Integrated Analysis of Copy Number Variations and Gene Expression Profiling in Hepatocellular carcinoma

Chenhao Zhou1,3, Wentao Zhang1,3, Wanyong Chen1,2,3, Yirui Yin1,3, Manar Atyah1,3, Shuang Liu1,3, Lei Guo1,3, Yi Shi4, Qinghai Ye1,3, Qiongzhu Dong2,5 & Ning Ren1,2,3

Hepatocellular carcinoma (HCC) is one of the top three types of fatal cancer worldwide. To identify CNV-driven differentially expressed genes (DEGs) in HBV related HCC, this study integrated analysis of copy number variations (CNVs) and gene expression profiling. Significant genes in regions of CNVs were overlapped with those obtained from the expression profiling. 93 CNV-driven genes exhibiting increased expression in the duplicated regions and 45 showing decreased expression in the deleted regions were obtained, which duplications and deletions were mainly documented at chromosome 1 and 4. Functional and pathway enrichment analyses were performed using DAVID and KOBAS, respectively. They were mainly enriched in metabolic process and cell cycle. Protein-protein interaction (PPI) network was constructed by Cytoscape, then four hub genes were identified. Following, survival analyses indicated that only high NPM1 expression was significantly and independently associated with worse survival and increased recurrence in HCC patients. Moreover, this correlation remained significant in patients with early stage of HCC. In addition, we showed that NPM1 was overexpressed in HCC cells and in HCC versus adjacent non-tumor tissues. In conclusion, these results showed that integrated analysis of genomic and expression profiling might provide a powerful potential for identifying CNV-driven genes in HBV related HCC pathogenesis.
Several studies have been conducted through integrated analysis of CNVs and gene expression profiling in HCC, but they were limited to the use of small sized tumor samples or relatively lower-resolution platforms. In this study, we applied a whole-genome SNP 6.0 array to analyze CNVs of the 33 paired HBV related HCC and non-tumor tissues. The gene expression profiling data were obtained from our previous studies (GSE14520).

By integrating the analysis of CNVs and gene expression profiling to identify CNV-driven DEGs, we may light further insights of HBV related HCC development at a molecular level, and explore a clinically useful candidate gene for diagnosis, prognosis, and drug targets.

Results

Identification of significant CNVs in HCC genomes. We analyzed the hybridization signal intensities of 33 paired HCC and non-tumor tissues to identify regions of CNVs. A total of 13,839 CNVs were identified in the 33 HCC genomes, including 5,457 copy number deletions (mean size, 349.1 kb) and 8,382 copy number duplications (mean size, 419.0 kb) (Supplementary Table 1). CNVs were scattered across chromosome 1 to 22, and both of the highest duplication and deletion were found in chromosome 1. The second highest number of duplications and deletions were documented at chromosome 5 and 4, respectively. HCC genomes had a mean of 419 CNVs, and copy number duplications were more commonly observed than deletions (1.5:1). It was found that regions of >100 kb long had the most copy number deletions and duplications (Fig. 1a, b).

To find potential HCC-related significant CNVs, we further evaluated CNVs using the following standard: the gene of CNVs was present in at least 10% (4 samples) samples. Accordingly, a total of 2,912 significant CNV genes were obtained, including 875 deletions and 2,037 duplications (Supplementary Table 2 and 3).

Analysis of gene expression profiling. The total number of samples analyzed was 30 paired HCC and non-tumor tissues from our previous study (GSE14520). The baseline characteristics of these patients were similar to the 33 patients whose paired HCC and non-tumor tissues analyses were carried out with a whole-genome SNP 6.0 array (Table 1). All patients of these two cohorts were infected with hepatitis B virus. In total, 965 genes were differentially expressed by at least two-fold with statistical significance ($P < 0.05$). Of these, 389 genes were found to be up-regulated while 576 genes were found to be down-regulated.

Integrative analysis of CNVs and gene expression profiling to identify CNV-driven DEGs. To identify driver genes in regions of CNVs, we performed an integrated analysis of CNVs and gene expression profiling. A total of 793 genes with only gene expression changes and 2740 genes with CNV changes, but no changes in transcript levels, were observed. Furthermore, 138 CNV-driven genes were identified with the same expressional tendencies, including 93 CNV-driven genes exhibiting increased expression in the duplicated regions and 45 showing decreased expression in the deleted regions (Supplementary Table 4). The highest number of duplications was documented at chromosome 1, while the highest number of deletions was recorded at chromosome 4. Hierarchical clustering of the normalized gene expression values for the 138 CNV-driven genes was generated and visualized via a heat map, where distinctive expression patterns can be observed. The clustered heat map of expression values for 138 DEGs in the regions of CNVs can effectively distinguish tumor and non-tumor samples (Fig. 1c).
Functional and pathway enrichment analysis of CNV-driven DEGs. To gain further insights into the function of identified CNV-driven DEGs, functional and pathway enrichment analyses were performed using DAVID and KOBAS, respectively. According to Go analysis results, the down-regulated CNV-driven DEGs were significantly enriched in biological processes (BP), including ethanol oxidation, xenobiotic metabolic process and metabolic process; the up-regulated CNV-driven DEGs were significantly enriched in positive regulation of translation, cell division and DNA damage response (Table 2). On the basis of cellular component (CC), the down-regulated CNV-driven DEGs mostly assembled at the cytosol, extracellular exosome and apical plasma membrane, while up-regulated CNV-driven DEGs assembled at the pathways of membrane, nucleoplasm and cytoplasm (Table 2). In addition, for molecular function (MF), the down-regulated CNV-driven DEGs prominently accumulated in alcohol dehydrogenase activity and serine-type endopeptidase activity, and up-regulated CNV-driven DEGs were enriched in protein binding, poly(A) RNA binding and cadherin binding involved in cell-cell adhesion (Table 2).

