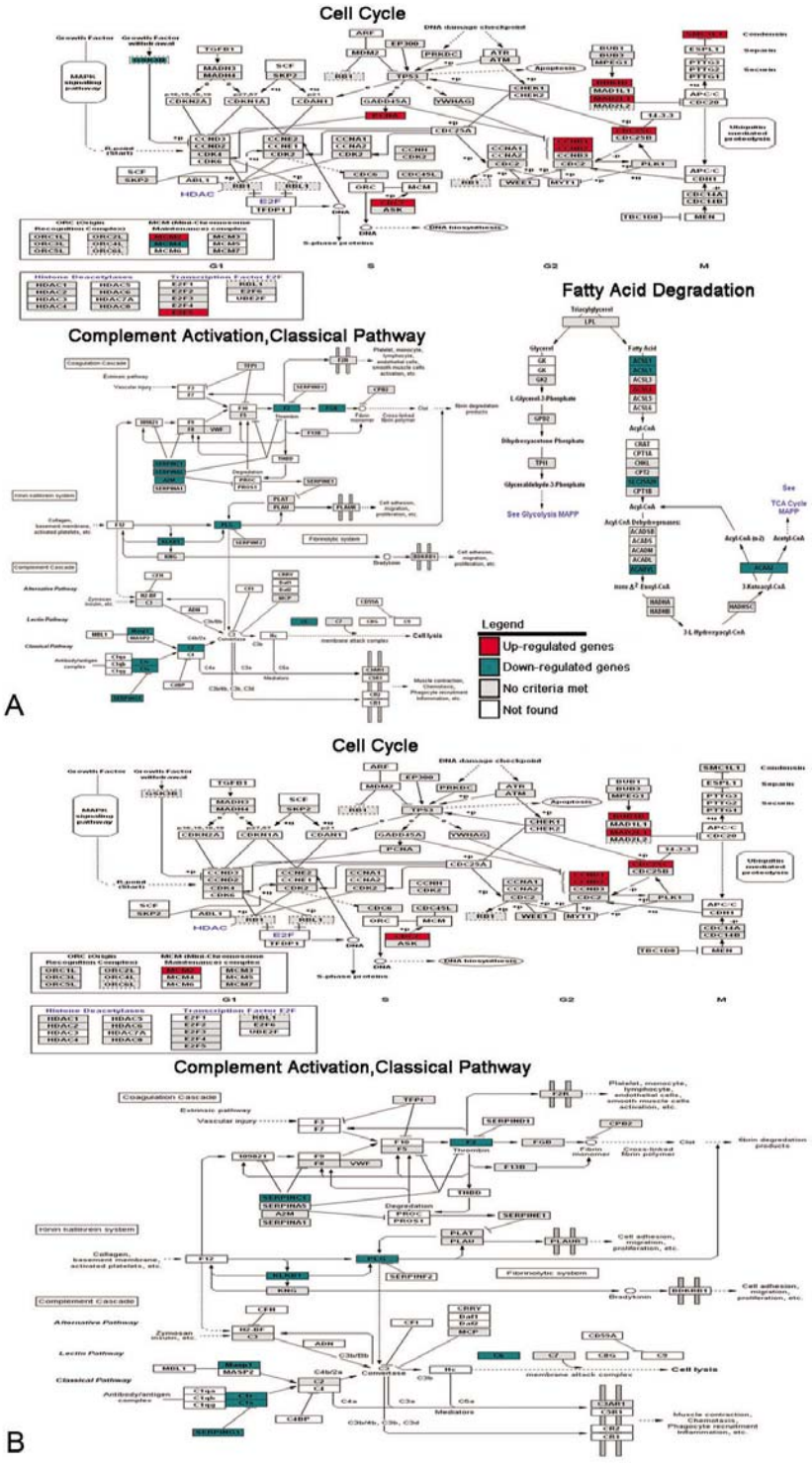

Figure 2. GenMAPP analysis of pathways involved in HCC carcinogenesis and tumor progression. Our expression data are integrated into GenMAPP pathways. Color-coding of genes is as follows: Red, overexpression; green, underexpression; Gray, neither of the above criteria met; White, gene not found on the array. *(A)*, three major pathways significantly altered in HCCs relative to nontumor liver tissues: Cell_cycle, Complement_and_Coagulation_Cascades, and Fatty_Acid_Degradation *(P*<0.05*). (B),* two major pathways significantly altered in poorly-differentiated HCCs relative to well-differentiated HCCs: Cell_cycle, and Complement_and_Coagulation_Cascades *(P*<0.05*)*.

