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Identification of cancer-related gene network in hepatocellular carcinoma by combined bioinformatic approach and experimental validation

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ABSTRACT

HCC (hepatocellular carcinoma) is a highly aggressive malignancy that cause a mass of deaths world widely. We chose gene expression datasets of GSE27635 and GSE28248 from GEO database to find out key genes and their interaction network during the progression and metastasis of HCC. GEO2R online tool was used to screen differentially expressed genes (DEGs) between tumor and peri-tumor tissues based on these two datasets. The identified differentially expressed genes were prepared for further analysis such as GO function, KEGG pathway, PPI network analysis using Database for Annotation, Visualization and Integrated Discovery (DAVID) and Retrieval of Interacting Genes (STRING). Two modules were constructed by MOCDE plugin in Cytoscape and 21 genes were selected as hub genes during this analysis. The expression heatmap and GO function of hub genes were performed using R pheatmap package and BiNGO plugin in Cytoscape respectively. Six hub genes including CDC25 A, CDK1, HMMR, MYBL2, TOP2A were recollected for survival analysis and their expression was validated using Kaplan Meier-plotter and GEPIA website. We also investigated the DEGs between metastasis and non-metastasis tissues and two genes (NQO1 and PTHLH) are highly associated with the metastasis in HCC. Further verification using woundhealing and transwell assay confirmed their ability to mediate cell migration and invasion. In summary, our results obtained by bioinformatic analysis and experimental validation revealed the dominant genes and their interaction networks that are associated with the progression and metastasis of HCC and might serve as potential targets for HCC therapy and diagnosis.

1. Introduction

HCC (hepatocellular carcinoma) has become an aggressive tumor, with approximately 782,500 new cases and 745,500 deaths in 2012 [1], which accounts for the second most common mortality of cancer-related death worldwide [2]. Although surgical resection and other therapies may improve the patients' overall survival, the 5-year recurrence remains extremely high ranging from 80% to 90% [3]. For the advanced HCC, the multi-target tyrosine kinase inhibitor (TKI) sorafenib, which acts as an anti-angiogenic and anti-proliferative drug, is capable of prolonging life expectation. But the effect is limited because the median survival is minimally extended with 3 months [4]. Thus, novel approaches should be explored to find out more disease-related genes and subsequently make breakthrough both in early diagnosis and the therapy of HCC.

The development of HCC is a multistep process involving a variety of genome and transcriptome deregulation. Some previous studies have classified HCC into two main groups based on the molecular discrepancy: the proliferation group and the non-proliferation group [5]. the proliferation groups characterized by activation of survival signaling pathways such as the PI3K-AKT [6], RAS-MAPK [7] and MET [8] cascades, chromosomal instability, FGF19 amplification as well as the inactivation of TP53 (9). The non-proliferation group, however, is characterized by aberrant activation of WNT signaling pathway attributed to the mutation of CTNNB1 and high incidence of TERT promoter mutation [10]. Moreover, many mi-RNAs have been reported to

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play a pivotal role in the enormous biological processes of HCC such as proliferation, apoptosis, and metastasis. For example, the inhibition of miR-25 could enhance apoptosis of HCC cells caused by TRAIL though PTEN/PI3K/AKT/BAD pathway [11], whereas its over-expression could facilitate the EMT formation by suppressing RhoGDI1 [12].

Admittedly, the rapid progression of high throughout strategies makes it possible to find out more HCC-related genes. However, it is inappropriate to investigate their functions as a single factor rather than a whole network. A certain biological function can rarely be attributed to one gene or a few genes and cannot be predicted by studying the properties of isolated components. In contrast, most biological functions arise from interactions among many components. Previous work on interaction networks in HCC cell lines identified many interactions among proliferation, apoptosis-related proteins, suggesting a 'module of genes' be highlighted in the diagnosis and prediction of metastasis in HCC [13].

Hence, in this study, we are the first to use GEO2R online tool to search HCC-related differentially-expressed genes in GSE27635 and GSE28248 from GEO (Gene Expression Ominibus). Furthermore GO function and KEGG pathways of these genes were analyzed by DAVID, while PPI network within two important modules were also established to obtain hub genes in the progression of HCC. Subsequently, Cytoscape was used to explore the GO function of hub genes, Kaplan Meier Plotter online database was applied to analyze the correlation between the hub genes and overall survival, and GEPIA web tool based on TCGA database was used to compare the different expression of hub genes between tumor and normal tissues. Meanwhile, we also screened out differentially expressed genes that may partially attributed to bone metastasis or lymphatic metastasis from the two data sets and overlapped them with the co-differentially expressed genes to obtain metastasis relevant genes in HCC. Finally, wound healing and transwell assay were implemented to verify their ability to affect metastasis in HCC development.