As for KEGG pathway enrichment analysis, thirteen enriched key pathways were presented in the down-regulated CNV-driven DEGs, including chemical carcinogenesis and several metabolic pathways, such as metabolism of xenobiotics by cytochrome P450, tyrosine metabolism, and retinol metabolism (Fig. 2a). In addition, for molecular function (MF), the down-regulated CNV-driven DEGs prominently accumulated in alcohol dehydrogenase activity and serine-type endopeptidase activity, and up-regulated CNV-driven DEGs were enriched in protein binding, poly(A) RNA binding and cadherin binding involved in cell-cell adhesion (Table 2).

As for KEGG pathway enrichment analysis, thirteen enriched key pathways were presented in the down-regulated CNV-driven DEGs, including chemical carcinogenesis and several metabolic pathways, such as metabolism of xenobiotics by cytochrome P450, tyrosine metabolism, and retinol metabolism (Fig. 2a). In addition, eight KEGG pathways were significant for the up-regulated CNV-driven DEGs, such as cell cycle, DNA replication, and Hippo signaling pathway (Fig. 2b).

PPI network construction and module screening from the PPI network. The STRING database was used to analyze the PPI relationships of the CNV-driven DEGs. The PPI network of the CNV-driven DEGs consisted of 81 nodes and 103 edges, including sixteen down-regulated and 65 up-regulated CNV-driven DEGs (Fig. 3). Node degrees ≥ eight was selected as the threshold. Heat shock protein 90 alpha family class B member 1 (HSP90AB1), ribosomal protein L8 (RPL8), nucleophosmin (NPM1), and mini-chromosome maintenance complex component 3 (MCM3) were selected as the hub genes.

We then screened the significant module of the PPI network using plug-ins MCODE. In our study, four nodes were involved in this module and one hub gene with higher degrees, including eukaryotic translation initiation factor 3 subunit E (EIF3E), poly(A) binding protein cytoplasmic 1 (PABPC1), eukaryotic translation initiation factor 3 subunit H (EIF3H), and RPL8. (Fig. 3) Interestingly, all of these four genes were located at chromosome

| Clinical variable — no. (%) | Cohort 1 (N = 33) | Cohort 2 (N = 30) | P value* |
|---------------------------|------------------|------------------|---------|
| Gender                    |                  |                  |         |
| Male                      | 30(90.9)         | 25(83.3)         | 0.462   |
| Female                    | 3(9.1)           | 5(16.7)          |         |
| Age                       |                  |                  |         |
| ≥50 years                 | 17(51.5)         | 15(50)           | 1.000   |
| <50 years                 | 16(48.5)         | 15(50)           |         |
| AFP                       |                  |                  |         |
| >300 ng/mL                | 12(36.4)         | 18(60)           | 0.073   |
| ≤300 ng/mL                | 21(63.6)         | 12(40)           |         |
| ALT                       |                  |                  |         |
| >50 U/L                   | 12(36.4)         | 15(50)           | 0.316   |
| ≤50 U/L                   | 21(63.6)         | 15(50)           |         |
| Cirrhosis                 |                  |                  |         |
| Yes                       | 31(93.9)         | 28(93.3)         | 1.000   |
| No                        | 2(6.1)           | 2(6.7)           |         |
| Tumor size                |                  |                  |         |
| >5 cm                     | 15(45.5)         | 14(46.7)         | 1.000   |
| ≤5 cm                     | 18(54.5)         | 16(53.3)         |         |
| Multinodular              |                  |                  |         |
| Yes                       | 12(36.4)         | 6(20)            | 0.174   |
| No                        | 21(63.6)         | 24(80)           |         |
| BCLC staging              |                  |                  |         |
| 0-A                       | 21(63.6)         | 20(66.7)         | 1.000   |
| 0-C                       | 12(36.4)         | 10(33.3)         |         |
| TNM staging               |                  |                  |         |
| I                         | 13(39.4)         | 14(46.7)         | 0.616   |
| I–III                     | 20(60.6)         | 16(53.3)         |         |

