Original Article

Alternatively activated NUSAP1 promotes tumor growth and indicates poor prognosis in hepatocellular carcinoma

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Background: Hepatocellular carcinoma (HCC) is one of the most common malignancies with high mortality. The key genes involved in initiation and development of HCC is not entirely clear.

Methods: We performed a meta-analysis of available transcriptome data from 6 independent HCC datasets [5 datasets from the Gene Expression Omnibus (GEO) and 1 dataset from The Cancer Genome Atlas (TCGA)]. The associations of the nucleolar and spindle-associated protein 1 (NUSAP1) expression level with clinicopathological factors and survival times were analyzed. Two representative HCC cell models were built to observe the proliferation capacity of HCC cells when NUSAP1 expression was inhibited by shNUSAP1.

Results: Based on the transcriptome and survival data in the GEO and TCGA databases, *NUSAP1* gene was markedly upregulated in HCC. High expression of NUSAP1 in HCC is related to the iCluster1 molecular subgroup, poor survival, poor tumor differentiation and TNM stage. Additionally, pathway analysis based on RNAseq data suggested that NUSAP1 could activate the expression of genes involves in cell proliferation. Furthermore, downregulation of NUSAP1 expression could significantly inhibit the proliferation of SMMC-7721 and Huh7 cells *in vitro*.

Conclusions: Our study provides evidence that NUSAP1 may serve as a candidate prognostic marker and a target for future therapeutic intervention in HCC.

Keywords: Nucleolar and spindle-associated protein 1 (NUSAP1); tumor growth; hepatocellular carcinoma (HCC)

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Introduction

Primary liver cancer is the fifth most common cancer type around the world, with increasing incidence in the past several decades (1). According to the statistics from GLOBOCAN 2012, approximately 782,500 new cases and 745,500 deaths occurred globally due to liver cancer; however, approximately 50% of all cases were in China (2). Previous studies have found that the high incidence of primary liver cancer in China was associated

with the increasing prevalence of chronic infection with hepatitis B virus (HBV) and hepatitis C virus (HCV) (2-5). Hepatocellular carcinoma (HCC), originating in hepatocytes, is the major histologic subtype of liver cancer and accounts for ~80% of primary liver cancer cases. Currently, there are various types of HCC therapies, such as hepatic resection, chemotherapy, immunotherapy, radiofrequency ablation or alcohol ablation, and liver transplantation (6-8). Although these therapies have improved the survival of HCC patients compared to that observed in the last decade, the overall prognosis is still poor. Thus, underlying molecular mechanism investigation, novel biomarker identification and recurrence prediction are urgent goals to improve the early diagnosis and prognosis of HCC (7).

Microarrays can detect hundreds to millions of different molecules at the same time. Microarray analysis is a rapid technique that plays an indispensable role in the human genome project (HGP). With advancements in science and scientific techniques, microarray analysis has been employed for various studies, for example, large-scale genotyping, new gene discovery, mutation identification, and, in particular, cancer genome studies (9). Microarray analysis has become one of the major methods for finding the origin and progression of cancer using data from the whole genome (10). Currently, microarrays are successfully being used in the discovery of new biomarkers in most human cancers, such as breast cancer (11), prostate cancer (12), lung cancer (13) and HCC (14). Although gene profiling is a powerful tool, the differentially identified genes (DEGs) identified in one study often cannot be identified in another study and can easily be influenced by confounding factors either because of sample selection or due to different analysis platforms. Hence, genome-wide meta-analyses are necessary to summarize similar gene-based data from multiple databases.

In this study, we performed an integrated analysis based on HCC mRNA profiles from the Gene Expression Omnibus (GEO) and The Cancer Genome Atlas (TCGA) databases to identify potential biomarkers of HCC on a genome-wide scale. Eleven proliferation-related DEGs (*BBOX1*, *CENPA*, *DLGAP5*, *FOXM1*, *KIF20A*, *KIF4A*, *MKI67*, *NCAPG*, *NUSAP1*, *SFN*, and *TPX2*) were identified at the in silico stage. Among these DEGs, nucleolar and spindle-associated protein 1 (*NUSAP1*) gene was one of the overexpressed genes in HCC and displayed a high likelihood of being involved in HCC progression. This study mainly focused on exploring the effects of NUSAP1 in HCC.

Methods

Microarray data collection, comparison and meta-analysis

Because small sample sizes and different microarray analyses may lead to errors during the screening process, the results may not be completely reliable. A meta-analysis is a statistical analysis method that combines multiple studies to analyze a larger population to obtain more accurate and comprehensive results. The GEO (https://www.ncbi.nlm.nih.gov/geo/) is a national public database that includes high-throughput gene expression, array- and sequence-based data (15). In this study, eligible gene expression data were obtained from the GEO (Affymetrix Human Genome U133 Plus 2.0 Array). Then, a meta-analysis was performed to identify the DEGs based on these datasets.