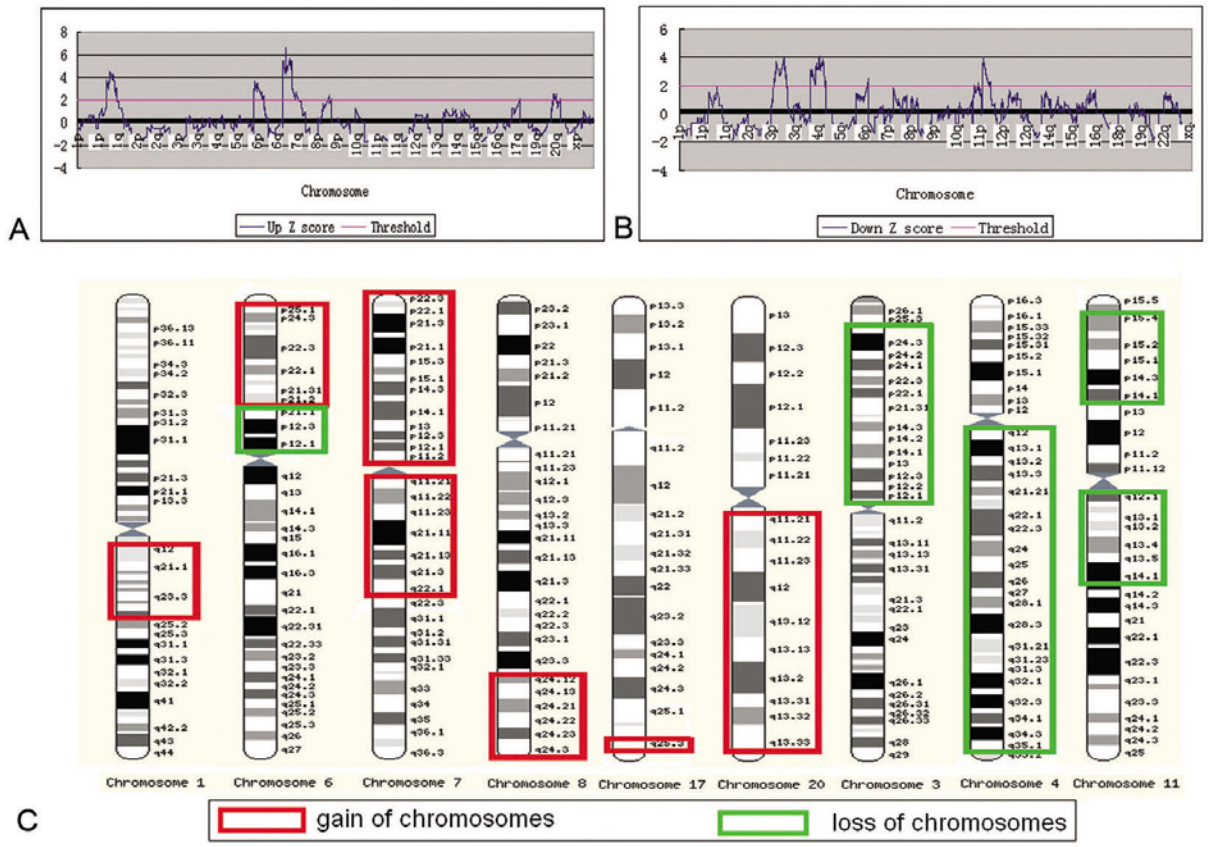

Figure 3. Frequent cytogenetic aberrations identified using LS-CAP analysis ($Z \geq 1.96$). 12 regions of cytogenetic aberrations identified HCCs. *(A),* Z statistic for frequent gain of chromosomes. *(B)*, Z statistic for frequent loss of chromosomes. *(C)*, precise localizations of cytogenetic aberrations. Red indicates gain of chromosomes; green indicates loss of chromosomes.

differentiation might be involved in HCC carcinogenesis and tumor progression.

5. DISCUSSION

We analyzed gene expression profiles in HCC using cDNA microarrays. Selection of appropriate control is very important for microarray study (58). The debatable issue is whether corresponding noncancerous liver tissues (18) or pooled normal liver tissues (11) should be used. Influence of individual variations can be excluded using corresponding noncancerous liver tissues; however, noncancerous tissues may be genetically altered. Clustering analysis among multiple samples can be facilitated using pooled normal liver tissues; yet influence of individual variations can't be completely excluded with limited normal tissues. Thus, combination of the two strategies can help minimize disadvantages of control selection. Here, we analyzed gene expression profiles not only between 33 HCCs and their corresponding noncancerous liver tissues, but also between 25 HCCs and pooled normal liver tissues. 416 common DE genes in HCC were identified in both data sets, indicating consistent results from the two references.