2. Materials and methods

2.1. Microarray data

Two data sets GSE27635 and GSE28248 were chosen from GEO database [14] (http://www.ncbi.nlm.nih.gov/geo) based on the same GPL5858 platform, DASL Human Cancer Panel by Gene. The probes were converted into the corresponding gene symbol according to the annotation information in the platform. GSE27635 contains 48 pairs of intratumoral and peritumoral formalin-fixed, paraffin-embedded (FFPE) tissue from hepatocellular carcinoma (HCC) patients with and without bone metastases. GSE28248 consists of 40 pairs of intratumoral and peritumoral formalin-fixed, paraffin-embedded (FFPE) tissue from hepatocellular carcinoma (HCC) patients with and without lymphatic metastases.

2.2. Identification of DEGs

DEGs (differentially expressed genes) between tumor and peritumor tissues from the two datasets were screened out by the use of GEO2R (http://www.ncbi.nlm.nih.gov/geo/geo2r), and the respective DEGs from separate data sets were overlapped with VENN (http://bioinformatics.psb.ugent.be/webtools/Venn/) to detect the co-differentially-expressed genes. The adjust p values, Benjamini and Hochberg false discovery rate were used to provide a balance between discovery of statistically significant genes and limitation of false-positives. Probe sets without corresponding gene symbols or genes with more than one probe set were removed or averaged. The adjust p value < 0.05 and $|\log FC| \geq 1$ were set as the cut off criterion.

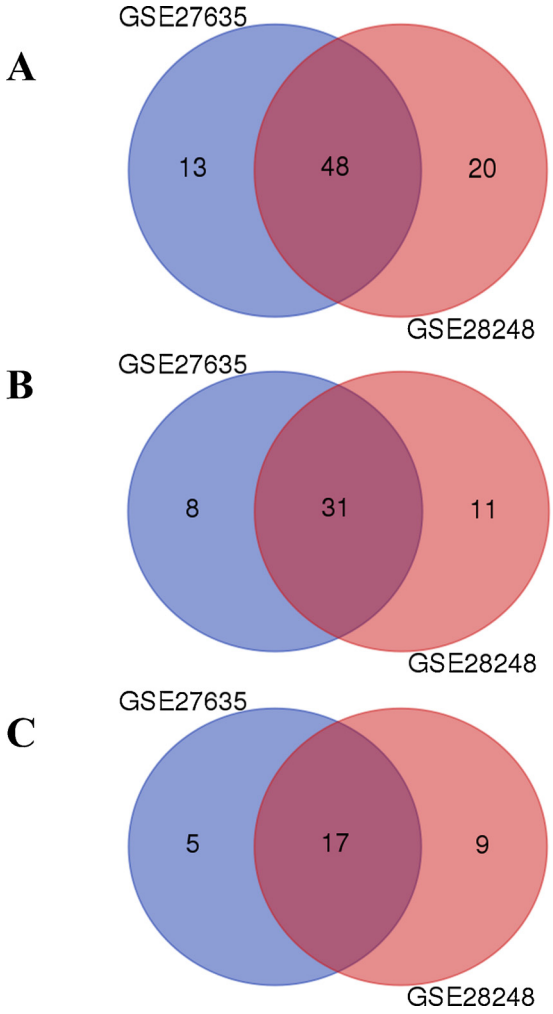

Fig. 1. Venn diagram demonstrated co-differentially-expressed genes between tumor and peri-tumor tissues among GSE27635 and GSE28248. (A) Total co-differentially-expressed genes. (B) Up-regulated co-differentially-expressed genes. (C) Down-regulated co-differentially-expressed genes.

2.3. Gene ontology and KEGG pathway analysis of DEGs

Gene ontology (GO) analysis is a tool to annotate genes and gene products identifying their characteristic biological functions based on the high-throughput genome or transcriptome data [15]. Kyoto Encyclopedia of Genes and Genomes (KEGG) is a collection of databases dealing with genomes and biological pathways [16]. The Database for Annotation Visualization and Intergrated Discovery (DAVID; http://david.ncifcrf.gov) (version 6.8) [17] integrating the information of biological functions of genes and analysis tools was used to unravel the GO and KEGG pathway of identified co-DEGs.