Table 1. Clinical Characteristics of Patients in two Cohorts at the Time of Surgery. *Fisher’s exact tests; P value < 0.05 was considered statistically significant. Abbreviations: AFP, alpha-fetoprotein; ALT, alanine aminotransferase; BCLC, Barcelona Clinic Liver Cancer; TNM, tumor-nodes-metastases.
| Expression | Category     | Term/gene function                                | Count | P value      |
|------------|--------------|--------------------------------------------------|-------|--------------|
| Down-regulated | GOTERM_BP_DIRECT | GO:0006069~ethanol oxidation                     | 3     | 3.97E-04     |
|             | GOTERM_BP_DIRECT | GO:0006805~xenobiotic metabolic process          | 4     | 9.72E-04     |
|             | GOTERM_BP_DIRECT | GO:0006508~proteolysis                           | 6     | 0.006776     |
|             | GOTERM_BP_DIRECT | GO:0008152~metabolic process                     | 4     | 0.00949      |
|             | GOTERM_BP_DIRECT | GO:0051919~positive regulation of fibrinolysis   | 2     | 0.009971     |
|             | GOTERM_CC_DIRECT | GO:0005829~cytosol                               | 17    | 0.002497     |
|             | GOTERM_CC_DIRECT | GO:0070062~extracellular exosome                 | 13    | 0.02121      |
|             | GOTERM_CC_DIRECT | GO:0016324~apical plasma membrane                | 4     | 0.031988     |
|             | GOTERM_MF_DIRECT | GO:0004024~alcohol dehydrogenase activity, zinc-dependent | 3     | 9.64E-05     |
|             | GOTERM_MF_DIRECT | GO:0004252~serine-type endopeptidase activity    | 6     | 3.21E-04     |
|             | GOTERM_MF_DIRECT | GO:0016491~oxidoreductase activity               | 5     | 0.001768     |
|             | GOTERM_MF_DIRECT | GO:0004060~arylamine N-acetyltransferase activity | 2     | 0.007705     |
|             | GOTERM_MF_DIRECT | GO:0030492~hemoglobin binding                    | 2     | 0.01026      |
| Up-regulated | GOTERM_BP_DIRECT | GO:0045727~positive regulation of translation    | 4     | 0.003197     |
|             | GOTERM_BP_DIRECT | GO:0051301~cell division                         | 7     | 0.010732     |
|             | GOTERM_BP_DIRECT | GO:0042769~DNA damage response, detection of DNA damage | 3     | 0.015911     |
|             | GOTERM_BP_DIRECT | GO:0090306~spindle assembly involved in meiosis  | 2     | 0.015999     |
|             | GOTERM_BP_DIRECT | GO:0006695~cholesterol biosynthetic process      | 3     | 0.017632     |
|             | GOTERM_CC_DIRECT | GO:0016020~membrane                              | 26    | 4.42E-05     |
|             | GOTERM_CC_DIRECT | GO:0005654~nucleoplasm                           | 29    | 1.19E-04     |
|             | GOTERM_CC_DIRECT | GO:0005737~cytoplasm                             | 43    | 2.37E-04     |
|             | GOTERM_CC_DIRECT | GO:0005829~cytosol                               | 32    | 2.73E-04     |
|             | GOTERM_CC_DIRECT | GO:0000785~chromatin                            | 5     | 0.001167     |
|             | GOTERM_MF_DIRECT | GO:0005515~protein binding                       | 53    | 1.60E-05     |
|             | GOTERM_MF_DIRECT | GO:0044822~poly(A) RNA binding                   | 18    | 7.10E-05     |
|             | GOTERM_MF_DIRECT | GO:0051082~unfolded protein binding              | 4     | 0.018488     |
|             | GOTERM_MF_DIRECT | GO:0098641~cadherin binding involved in cell-cell adhesion | 6     | 0.018571     |

Table 2. Gene ontology analysis of CNV-driven DEGs associated with HCC. If there were more than five terms enriched in this category, top five terms were selected according to P value. Count: the number of enriched genes in each term. DEGs, differentially expressed genes; GO, gene ontology; CNV, copy number variation.

---

Figure 2. Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment scatter plot of DEGs. The y-axis represents the name of the pathway, and the x-axis represents the Rich factor. Dot size represents the number of different genes and the color indicates the q-value. (a) Down-regulated CNV-driven DEGs Enrichment analysis results. (b) Up-regulated CNV-driven DEGs Enrichment analysis results.
8. Functional and KEGG pathway enrichment analysis revealed that genes in this module were mainly associated with translational initiation, RNA transport, and hepatitis C pathway (Supplementary Table 5).

Validation studies of the hub CNV-driven DEGs in large cohort of patients with HCC. We assessed the prognostic value of four hub CNV-driven DEGs through the data of 180 HCC patients from our previous study (GSE14520), and follow-up data of 30 HCC patients who were involved in integrated analysis with CNVs were not included. Patients were divided into a high expression group and a low expression group, using the mean expression levels of the hub genes as the cutoff point. According to the low and high expression of each hub gene, overall survival (OS) and time to recurrence (TTR) for patients with HCC were obtained.

Obviously, the Kaplan–Meier analysis revealed that individuals with high intra-tumor expression levels of NPM1 had a significantly worse prognosis than those with low intra-tumor expression levels of NPM1, as reflected by the OS and TTR ($P = 0.0257$ and $P = 0.0115$, respectively; Fig. 4). NPM1 was located at chromosome 5. To further evaluate the prognostic value of NPM1 for HCC patients, univariate and multivariate analyses were performed with the clinic-pathological characteristics and NPM1 (Table 3). In the univariate analysis, serum alpha-fetoprotein (AFP) level, tumor-nodes-metastases (TNM) stage, and Barcelona Clinic Liver Cancer (BCLC) stage were revealed to significantly associated with OS and TTR of HCC patients. Gender was also revealed to be significantly associated with TTR of HCC patients. NPM1 was also significantly associated with both OS and
mRNA levels of NPM1 in human HCC cells. Interestingly, we found that NPM1 expression was remarkably up-regulation of NPM1 was observed in 75.76% (25/33) of HCC patients (Fig. 5a). Moreover, we detected the expression pattern of NPM1 in 33-paired HBV related HCC tumor and adjacent non-tumor tissues. Our results showed that an expression pattern of NPM1 in HCC tissues, qRT-PCR was utilized to detect the messenger RNA (mRNA) levels was frequently overexpressed in human HCC.

Validation of the expression pattern of NPM1 in HCC tissues and cell lines. To investigate the expression pattern of NPM1 in HCC tissues, qRT-PCR was utilized to detect the messenger RNA (mRNA) levels of NPM1 in 33-paired HBV related HCC tumor and adjacent non-tumor tissues. Our results showed that an up-regulation of NPM1 was observed in 75.76% (25/33) of HCC patients (Fig. 5a). Moreover, we detected the mRNA levels of NPM1 in human HCC cells. Interestingly, we found that NPM1 expression was remarkably higher in HCC cells compared to nonmalignant liver cells (L02) (Fig. 5b). Our results showed that NPM1 mRNA was frequently overexpressed in human HCC.