We used slot blot to validate four DE genes in HCC, two up-regulated genes (*SPP1* and *DAP3*) and two down-regulated genes (*ALDH2* and *ADH4*). Osteopontin (SPP1) was up-regulated in HCC, especially in poorly-differentiated HCC. SPP1 is a potential diagnostic marker because it can be found in all bodily fluids and it plays a role in anti-apoptosis, cell adhesion and migration. It has been reported that SPP1 may support metastasis in HCC (9). Interestingly, we found that death associated protein 3 (DAP3) was up-regulated in HCC. DAP3 overexpression has been reported in invasive glioblastoma (59), which has not been reported in HCC before. Although function of full-length DAP3 protein has been described as induction of apoptosis, NH(2)-terminal fragment can act in a negative way resulting in protection from apoptosis. Thus, DAP3 may confer apoptosis-resistance in HCC. Moreover, reason for DAP3 overexpression may be that it maps to frequent cytogenetic gain of 1q21-q22 in HCC. Both down-regulated genes, aldehyde dehydrogenase 2 (ALDH2) and alcohol dehydrogenase 4 (ADH4), are associated with alcohol metabolism, which are consistent with previous reports that detoxification related genes are always down-regulated in HCC (18).

We identified a gene expression signature based on 9 most discriminatory genes (*MCM2, CCNB1, SPP1, CDC7, SMC4L1, BIRC5, ASNS, CCT6A,* and *KNTC1*), which was able to predict differentiation degree of HCC