2.4. PPI network and module establishment

Search Tool for the Retrieval of Interacting Genes (STRING; http://string-db.org) is a web tool for elucidating protein–protein interaction (PPI) [18]. Cytoscape is an open-access software designed to visualize molecular interaction networks [19]. These tools were used to evaluate the protein interactions and hub genes among co-differentially expressed genes from the two GSEs. Combined score > 0.4 was deemed to be significant. In addition, the Molecular Complex Detection (MCODE) app plugin of Cytoscape was applied to verify important modules with the following standard: degree cutoff = 2, node score cutoff = 0.2, k-core = 2, and max. depth = 100.

Table 1
The GO function of up-regulated co-differentially-expressed genes.

Category	Term	Count	%	PValue	Genes	FDR
Upregulat						
GOTERM_BP_DIRECT	GO:0000082~G1/S transition of mitotic cell cycle	5	16.12903	3.11E-05	CDC25 A, TYMS CDK1, CDKN2A CDKN2C	0.046625
GOTERM_BP_DIRECT	GO:0030335~positive regulation of cell migration	6	19.35484	1.71E-05	NOTCH1, TGFBR1 PDGFA, HBEGF CDH13, PDGFRB	0.025556
GOTERM_MF_DIRECT	GO:0019901~protein kinase binding	7	22.58065	4.43E-05	MSH2, E2F1 CDC25 A, CCNA2 PDGFRB, CDKN2A CDKN2C	0.05313
GOTERM_MF_DIRECT	GO:0019899~enzyme binding	7	22.58065	2.24E-05	NOTCH1, MSH2 PDGFRB, HOXA9 BIRC5, XRCC1 TOP2A	0.026893
GOTERM_CC_DIRECT	GO:0005654~nucleoplasm	19	61.29032	2.64E-08	E2F1, CDK1 RBBP5, MSH2 AFF3, BIRC5 MYBL2, XRCC1 CDC25 A, MEN1 PTHLH, TYMS NOTCH1, CDKN2A TOP2A, CCNA2 TERT, BARD1 SMARCA4	2.95E-05
KEGG_PATHWAY	hsa04110:Cell cycle	6	19.35484	2.77E-05	CDC25 A, CCNA2 CDK1, CDKN2A CDKN2C, E2F1	0.027284

Table 2
The GO function of down-regulated co-differentially-expressed genes.

Category	Term	Count	%	PValue	Genes	FDR
Downregulated						
GOTERM_BP_DIRECT	GO:0042981~regulation of apoptosis	8	47.05882	1.08E-05	CASP10, BCL2 ESR1, IL1B IGF1, NGFR GAS1, TNFSF8	0.017
GOTERM_BP_DIRECT	GO:0043067~regulation of programmed cell death	8	47.05882	1.15E-05	CASP10, BCL2 ESR1, IL1B IGF1, NGFR GAS1, TNFSF8	0.018146

2.5. Heatmap drawing, GO and clinical analysis of hub genes

Based on the PPI network, we choosed hub genes based on following criterions that are degree > 10 and genes included in both two modules. Heatmaps of hub genes in GSE27635 and GSE28248 were drawn by the use of pheatmap package in R [20]. Survival analysis was achieved using Kaplan Meier-plotter (KM plotter, http://kmplot.com/analysis/) [21]. GO function of hub genes was assessed by BiNGO plugin of Cytoscape [22]. The boxplots of hub genes indicating the expression comparison between tumor and normal tissues in HCC were performed with GEPIA (http://gepia.cancer-pku.cn/index.html) which is a novel interactive web server for analyzing RNA sequencing data from TCGA database [23].

2.6. Screen of metastasis related genes

We compared gene profiling in HCC patients with or without bone metastasis by using GSE27635 dataset to obtain the bone metastasis-related genes, and compared the gene profiling in HCC patients with or without lymphatic metastasis to obtain the lymphatic metastasis-related genes. The analysis was performed using GEO2R. All the screened genes were overlapped with co-DEGs to determine general metastasis related genes.