TCGA data extraction and analysis

After identifying DEGs using the method described above, we analyzed the effects of the genes in patients and their association with survival. mRNA expression and clinical data from TCGA were extracted from cBioPortal (www.cbioportal.org). Then, the data were preprocessed and analyzed by R. A violin plot was constructed to show the differences in specific gene expression between HCC tissues and nontumor tissues. Kaplan-Meier plots were constructed to demonstrate the relationship between the dysregulated genes and patient survival. In addition, various clinicopathological characteristics, including age at initial pathologic diagnosis, sex, TNM stage, histologic grade and pathologic stage, were investigated. The data was quantified and analyzed in R, and Chi-square test was performed.

Short hairpin RNA (shRNA) sequencing and data analysis

shRNA sequencing was conducted to investigate differential gene expression in HCC by knocking down genes with shRNAs. The shRNA sequencing data were uploaded and analyzed using a website (https://ncedu.shinyapps.io/shrna/). MDS, BCV, MAPlot, and two files (QC.csv and CPM.csv) were obtained from the website. Then, logFC and adjust P values [$-\log_{10}(P)$] were plotted in a volcano plot by using Excel 2016. To elucidate the possible molecular mechanisms regulated by the DEGs in HCC, we conducted gene set enrichment analysis (GSEA) to identify significantly enriched pathways. Data were uploaded and interpreted using the WEB-based GEne SeT AnaLysis Toolkit (http://www.webgestalt.org/option.php).

shRNA sequence construction and transfection

shNUSAP1 and shFoxM1 expression vectors were purchased from GeneChem (Shanghai, China). The target sequences of the above shRNA expression constructs are as follows: shNUSAP1#1: CCGGGAGGGCAACCAAGTTGTTAAACTCGAGTTTAACAACTTGGTTGCCCTCTTTTTTG; shNUSAP1#2: CCGGGAGCACCAAGAAGCTGAGAATCTCGAGATTCTCAGCTTCTTGGTGCTCTTTTTTG; and shNUSAP1#3: CCGGGAACCACACAAAGGAAAGCTACTCGAGTAGCTTTCCTTTGTGTGGTTCTTTTTTG. The positive control shFoxM1 sequence is as follows: CCGGGCCAATCGTTCTCTGACAGAACTCGAGTTCTGTCAGAGAACGATTGGCTTTTT.

Transient transfection of cells was performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Cell proliferation assay

HCC cells were cultured in DMEM with 10% (v/v) FBS (Gibco). Culture flasks were placed in an incubator (37 ℃, 5% CO_2) and subcultured every 3 days. To perform cell proliferation assay, we divided HCC cells into 24-well plates at a density of 5×10^3 cells/well and cultured them with 0.5 mL DMEM (with 10% FBS) for 24 h. Then, the cells were transfected with constructed shRNA expression vectors. After transfection, the bromodeoxyuridine (BrdU) assay was performed according to the manufacturer's protocol for the BrdU colorimetric cell proliferation assay kit (cat# 1647229; Roche). For clonogenic assay, HCC cells (3×10^3 per well) were plated in 6-well plates and cultured for 2 weeks. All plates were stained with 0.5% crystal violet (w/v), and the colony numbers were determined. Cell cycle distribution was analyzed by propidium iodide staining and flow cytometry.

RNA purification and quantitative RT-PCR

For real-time PCR, total RNA (1 μg) was subjected to reverse transcription using a reverse transcription reagent kit (Applied Biosystems, Foster City, CA, USA). Quantitative real-time PCR was performed with an ABI Step Plus One Sequence Detection System (Applied Biosystems). The specific primers used for PCR amplification are as follows: NUSAP1-F: AAGCAAGTTTGTCTCGTCC, NUSAP1-R: GAGATGGGGTTGTTTGTAAG, GAPDH-F: TGACTTCAACAGCGACACCA, and GAPDH-R: CACCCTGTTGCTGTAGCCAAA. All experiments were repeated at least three times with consistent results.

Statistical analysis

Data were assessed using Student's *t*-test or one-way ANOVA followed by Fisher's multiple range test.

Results

Integrated analysis identified 11 proliferation-related genes that were significantly overexpressed in HCC

In this study, we identified five publicly available HCC datasets (GSE14520, GSE41804, GSE45267, GSE60502 and GSE6764) and manually extracted the data for 650 liver samples (including 341 HCC and 309 normal tissues; *Table S1*) from the GEO database. To scan for robust HCC-associated biomarkers across multiple independent cohorts, we performed two meta-analyses to detect DEGs between HCC and normal tissues. In total, 580 candidates represented by 13,021 transcripts displayed a considerable change in expression ($P<0.0001$) in at least one meta-analysis. As shown in *Figure 1A*, many DEGs (6.38%, 37/580) were common across both analyses. This number was over seven times higher than that expected from each analysis (5 genes, χ^2 test, $P<10^{-4}$). This result indicated the reliability of the meta-analyses in the detection of biomarkers of HCC.