Collectively, these data suggested that high expression of NPM1 was a valuable index for dismal prognosis in HBV related HCC patients.

Discussion
The pathogenesis of HCC is a multi-step process, and molecular alterations at both genetic and epigenetic levels have been shown to drive hepatocarcinogenesis. Therefore, understanding the etiological risk factors and molecular mechanisms of HCC progression is of a critical importance for prevention, diagnosis, and treatment. In the present study, we performed an integrated analysis of CNVs and gene expression profiling to identify the

| Factors                       | OS Univariate | OS Multivariate | TTR Univariate | TTR Multivariate |
|-------------------------------|---------------|-----------------|----------------|------------------|
|                               | P             | HR (95% CI)     | P              | HR (95% CI)      |
| Gender (female vs. male)      | 0.098         | NA              | 0.031          | NA               |
| Age (<50 y vs. ≥50 y)         | 0.640         | NA              | 0.525          | NA               |
| HBV(AVR-CC vs. CC)            | 0.593         | NA              | 0.484          | NA               |
| ALT (>50 U/L vs. ≤50 U/L)     | 0.428         | NA              | 0.071          | NA               |
| AFP (>300 ng/mL vs. ≤300 ng/mL)| 0.041         | NS              | 0.391          | NA               |
| Cirrhosis (yes vs. no)        | 0.064         | NA              | 0.193          | NA               |
| Tumor size (>5 cm vs. ≤5 cm)  | 0.065         | NA              | 0.459          | NA               |
| Multinodular (yes vs. no)     | 0.056         | NA              | 0.281          | NA               |
| TNM stage (III vs. II vs. I)  |               |                 |               |                  |
| Overall                       | <0.001        | NS              | <0.001         | 0.012            |
| II vs. I                      | 0.021         | NS              | 0.001          | 1.993 (1.210–3.282) |
| III vs. I                     | <0.001        | NS              | <0.001         | NS               |
| BCLC stage (C vs. B vs. A vs. 0) |               |                 |               |                  |
| Overall                       | <0.001        | <0.001          | <0.001         | 0.002            |
| A vs. 0                       | 0.064         | NA              | 0.128          | NA               |
| B vs. 0                       | 0.013         | 6.747 (1.394–32.658) | 0.018 | 0.010 | 3.181 (1.055–9.596) | 0.040 |
| C vs. 0                       | <0.001        | 27.456 (6.169–122.201) | <0.001 | <0.001 | 11.855 (3.208–34.810) | <0.001 |
| NPM1 (high vs. low)           | 0.028         | 2.197 (1.298–3.721) | 0.003 | 0.013 | 1.846 (1.207–2.822) | 0.005 |

Table 3. Univariate and multivariate analysis of factors associated with survival and recurrence in 180 HCCs. Univariate analysis was calculated by the Kaplan–Meier method (log-rank test). Multivariate analysis was done using the Cox multivariate proportional hazard regression model with stepwise manner. OS, overall survival; TTR, time to recurrence; AVR-CC, active viral replication chronic carrier; CC, chronic carrier. TNM, tumor-nodes-metastases; HR, hazard ratio; CI, confidential interval; NA, not adopted; NS, not significant.
DEGs with alterations in genomic segments. We analyzed the CNVs by using high-resolution SNP 6.0 arrays and gene expression profiling in 33 paired and 30 paired tissues respectively. All of patients selected were hepatitis B virus surface antigen (HBsAg) positive. To improve the accuracy of screening out CNV-driven DEGs, the baseline characteristics of the two cohort patients were similar.

In our study, a total of 138 CNV-driven DEGs were identified with the same expression tendencies, including 93 CNV-driven DEGs exhibiting increased expression in the duplicated regions and 45 showing decreased expression in the deleted regions. The highest number of duplications and deletions were documented at chromosome 1 and 4, respectively, which is in accordance with previous studies. It was reported that the duplication of chromosome 1q can be detected in 58–78% of HCC patients, and the deletion of chromosome 4q may be involved in hepatitis B virus-related hepatocarcinogenesis and the elevation of serum alpha-fetoprotein7,15. Functional enrichment analysis of the 93 up-regulated CNV-driven DEGs showed that they were mainly involved in positive regulation of translation, cell division and DNA damage response, which were very closely related to tumor, while the 45 down-regulated CNV-driven DEGs were mainly enriched in ethanol oxidation, xenobiotic metabolic process and metabolic process. To our knowledge, defects in asymmetric cell division can lead to the formation of tumors24. Meanwhile, it was reported that DNA damage response was a double-edged sword in cancer prevention and cancer therapy25. At present, several studies have reported that ethanol oxidation would delay or impair the replication of hepatocytes18,19. Furthermore, pathway enrichment annotation showed that the up-regulated CNV-driven DEGs were mainly involved in cell cycle, DNA replication, and Hippo signaling pathway; and the down-regulated CNV-driven DEGs were involved in chemical carcinogenesis and several metabolic pathways. It was consistent with our knowledge that the main course for cancer development and progression was the defective function of cell cycle and DNA replication30,31. The Hippo signaling pathway was reported to be a critical regulator of mammalian liver growth and a potent suppressor of liver tumor formation23. What’s more, chemical carcinogenesis and metabolic pathways were also very important in the development of HCC. Therefore, closely monitoring these signaling pathways may help us better diagnosis and predict the progression of HCC.