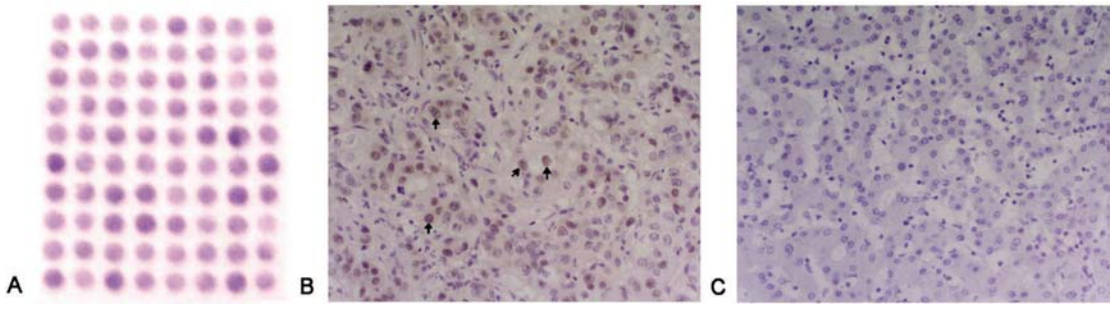

Figure 4. Expression of MCM2 protein in HCC using tissue microarray-based immunohistochemistry analysis. *(A)*, photomicrograph of HCC tissue microarray illustrating the array containing numerous small 1.5-mm cores of tissues. *(B)*, representative HCC tissue showing strong nuclear staining of MCM2 in cancer cells (*arrows*). *(C),* representative normal liver tissue, where MCM2 staining is undetectable. (*B* and *C*: magnification, x200)

samples. MCM2, CCNB1, CDC7, SMC4L1, BIRC5, and KNTC1 play roles in cell proliferation and cell cycle; while SPP1 and BIRC5 play roles in cell proliferation and anti-apoptosis. For validation of predictive performance, we successfully used the 9-gene expression signature to reciprocally predict the differentiation degree of 33 HCCs and 25 HCCs. The 9-gene expression signature could be used to help classification and prognosis of HCCs associated with differentiation degree. Furthermore, gene expression profiles of corresponding noncancerous liver tissues and pooled normal liver tissues are similar in some degree. Both of them can be used as appropriate references. Kaplan-Meier survival data indicated that poorly-differentiated HCC patients had substantially shorter survival than well-differentiated HCC patients. Notably, the 9-gene expression signature was a more powerful predictor of survival of HCC patients than standard systems based on clinical and histopathological criteria.

GO biological process and pathway analysis of DE genes involved in HCC carcinogenesis and tumor progression revealed that up-regulated genes are mainly associated with cell cycle, while down-regulated genes are mainly associated with immune response, which is consistent with previous report (18). Cell cycle-related genes were important because they might be directly bound up with tumor development. Furthermore, groups of DE genes in poorly-differentiated HCCs relative to well-differentiated HCCs were more important because they correlated with tumor progression and poor prognosis, which was insufficiently studied before. Some of these genes might be effective anticancer targets, such as seven up-regulated genes associated with cell cycle pathway. Except for *CCNB1* (33), our work is the first report of the association of the other six genes (*MCM2, CCNB2, CDC7, CDC25C, BUB1B,* and *MAD2L1*) with poorly-differentiated HCC. Overexpression of these genes might contribute to activation of cell cycle pathway and play critical roles in HCC carcinogenesis and tumor progression.

Interestingly, among the seven up-regulated cell cycle-related genes, three genes (*MCM2, CCNB1* and *CDC7*) also appeared in the 9 most discriminatory genes. MCM2 and CCNB1 were chosen for further analysis. Reason for overexpression of MCM2 and CCNB1 may be that they map to frequent cytogenetic gains of 3q21 and 5q12 in HCC (5, 6), respectively; while their overexpression may drive selection for the chromosomal gains. Moreover, as both MCM2 and CCNB1 are cell cycle regulated (60), their overexpression are likely to be due to increased cell proliferation and cycling in cancer cells; while their overexpression may induce cell proliferation. MCM2 is one of the MCM proteins which are essential for initiating and elongating replication forks during S-phase (61). Moreover, MCM proteins affect chromosome structure, which is consistent with the evidence that most MCM proteins don't colocalize with DNA synthesis sites (62). CCNB1 complexes with CDC2 to form M-phase promoting factor (MPF), which is essential for G2/M phase transitions of cell cycle (63). Because MCM2 and CCNB1 have a direct effect on mitosis, their overexpression in HCC may lead to uncontrolled cell proliferation and tumorigenesis.