An additional independent dataset from TCGA provided an opportunity for confirming the primary biomarkers related to HCC. Only one gene (*CLGN*) did not show significant change in expression ($P>0.0001$) between 373 HCC and 50 normal tissues and was thus removed from the further analysis. Interestingly, among the 36 common DEGs, most (11 genes) were involved in "*cell cycle and division*". Most importantly, all of these proliferation-related DEGs were upregulated in HCC tissues (*Figure 1B*), and most of them had been demonstrated to have crucial roles in HCC progression [including FOXM1 (16), CAP2 (17), KIF20A (18), KIF4A (19) and DLGAP5 (20)] in previous reports. Therefore, based on an integrated analysis of 6 independent HCC datasets (5 GEO datasets and 1 TCGA dataset) consisting of 714 HCC cases and 359 normal liver samples, we identified eleven proliferation-related DEGs

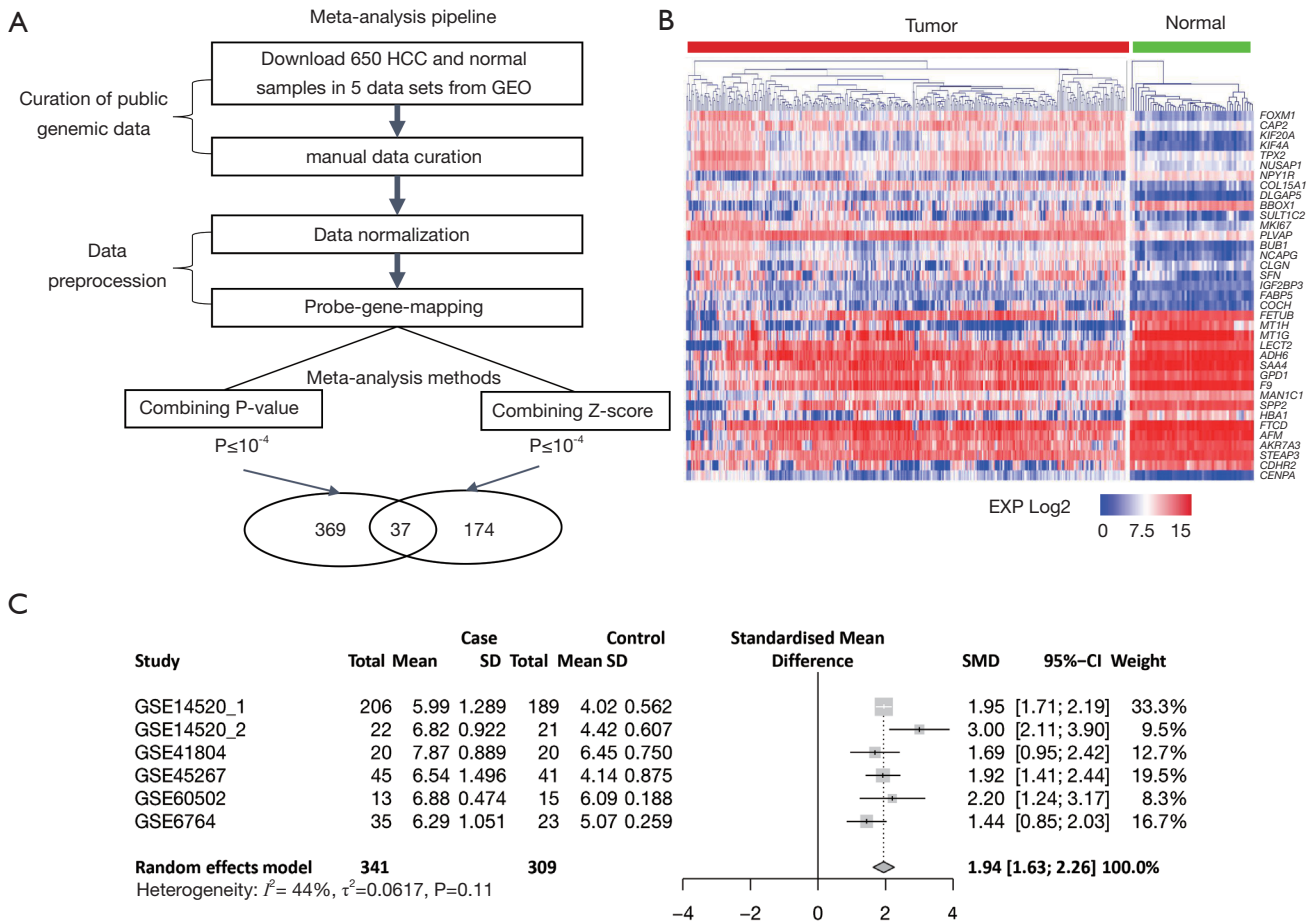

Figure 1 Summary of integrate-analysis based on GEO and TCGA datasets. (A) Meta-analysis pipeline using GEO datasets. In silico bioinformatics data analysis workflow consisted of the curation of five publicly available datasets. (B) The 37 genes cluster HCC versus normal across each dataset. Each column is a sample and each row is the expression level of a gene. The color scale represents the raw Z-score ranging from blue (low expression) to yellow (high expression). Dendrograms above each heatmap correspond to the Pearson correlation-based hierarchical clustering of samples by expression of the 37 genes. (C) Forest plot of NUSAP1 expression across all meta-analysis datasets. GEO, Gene Expression Omnibus; TCGA, The Cancer Genome Atlas; HCC, hepatocellular carcinoma; NUSAP1, nucleolar and spindle-associated protein 1.

(*BBOX1*, *CENPA*, *DLGAP5*, *FOXM1*, *KIF20A*, *KIF4A*, *MKI67*, *NCAPG*, *NUSAP1*, *SFN*, and *TPX2*) that were significantly and consistently overexpressed in HCC tissues across all datasets. Although we speculated that NUSAP1 likely participates in HCC development and progression, the function of NUSAP1 in HCC remained unknown.

Overexpression of NUSAP1 in HCC is related to clinicopathological features

NUSAP1, a 55-kDa microtubule-associated protein (MAP), is one of the members essential for the arrangement and functioning of the mitotic spindle (21). Our meta-analysis based on the five GEO datasets indicated that the expression level of *NUSAP1* in HCC was higher than that in the nontumor tissues ($P=1.87\times10^{-73}$). In addition, forest plots illustrated that these results were stable without heterogeneity ($I^2=44\%$, $\tau^2=0.0617$, P=0.11, *Figure 1C*). Furthermore, TCGA transcriptome data confirmed the significant differential expression of *NUSAP1* between tumor and nontumor tissues (*t*-test =8.93, $P=7.17\times10^{-16}$, *Figure 2A*).

To explore the relationship between *NUSAP1* and HCC clinicopathological features, we analyzed the expression profiles of HCC patients and clinical data from TCGA.