In this study, PPI network with CNV-driven DEGs were also constructed. EIF3E, PABPC1, EIF3H, and RPL8 were screened from the PPI network as the most significant module. EIF3 is the largest and most complex of the initiation factors, consisting of 13 putative protein subunits. EIF3 and its subunits regulate translation of a subset of mRNAs involved in many cellular processes, such as proliferation, apoptosis, DNA repair, and cell cycle24,25. EIF3E (called Int-6) was detected to be at low levels in non-small cell lung carcinomas, which suggested that EIF3E may function as a tumor suppressor25. Moreover, previous studies suggested that overexpression of EIF3H was associated with advanced cancer stages and poor prognosis in prostate and liver cancer26,27. Zhang et al. reported that PABPC1 was aberrantly expressed in HCC, especially in high grade HCC. And they also found that PABPC1 played a role as an oncogene in HCC, which accelerated the cell proliferation and promoted entry into the S Phase and progression to the G2/M phase28,29. RPL8, a member of the L2P family of ribosomal proteins, is a component of the 60 S ribosomal subunit in eucaryotic cells. The duplication of RPL8 was reported to be associated with the pathogenesis of osteosarcoma30, but the biological functions of RPL8 in HCC remains unclear. Moreover, all of these four genes were located at chromosome 8. It was reported that the duplication of chromosome 8 was a frequently detected genomic alteration in HCC (48–66%), and may confer a selective growth advantage on HCC cells31. Furthermore, functional and KEGG pathway enrichment analysis revealed that genes in this module were mainly associated with translational initiation, which was closely involved in the progression of HCC.

Additionally, a total of four DEGs were selected as the top degree hub genes from the PPI network, including HSP90AB1, RPL8, NPM1, and MCM3. Through analyzing the clinical relevance of these hub genes in HCC, only NPM1 was found to be significantly correlated with OS and TTR. NPM1, copy number duplication on chromosome 5 in our study, was overexpressed in primary HCC tissues compared to those in non-tumor tissues. To date, NPM1 is an abundant phosphoprotein which moves between the nucleus and the cytoplasm32,33. Previous studies showed that NPM1 is a multifunctional protein involved in many cellular processes such as DNA repair and ribosome biogenesis34,35. To our knowledge, genetic alterations of NPM1 have been found in various hematological

Figure 5. Up-regulation of NPM1 in HCCs. (a) NPM1 mRNA expression in 33 paired HCC tumor and adjacent non-tumor tissues. (b) mRNA levels of NPM1 were quantified in four HCC cells and a non-malignant liver cell (L02). GAPDH was acted as an internal control. **P < 0.01.
malignancies, such as acute myeloid leukemia. Meanwhile, the expression level of NPM1 was reported to be overexpressed in many solid tumors and correlated with the progression and recurrence of tumor. In the present study, the clinically relevant data presented showed that patients with HCC and high tumor NPM1 expression levels had decreased OS times and earlier TTR compared to those with low tumor NPM1 expression levels. Those findings are consistent with previous studies. In the meanwhile, NPM1 expression could stratify HCC patients by survival analyses in BCLC stage (0-A) and TNM stage (I-II) subgroups, which might redefine risk stratification of HCC patients. In addition, we found that copy number duplications of NPM1 were presented in four patients whose BCLC stages were A and TNM stages were I. It represented that the genetic changes of NPM1 occurred in the early stages of HCC. It was believed that the genetic changes of early liver cancer were relatively more favorable for early clinical diagnosis and predicting prognosis.

Many target proteins, including ARF, p53, p65, and NF-κB, were thought to be involved in the regulation of NPM1. A study showed that NF-κB required the chaperone-like function of NPM1 for DNA binding. Moreover, NPM1 was required for efficient inflammatory gene expression induced by tumor necrosis factor alpha (TNF-α) and lipopolysaccharide in fibroblasts and macrophages. In addition, it can be seen from the PPI network that FANCC (Fanconi anemia complementation group C), a DNA repair gene, was closely associated with NPM1. FANCC was a down-regulated CNV-driven gene in HCC samples, while NPM1 was the up-regulated CNV-driven gene. This may suggest that DNA repair defects/deficiencies may be important in the generation of CNVs.

In conclusion, this study provided an integrated analysis of CNVs and gene expression profiling to identify CNV-driven DEGs in HBV related HCC. EIF3E, PABPC1, EIF3H, and RPL8 which were all located at chromosome 8 from the module analysis, to the best of our knowledge, were demonstrated to be related to the progression of HCC. Furthermore, NPM1, the hub gene located at chromosome 5, was identified to be associated with the overall and recurrence-free survival time in postoperative patients with HCC, indicating that NPM1 may be a novel marker for predicting prognosis and a therapeutic target for HBV related HCC. However, more in-depth studies are needed to further verify the mechanisms between CNVs and gene expression profiling.

Methods

Patient selection. Thirty-three primary HCC tumors and paired adjacent non-tumor tissues stored at −80 °C were obtained from patients who underwent hepatectomy during the period from January to July 2009 at Zhongshan Hospital, Fudan University. The inclusion and exclusion criteria of the patient cohort include (1) no patients have received systemic or local treatment before operation, (2) no cases with extrahepatic metastases, (3) all patients having a distinctive postoperative pathologic diagnosis of HCC, (4) all patients undergoing primary and curative liver resection, (5) HBsAg (+); (6) and with complete clinic-pathologic and follow-up data. This study was approved by the Ethics Committee of Zhongshan Hospital, Fudan University and was conducted in accordance with the relevant guidelines. Written informed consent was obtained from all participants.