Recent studies have shown that MCM2 and CCNB1 are overexpressed in various tumors but present at low levels in normal tissues, indicating that they may be specific anticancer targets (34, 64). In HCC research, increased MCM2 mRNA levels have been reported (65). Here, we used for the first time TMAs to validate MCM2 protein overexpression in HCC, especially in poorly-differentiated HCC. We did not validate CCNB1 since its protein overexpression related to poor-differentiation in HCC has been reported (33). Therefore, MCM2 and CCNB1 may be potential diagnostic and therapeutic targets involved in HCC carcinogenesis and tumor progression.

In addition, we used gene-expression microarray data to predict cytogenetic changes that frequently occur in HCCs, especially in poorly-differentiated HCCs using LS-CAP approach previously reported (55). Previous CGH studies about genomic aberrations in HCCs showed that gains were prevalent on chromosome regions 1q, 3q, 5q, 6p, 7p, 7q, 8q, 17q and 20q, while the most frequent losses occurred on lp, 3p, 4q, 6q, 8p, 11p, 11q, 13q, 14q, 16q and 17p (5, 66, 67). In this study, we successfully identified the regions of frequent gains of 1q, 6p, 7p, 7q, 8q, 17q and 20q, and the regions of frequent losses of 3p, 4q, 11p and 11q as previously reported. We also detected loss of chromosome 6p11.2-p21.1 that has not been implicated by previous reports. Interestingly, 6p21.2-p25.2 and 6p11.2-p21.1 were

identified to be regions of frequent gains and losses, respectively. Furthermore, cytogenetic changes in poorly-differentiated HCCs were also identified using the same approach. Notably, gains of chromosome 7p11.1-p22.3 and 8q24.1-q24.3 were identified in poorly-differentiated HCCs, but not in well-differentiated HCCs, which might be associated with degree of differentiation and tumor progression. Some oncogenes and anti-oncogenes, which expressed differentially in this study, locate on the regions identified by LS-CAP. For instance, oncogenes DEK, RAC1, BIRC5 and STK6 which were up-regulated respectively map to frequent cytogenetic gains of 6p23, 7p22, 17q25 and 20q13.2-q13.3; and tumor suppressor HYAL2 which was down-regulated maps to frequent cytogenetic loss of 3p21.3. Therefore, we assumed that the altered expression of these tumor-related genes might result from the frequent chromosomal aberrations.

In conclusion, we have identified a 9-gene expression signature, which was able to predict differentiation degree of HCC samples and was a more powerful predictor of survival of HCC patients than standard systems based on clinical and histopathological criteria. We detected two regions of cytogenetic changes only in poorly-differentiated HCCs using the expression data. Our studies also show that MCM2 and CCNB1 may be potential biomarkers involved in HCC carcinogenesis and tumor progression.

6. ACKNOWLEDGMENT

Meiqian Sun and Gang Wu equally contributed to this article. This study supported by grants from the China 863 project (2006AA02Z324). We thank Xianmin He, Qin Wei, Xueqing Guo, Shizhong Han, Guifen He and Yinghua Lu for helpful discussions.

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Abbreviations: ADH4: alcohol dehydrogenase 4; AFB1: aflatoxin B1; ALDH2: aldehyde dehydrogenase 2; CCNB1: cyclin B1; DAB: diaminobenzidine; DAP3: death associated protein 3; DE: differentially expressed; EASE: Expression Analysis Systematic Explorer; FDA: Fisher discriminant analysis; FDR: false discovery rate; GenMAPP: Gene Microarray Pathway Profiler; GO: Gene Ontology; HCC: hepatocellular carcinoma; IHC: immunohistochemistry; LS-CAP: locally un-weighted smoothing cytogenetic aberrations prediction; MCM2: minichromosome maintenance protein 2; MPF: M-phase promoting factor; SPP1: secreted phosphoprotein 1; SPSS: SigmaStat statistical software package; TMA: tissue microarray

Key Words: Hepatocellular Carcinoma, Expression Profiling, DNA Microarray, Tissue Microarray, MCM2

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