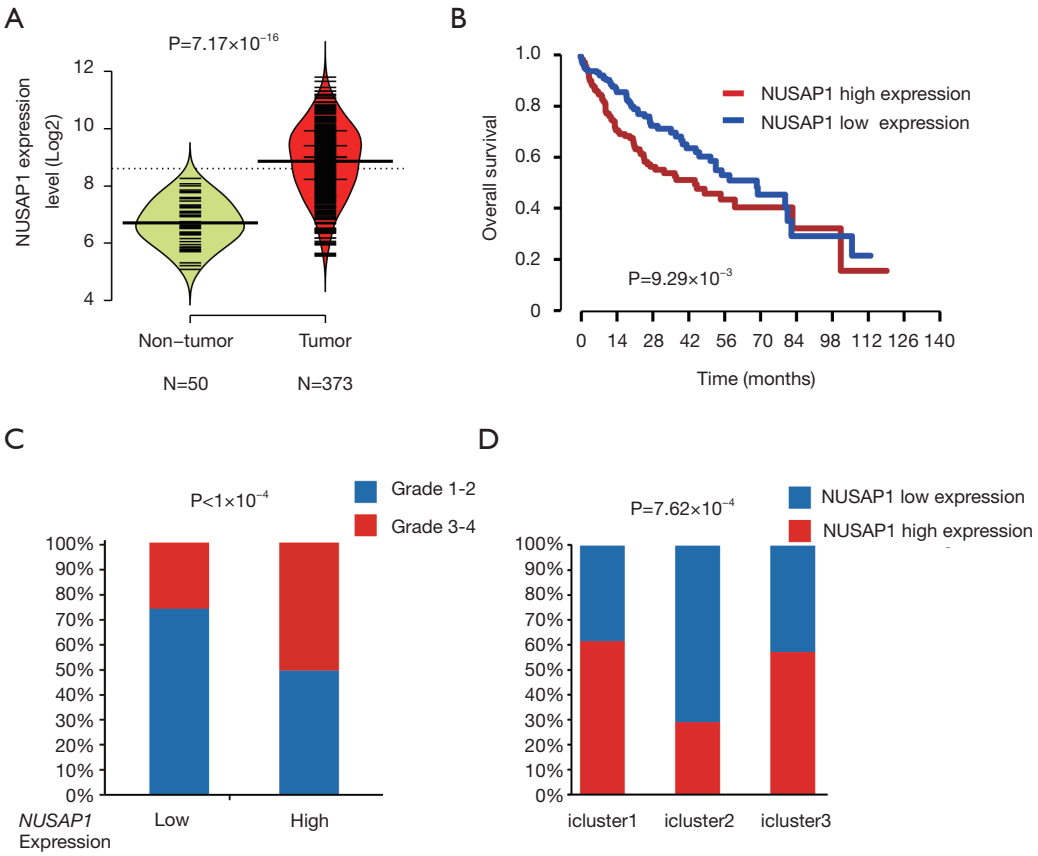

Figure 2 The association between NUSAP1 highly expression and clinical data analysis. (A) The expression of NUASP1 expression in tumor and para-tumor tissues. According to the TCGA data, NUSAP1 expression up-regulated in tumor tissues compared to non-tumor tissues; (B) the association between NUSAP1 expression and patients' survival. The overall survival curve showed that high NUSAP1 expression leads to poor prognosis, while low NUSAP1 expression patients have longer survival time; (C) the relationship of NUSAP1 expression with various clinical pathological features; (D) summary of the relationship between iCluster and NUSAP1 expression. TCGA, The Cancer Genome Atlas; NUSAP1, nucleolar and spindle-associated protein 1.

In total, 336 patients were included in this analysis, and the cutoff value of *NUSAP1* expression was defined by the median expression. As shown in *Figure 2B*, high expression of *NUSAP1* was significantly associated with poor survival in HCC patients (P=0.00929). In addition, we explored the relationships between the expression level of *NUSAP1* and clinicopathological features in HCC (*Table 1*). The data implied that high *NUSAP1* expression was associated with tumor histologic grade ($P<1\times10^{-4}$, *Figure 2C*), tumor TNM stage (P=0.044), and patient age (P=0.018, *Table 1*).

Furthermore, NUSAP1 expression was associated with multiplatform integrative molecular subtyping performed by TCGA Research Network. Patients with different pathological and molecular characteristics were divided into three major groups (iCluster1, iCluster2 and iCluster3), and iCluster1 was directly correlated with the abnormal activation of cell proliferation (22). We integrated the expression profiles and iCluster data for HCC patients and found that patients with high NUSAP1 expression were enriched in iCluster1 (*Figure 2D*). This result implied that the high expression of *NUSAP1* can activate cell proliferation.

In summary, high NUSAP1 expression plays a vital role in HCC occurrence and development and is closely associated with patient prognosis. NUSAP1 might thus be used as a cancer therapeutic target site in the future.

Downregulation of NUSAP1 suppresses the expression of genes involved in cell proliferation

To investigate the biological significance of NUSAP1

Table 1 Association of NUSAP1 expression levels with clinicopathologic characteristics in HCC (TCGA-cohort)

Clinicopathologic measurement data	NUSAP1 expression (n=336)				P value
	Low (n=168)		High (n=168)		
	Count	%	Count	%	
Age					0.018
≤50 years	28	8.3	46	13.7	
>50 years	140	41.7	122	36.3	
Gender					0.060
Male	123	36.6	107	31.8	
Female	45	13.4	61	18.2	
TNM stage					0.044
Stage I–II	134	39.9	118	35.1	
Stage III–IV	34	10.1	50	14.9	
Histologic grade					0.000
G1–G2	124	36.9	83	24.7	
G3–G4	44	13.1	85	25.3	

TCGA, The Cancer Genome Atlas; HCC, hepatocellular carcinoma; NUSAP1, nucleolar and spindle-associated protein 1.

expression in cancer cells, we downloaded RNA-Seq data from ENCODE to identify DEGs between HepG2_Control and HepG2_shNUSAP1 cells. Overall, 656 candidate genes, consisting of 264 upregulated genes and 392 downregulated genes, exhibited considerable change (P<0.05, *Figure 3A*). To further explore the biological significance of NUSAP1 in gene expression in HCC, we analyzed DEGs by overrepresentation analysis (ORA). As shown in *Figure 3B*, the DEGs were significantly enriched in 10 functional pathways (FDR <0.001), including 1 pathway that was related to cell proliferation and 3 pathways that were associated with metabolism. Most importantly, cell proliferation (GO:0008283) was included the largest number of candidate DEGs (85 genes, *Figure 3C*). Consistent with the clinicopathological data analysis, these results further confirmed that NUSAP1 plays vital roles in cell proliferation and cell cycle.