The gene expression profiles and clinical follow-up data of GSE14520, as reported in our previous studies, were acquired from the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database. The GSE14520 dataset contained 210 paired HCC and non-tumor samples. In the process of integrating the analysis of CNVs and gene expression profiling, thirty patients, whose baseline characteristics were similar to the thirty-three patients, were selected. And another 180 patients of GSE14520 were selected to validate the importance of CNV-driven genes.

SNP array analysis and data preparation. DNA was isolated using the QIAamp DNA Mini Kit according to the manufacturer's protocol. DNA was extracted, amplified and hybridized onto an Affymetrix Genome-Wide human SNP array 6.0 according to the manufacturer's instructions (Affymetrix Inc.). First, R packages of hapmapspn6, oligoClasses, crlmm and HMM were used to read the primary data (in CEL format) of Affy SNP 6.0 array; in addition to spotting the sites of SNPs. Second, R package of pd.genomewidesnp.6 was used to obtain the annotations of sites of each SNP spotted. Third, affy2sv package along with penncnv software helped to obtain all information regarding CNVs. According to the genetic annotations obtained, we completed the awk procedure and got the genetic information within the CNV domains. Partek Genomics Suite, version 6.4 (Partek Inc., St. Louis, MO, USA) was also used for copy number analysis. Hidden Markov Model (HMM) algorithm was used to detect the regions of CNVs in the standard Partek workflow for paired samples. The overall hybridization quality was estimated by the call rate index obtained from Genotyping Console Software (GTC 3.0, birdseed algorithm using default parameter settings). The criteria to define significant CNVs were as follows: the duplication was set at above 3, while deletion was set at below 1; the gene of copy number duplications or deletions had occurred in at least 10% (4 samples) of the total samples; the overlapping common regions among multiple tumors were calculated.

Statistical analysis of gene expression profiling. Microarray data from GSE14520 datasets were directly downloaded from GEO. The detailed process of performing gene expression profiling has been published in our previous study. Briefly, raw CEL files were processed for background correction and quantile normalization (median scaling) using the robust multi-array averaging (RMA) method. The Affy package from Bioconductor and Affy probe annotation files (Affymetrix HT Human Genome U133A chips) were applied to preprocess the gene expression profile data. In the end, gene expression matrix was obtained. Thirty paired samples were selected to identify differentially expressed genes (DEGs).

The classical t test was applied in our analysis to identify DEGs between paired HCC and non-tumor samples. Statistically significant genes were identified using the mixed model analysis of variance with a false discovery rate (Benjamini–Hochberg test) adjusted P value of < 0.05 and fold-change values of −2 to 2.
**Integration of copy number variation and gene expression profiling.** To identify the significant driver genes that exhibited CNV and gene expression alterations, we integrated the data of significant CNVs and DEGs. Hierarchical clustering analysis of the CNV-driven DEGs was performed using the pheatmap package in R49.

**Functional and pathway enrichment analysis.** The Database for Annotation, Visualization and Integrated Discovery (DAVID, https://david.ncifcrf.gov/) is the most common tool to analyze the functional enrichment of genes45. Gene ontology (GO) enrichment analysis was performed for identified CNV-driven DEGs using DAVID online tool42. Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/) database shows the way that genes or other molecules act42. KEGG Orthology Based Annotation System (KOBAS, http://kobas.cbi.pku.edu.cn/index.php) is a web server for annotating and identifying enriched pathways and associated diseases for large gene lists44. KEGG pathway enrichment analysis for identified CNV-driven DEGs was conducted using KOBAS 2.0. The threshold for these analyses was set as P value < 0.05.

**Protein-protein interaction (PPI) network construction and module screening.** Search Tool for the Retrieval of Interacting Genes (STRING, http://www.string-db.org/) is an online tool and biological database that includes comprehensively predicted and known interaction information45. To evaluate the functional interactions among CNV-driven DEGs, we mapped the DEGs to STRING. The combined score >0.4 was used as the cut-off criterion to screen out the validated interactions. Then, PPI networks were constructed using the Cytoscape software (version 3.4.0, available at http://www.cytoscape.org/)46. Then, the plug-in Molecular Complex Detection (MCODE) was performed to screen modules of PPI network with the criteria as follows: MCODE scores >3 and number of nodes >347. The functional and pathway enrichment analysis of genes in the module were performed by DAVID and KOBAS, respectively. P < 0.05 was considered to be statistically significant.

**The Cancer Genome Atlas (TCGA) data acquisition.** A dataset including information about mRNA expression and clinical characteristics of HCC patients (n = 424 for TCGA hepatocellular carcinoma, gene expression by RNAseq with IlluminaHiSeq) was acquired from the TCGA website (https://tcga-data.nci.nih.gov/tcga/). The expression levels of NPM1 were collected for each case and divided into the high expression and the low expression groups using the mean expression levels of the gene as the cutoff point.

**Cell lines.** The human HCC cell lines, SMMC-7721, Huh7, and a normal hepatocyte cell line (LO2) were purchased from the cell bank of the Chinese Academy of Sciences (Shanghai, China). MHCC97H and MHCCCL3 were established at the Liver Cancer Institute, Zhongshan Hospital, Fudan University, Shanghai, China. Cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) (HyClone, Logan, UT, USA) and supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), penicillin (100 units/ml) and streptomycin (100 ug/ml) at 37 °C humidified incubator under an atmosphere of 5% CO2 in air.