NUSAP1 promotes proliferation and cell cycle progression in HCC cells

To explore the biological effects of NUSAP1 in HCC cell proliferation, we first detected NUSAP1 mRNA expression in 8 different HCC cell lines (HMCC97L, SMMC-7721, HepG2, Hep3B, BEL-7402, Huh7, SK-Hep1 and LM3). High expression of NUSAP1 was observed in SMMC-7721 and Hep3B cell lines, while low expression of NUSAP1 was detected in Huh7 and SK-Hep1 cell lines (*Figure 4A*). Subsequently, quantitative RT-PCR was used to evaluate the effectiveness of silencing NUSAP1 using three established shRNAs. shNUSAP1#2 could decrease the expression level of NUSAP1 to 11% (*Figure 4B*). SMMC-7721 and Huh7 cells were transfected with shNC, shNUSAP1 and shFoxM1 to establish shCtrl, shNUSAP1 and shPC groups, respectively. SMMC-7721 and Huh7 cells in the shCtrl group could divide normally (*Figure 4C*). FoxM1 is an indispensable gene for cell proliferation. Silencing of the *FoxM1* gene in proliferating cells inhibited cell (*Figure 4C*). Knockdown of NUSAP1 suppressed cell proliferation in the two HCC cell lines (*Figure 4C*). Because the inhibition of proliferation in SMMC-7721 cells was the most obvious after NUSAP1 gene silencing, we further verified the results in colony forming and cell cycle analyses. As shown in *Figure 4D*,*E*, the growth of SMMC-7721 cells was significantly inhibited by NUSAP1 knockdown. Consistent with this result, NUSAP1 knockdown inhibited cell cycle progression and arrested most cells at the G1 phase. Therefore, NUSAP1 silencing can inhibit HCC cell growth and proliferation by blocking cell cycle progression.

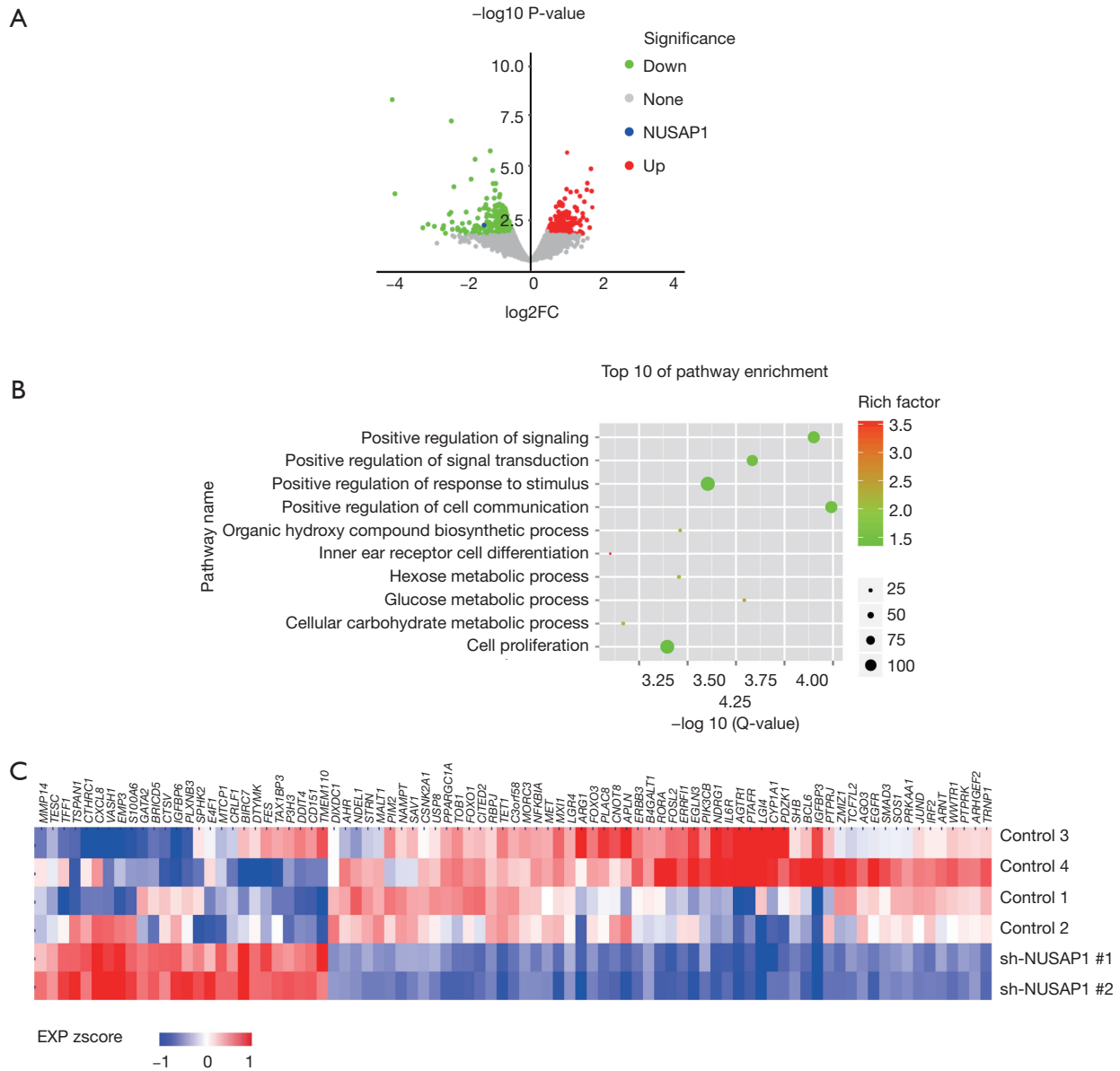

Figure 3 Differential expression genes (DEGs) and gene pathway enrichment analysis in NUSAP1 knockdown HepG2 cells. (A) RNA-Seq data of HepG2 cells knocking down or not knocking down the NUSAP1 gene were downloaded from the ENCODE project and analyzed for differential genes. Volcano plot was used to summarize DEGs between the knockdown and the control group. (B) The top 10 GO-pathways with DEGs over-represented. Negative log base 10 Q-values from pathway enrichment analysis was plot, and pathways were sorted by the P values of DEGs enrichment based on core-DEGs dataset. (C) Heat map of core-DEGs related to proliferation process in NUSAP1 knockdown HepG2 cells. The heat map shows that 85 genes were significantly differentially expressed in HepG2 cells after NUSAP1 knockdown. NUSAP1, nucleolar and spindle-associated protein 1.

Discussion

NUSAP1 is a recently identified 55-kDa MAP (23). Microtubules participate in the assembly of the mitotic spindle, which is crucial for ensuring the precise distribution of cellular material between two daughter cells (21). NUSAP1 is one of the proteins essential for mitotic spindle arrangement and function (24). By comparing gene expression patterns between proliferating and differentiating