**RNA isolation and quantitative reverse transcription PCR (qRT-PCR).** The total RNA was isolated from HCC tissues and cells with TRIzol reagent (Invitrogen, USA) and reverse-transcribed to cDNA using PrimeScript RT reagent kit (Takara, Japan). The qRT-PCR was performed for assessment of mRNA expression according to the manufacturer's protocols by an ABI Prism 7500 Sequence Detection system (Applied Biosystems, Foster City, CA, USA). Primers were designed as follows: NPM1, forward: 5′-CTGGGAGATCTCCAGGGTC-3′ and reverse: 5′-CGGCCCTTATTGTCACAAACCG-3′; GAPDH, forward: 5′-CTGGGCTACTGAGCACC-3′ and reverse: 5′-AAGTGGTCTGTAGGGAATG-3′. Then, the 2−ΔΔCT method was used to determine the relative gene expression levels. Triplicate qRT-PCR samples were performed in each assay.

**Statistical analysis.** Statistical analysis was performed with SPSS software (23.0; IBM, Armonk, NY, USA). Values are expressed as mean ± standard deviation (SD). The Student t test was used for comparisons between groups. Categorical data were analyzed by the chi-square or Fisher's exact tests. Overall survival (OS) and Time to recurrence (TTR) were analyzed using Kaplan-Meier’s method and the log-rank test. Variables associated with OS and TTR were identified using univariate Cox proportional hazards regression models. Significant factors in univariate analysis were further subjected to a multivariate Cox regression analysis in a stepwise manner. A two-tailed P < 0.05 was considered statistically significant.

**References**

1. Chen, W. et al. Cancer statistics in China, 2015. CA Cancer J Clin 66, 115–132 (2016).
2. Torre, L. A. et al. Global cancer statistics, 2012. CA Cancer J Clin 65, 87–108 (2015).
3. Cavazza, A. et al. Incidence, risk factors, and survival of hepatocellular carcinoma in primary biliary cirrhosis: comparative analysis from two centers. Hepatology 50, 1162–1168 (2009).
4. Kov, G. et al. The penetrance of copy number variations for schizophrenia and developmental delay. Biol Psychiatry 75, 378–385 (2014).
5. Shlien, A. & Malkin, D. Copy number variations and cancer. Genome Med 1, 62 (2009).
6. Stranger, B. E. et al. Relative impact of nucleotide and copy number variation on gene expression phenotypes. Science 315, 848–853 (2007).
7. Guan, X. Y. et al. Recurrent chromosome alterations in hepatocellular carcinoma detected by comparative genomic hybridization. Genes Chromosomes Cancer 29, 110–116 (2000).
8. Dore, M. P. et al. Genomic instability in chronic viral hepatitis and hepatocellular carcinoma. Hum Pathol 32, 698–703 (2001).
9. Miodrakawa, Y., Tang, W. & Sugiyama, Y. High-resolution mapping of copy number aberrations and identification of target genes in hepatocellular carcinoma. Biosci Trends 1, 26–32 (2007).
10. Patil, M. A. et al. Array-based comparative genomic hybridization reveals recurrent chromosomal aberrations and JAB1 as a potential target for 8q gain in hepatocellular carcinoma. Carcinogenesis 26, 2650–2657 (2005).
11. Zhang, Y. et al. Integrated Analysis of Genome-Wide Copy Number Alterations and Gene Expression Profiling of Lung Cancer in Xuanwei, China. PLoS One 12, e0169098 (2017).
12. Woo, H. G. et al. Identification of potential driver genes in human liver carcinoma by genomewide screening. Cancer Res 69, 4059–4066 (2009).
13. Chen, C. F. et al. Overlapping high-resolution copy number alterations in cancer genomes identified putative cancer genes in hepatocellular carcinoma. Hepatology 52, 1690–1701 (2010).
14. Ho, D. W., Lo, R. C., Chan, L. K. & Ng, I. O. Molecular Pathogenesis of Hepatocellular Carcinoma. Liver Cancer 5, 290–302 (2016).
15. Yeh, S. H. et al. Allelic loss of chromosome 4q21 approximately 23 associates with hepatitis B virus-related hepatocarcinogenesis and elevated alpha-fetoprotein. Hepatology 40, 847–854 (2004).
16. Neumuller, R. A. & Knoblich, J. A. Dividing cellular asymmetry: asymmetric cell division and its implications for stem cells and cancer. Genes Dev 23, 2675–2699 (2009).
17. Tian, H. et al. DNA damage response—a double-edged sword in cancer prevention and cancer therapy. Cancer Lett 358, 8–16 (2015).
18. Clemens, D. L., Calisto, L. E., Sorrell, M. F. & Tuma, D. J. Ethanol metabolism results in a G2/M cell-cycle arrest in recombinant Hep G2 cells. Hepatology 38, 383–393 (2003).
19. Clemens, D. L., Forman, A., Jerrells, T. R., Sorrell, M. F. & Tuma, D. J. Relationship between acetaldehyde levels and cell survival in ethanol-metabolizing hepatoma cells. Hepatology 35, 1196–1204 (2002).
20. Chin, R. et al. Modulation of MAPK pathways and cell cycle by replicating hepatitis B virus: factors contributing to hepatocarcinogenesis. J Hepatol 47, 325–337 (2007).
21. Cai, Y. N. et al. LRH-1/hB1F and HNF1 synergistically up-regulate hepatitis B virus gene transcription and DNA replication. Cell Res 13, 451–458 (2003).
22. Lu, L. et al. Hippo signaling is a potent in vivo growth and tumor suppressor pathway in the mammalian liver. Proc Natl Acad Sci USA 107, 1437–1442 (2010).
23. Yin, J. Y., Dong, Z. & Zhang, J. T. eIF3 Regulation of Protein Synthesis, Tumorigenesis, and Therapeutic Response. Methods Mol Biol 1507, 113–127 (2017).
24. Buttitta, F. et al. Int6 expression can predict survival in early-stage non-small cell lung cancer patients. Clin Cancer Res 11, 3198–3204 (2005).
25. Savinainen, K. I., Heleinus, M. A., Lehtonen, H. J. & Visakorpi, T. Overexpression of EIF3S3 promotes cancer cell growth. Prostate 66, 1144–1150 (2006).
26. Zhu, Q. et al. Elevated expression of eukaryotic translation initiation factor 3H is associated with proliferation, invasion and tumorigenicity in human hepatocellular carcinoma. Oncotarget 7, 49888–49901 (2016).
27. Zhang, H. et al. PABPC1 interacts with AGO2 and is responsible for the microRNA mediated gene silencing in high grade hepatocellular carcinoma. Cancer Lett 367, 49–57 (2015).
28. Yang, J. & Zhang, W. New molecular insights into osteosarcoma targeted therapy. Curr Opin Oncol 25, 398–406 (2013).
29. Borer, R. A., Lehner, C. F., Eppenberger, H. M. & Nigg, E. A. Major nucleolar proteins shuttle between nucleus and cytoplasm. Cell 56, 379–389 (1989).
30. Kokke, A. et al. Recruitment of phosphorylated NPM1 to sites of DNA damage through RNF8-dependent ubiquitin conjugates. Cancer Res 70, 6746–6756 (2010).
31. Murano, K., Okuwaki, M., Hisaoka, M. & Nagata, K. Transcription regulation of the rRNA gene by a multifunctional nuclear protein, B23/nucleophosmin, through its histone chaperone activity. Mol Cell Biol 28, 3114–3126 (2008).
32. Falini, B. et al. Cytoplasmic nucleophosmin in acute myelogenous leukemia with a normal karyotype. N Engl J Med 352, 254–266 (2005).
33. Grisendi, S., Mecucci, C., Falini, B. & Pandolfi, P. P. Nucleophosmin and cancer. Nat Rev Cancer 6, 493–505 (2006).
34. Cho, K. H., Cheng, A. J., Chang, P., Pan, T. L. & Yung, B. Y. Association of nucleophosmin/B23 mRNA expression with clinical outcome in patients with bladder carcinoma. Urology 64, 839–844 (2004).
35. Cheng, K. et al. The leukemia-associated cytoplasmic nucleophosmin mutant is an oncogene with paradoxical functions: Arf inactivation and induction of cellular senescence. Oncogene 26, 7391–7400 (2007).
36. Lin, J. Kato, M., Nagata, K. & Okuwaki, M. Efficient DNA binding of NF-kappaB requires the chaperone-like function of NPM1. Nucleic Acids Res 45, 3707–3723 (2017).
37. Plimack, E. R. et al. Defects in DNA Repair Genes Predict Response to Neoadjuvant Cisplatin-based Chemotherapy in Muscle-invasive Bladder Cancer. Eur Urol 68, 959–967 (2015).
38. Roessler, S. et al. A unique metastasis gene signature enables prediction of tumor relapse in early-stage hepatocellular carcinoma patients. Cancer Res 70, 10202–10212 (2010).
39. Wang, L. et al. DNA-seq analyses of multiple misestments of soybean: novel and alternative transcripts, evolutionary and functional implications. BMC Plant Biol 14, 169 (2014).
40. Jiao, X. et al. DAVID-WS: a stateful web service to facilitate gene/protein list analysis. Bioinformatics 28, 1805–1806 (2012).
41. Consortium, G. O. The Gene Ontology (GO) project in 2006. Nucleic Acids Res 34, D322–326 (2006).
42. Kanehisa, M. & Goto, S. KEGG: kyoto encyclopedia of genes and genomes. Nucleic Acids Res 28, 27–30 (2000).
43. Xie, C. et al. KOBAS 2.0: a web server for annotation and identification of enriched pathways and diseases. Nucleic Acids Res 39, W316–322 (2011).
44. Szklarczyk, D. et al. STRING v10: protein–protein interaction networks, integrated over the tree of life. Nucleic Acids Res 43, D447–452 (2015).
45. Shannon, P. et al. Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res 13, 2498–2504 (2003).
46. Bader, G. D. & Hogue, C. W. An automated method for finding molecular complexes in large protein interaction networks. BMC Bioinformatics 4, 2 (2003).

Acknowledgements
We thank Yaohui Li, a clinician of the Department of Urology Division of Zhongshan Hospital, Fudan University, for his advice on statistical analysis. This work was supported by grants from the National Natural Science Foundation of China (81472672, 81572301 and 81502487), the Shanghai Rising Star of Young Scientist Project (13QH1401000).

Author Contributions
Chenhao Zhou, Wentao Zhang and Wanyong Chen designed and performed experiments. Yirui Yin, Manar Atyah and Shuang Liu contributed to sample collection and processed the raw sequencing data. Wentao Zhang, Lei Guo and Yi Shi performed the bioinformatics analysis. Qinghui Ye prepared figures and tables. Chenhao Zhou and Wanyong Chen drafted the manuscript. Ning Ren and Qiongzhong Dong supervised the study and edited the manuscript. All authors read and approved the final manuscript.
Additional Information
Supplementary information accompanies this paper at doi:10.1038/s41598-017-11029-y

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

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2017