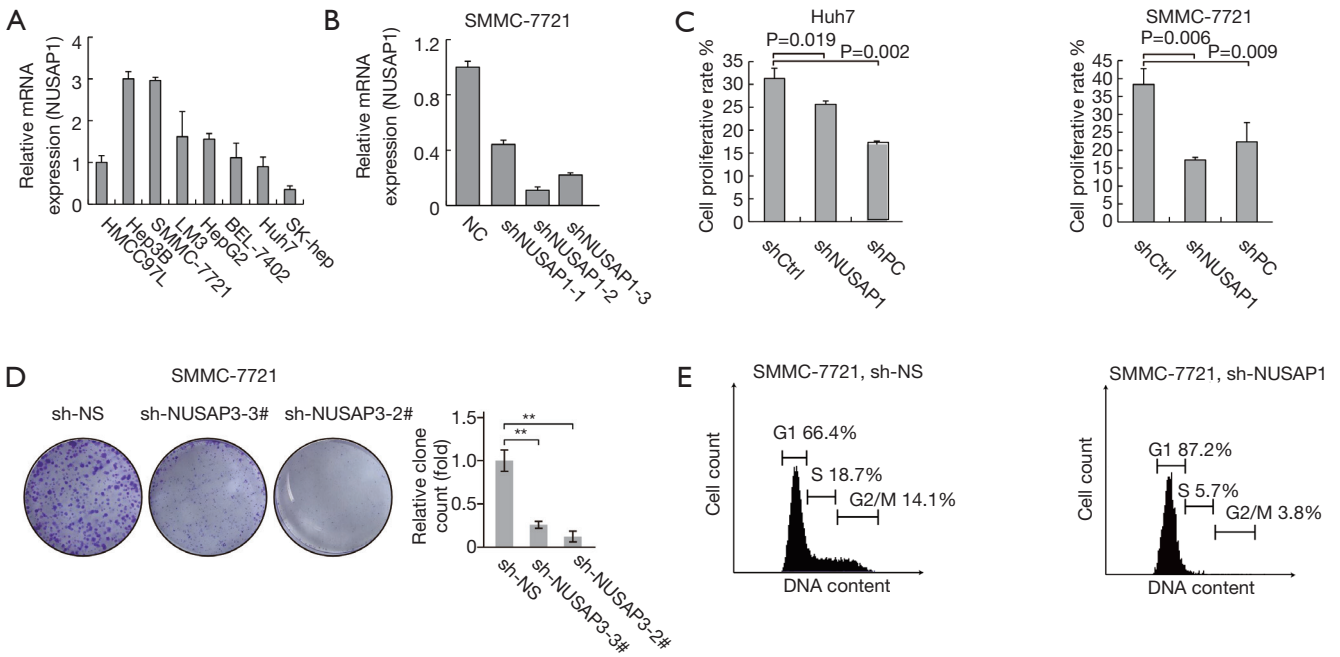

Figure 4 Cell proliferation assay in NUSAP1 knockdown HCC cell lines. (A) NUSAP1 expression in different HCC cell lines. NUSAP1 highly expressed in Hep3B, SMMCC-7721, LM3, HepG2. (B) The capability of different shRNAs down regulating NUSAP1 expression. (C) the cell proliferation rate in SMMC-7721 and Huh7 cell lines at 48 h after knocking down NUSAP1. After transfecting NUSAP1 shRNA into the two cell lines, NUSAP1 expression was down-regulated, and the cell proliferation in Huh7 and SMMC-7721cell lines was inhibited dramatically, compared to the shCtrl groups. (D) Representative clonogenic assay of SMMC-7721 cells expressing the indicated plasmids. Quantitative analysis was performed using ImageJ software. The bar graph shows the means ± SD, n=3, **, P<0.01. (E) SMMC-7721 cells transfected with the indicated plasmids for 48 h were subjected to cell cycle assays. HCC, hepatocellular carcinoma; NUSAP1, nucleolar and spindle-associated protein 1.

MC3T3E1 mouse osteoblasts *in vitro*, Raemaekers *et al.* [2003] identified NUSAP1 as a proliferation marker in cells (24). NUSAP1 expression is upregulated in dividing cells and reaches peak levels at G2 to M phase transition during the cell cycle (21,23,24). NUSAP1 overexpression leads to extended bundling of cytoplasmic microtubules during interphase. In contrast, the depletion of NUSAP1 in proliferating cells by RNA interference causes nuclear defects and disruptions in normal cell cycle progression (24). These data demonstrate the essential roles of NUSAP1 during cell proliferation.

Previous studies have found that NUSAP1 is actively expressed in some cancers such as prostate cancer (25), breast cancer (26), melanoma (27,28), and glioma (29), and *NUSAP1* gene expression is associated with cell proliferation, migration and invasion in some specific cancer types (26,30). Gordon *et al.* [2017] found that NUSAP1 accelerates the invasion and metastasis of prostate cancer by partly modulating downstream FAM101B, which is a TGFβ1 signaling effector, and participates in epithelial-mesenchymal transition (EMT) (31,32). Additionally, researchers have found that NUSAP1 is regulated by the Rb-E2F pathway in prostate cancer (25). These findings all imply the vital functions of NUSAP1 in the development and progression of various tumors. Nevertheless, the mechanisms of NUSAP1 in HCC remains unknown.

In our study, meta-analysis of five independent datasets validated high mRNA expression of NUSAP1 in HCC in a large sample (650 samples). As shown in the forest plot (*Figure 1C*), compared to that in nontumor samples, NUSAP1 was predominantly overexpressed in HCC samples [SMD =1.94, 95% CI, 1.63–2.26, I^2=44%]. Additionally, TCGA dataset confirmed the high expression of NUSAP1 in HCC (*Figure 1B*). On the other hand, the clinical data obtained from TCGA suggested that the overexpression of NUSAP1 in HCC is related to

clinicopathological features (such as poor survival and high tumor histologic grade and TNM stage). Overexpression of NUSAP1 was also associated with the iCluster1 subgroup defined by TCGA Research Network, implying the NUSAP1 could activate cell proliferation.

In addition, we established a cell model with NUSAP1 downregulation to explore the role of NUSAP1 in HCC. Pathway analysis based on RNAseq data suggested that NUSAP1 could active the expression of genes involves in cell proliferation. As shown in *Figure 4C*, NUSAP1 expression was downregulated after the transfection of NUSAP1-targeting shRNA into two cell lines, and compared to that in the shCtrl-transfected cells, proliferation was dramatically inhibited in shNUSAP1-trasfected Huh7 and SMMC-7721 cells, indicating that NUSAP1 promotes cell proliferation in HCC. Based on the results of this study and previous reports, more experiments should be performed to investigate the role of NUSAP1 in the regulation of HCC development and progression, and NUSAP1 might be a target for HCC therapy.

In conclusion, compared to that in nontumor tissues, NUSAP1 is highly expressed in HCC tumor tissues. Analysis of clinical data showed that HCC patients with high NUSAP1 expression have poor prognosis. In addition, the results of cell proliferation assays also proved that NUSAP1 plays a crucial role in HCC cell proliferation. Our findings indicate that NUSAP1 might be a novel diagnostic marker and a therapeutic target in HCC.

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr.2019.01.29). The authors have no conflicts of interest to declare.

Ethical statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Supplementary

Table S1 HCC datasets downloaded from NCBI GEO

GEO ID	Platforms	Total	Tumor	Non-tumor
GSE45267	HG-U133_Plus_2	86	45	41
GSE41804	HG-U133_Plus_2	40	20	20
GSE14520-2	HG-U133A_2	43	22	21
GSE14520-1	HT_HG-U133A	395	206	189
GSE60502	HG-U133A	28	13	15
GSE6764	HG-U133_Plus_2	58	35	23
Total		650	341	309

HCC, hepatocellular carcinoma; GEO, Gene Expression Omnibus.