2.7. Wound healing and transwell assay

HEP3B cell line was chosen for both assays. For wound healing assay, cells were plated in six-well plates with 1×10^5 cells per well. After transfection (50 nM), the cells were grown to 100% confluence. Then, a micropipette tip was applied to make a cross wound. Photographs were taken by a microscopy at 0 h, 24 h, 48 h, and 72 h after wounding. The transwell assay was performed using 24-well transwell chambers (Corning Incorporated, USA). Firstly, the inserts were coated with Matrigel (BD Bioscience, USA). After transfection (50 nM), 1×10^4 cells were suspended in 0.2 ml serum-free medium and added to the inserts. 0.6 ml medium with 20% FBS was added to the lower compartment as chemoattractant. After incubation for 24 h, cells on the lower surface were removed, fixed with 4% paraformaldehyde, and stained with 0.1% crystal violet. Three visual fields of 200× magnification were randomly selected and counted under light microscope.

3. Results

3.1. Identification of DEGs and co-DEGs in two GSEs

GSE27635 containing 48 pairs of intratumoral and peritumoral tissues with or without bone metastasis, and GSE28248 containing 40

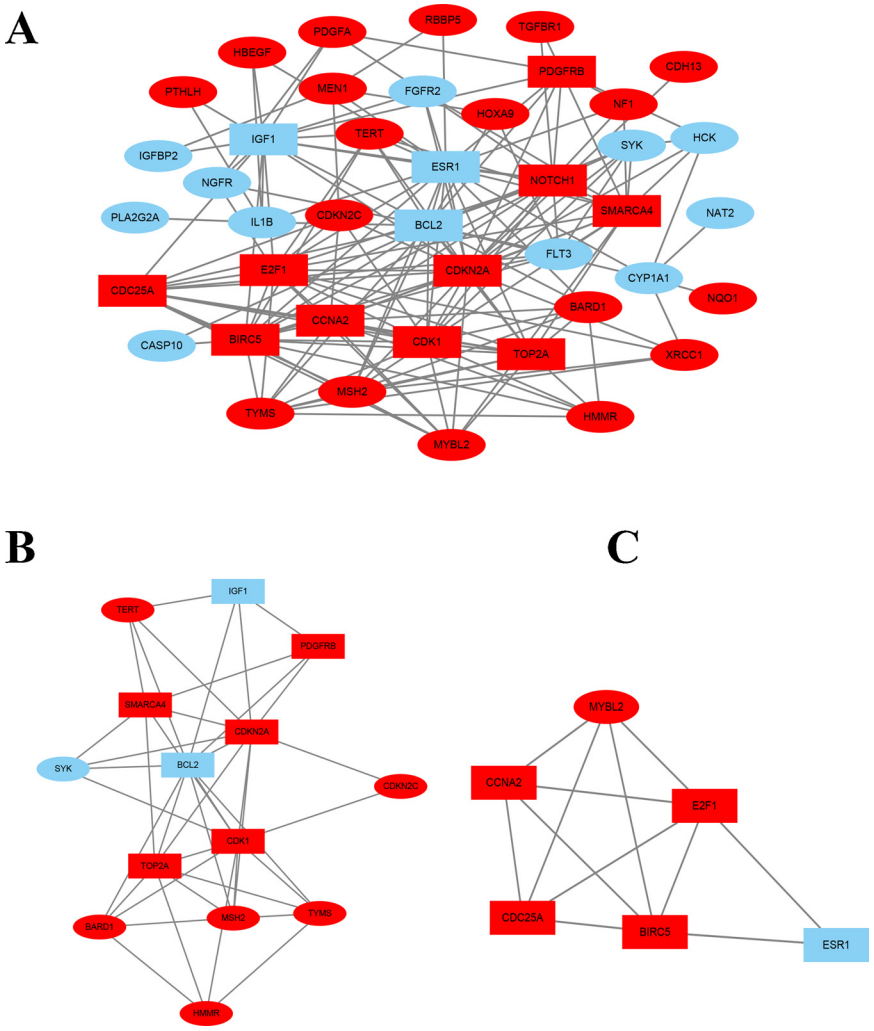

Fig. 2. PPI network construction of co-differentially-expressed genes. (A) PPI network was obtained by application of Cytoscape; the red boxes indicated up-regulated genes, while the blue ones indicated down-regulated genes. (B) (C) Two important modules were built based on the network.

pairs of intratumoral and peritumoral tissues with or without lymphatic metastasis were chosen for the subsequent analysis. A total of 61 and 68 DEGs (differentially expressed genes) between tumor and peritumor were detected in two dataset respectively and 48 genes of them are found in both two datasets (co-DEGs). Among these 48 genes, 31 was up-regulated and 17 was down-regulated (Fig. 1). Adjust p value < 0.05 and $|\log FC| > 1$ were set as the cut-off criterion.

3.2. GO and KEGG pathway analysis of co-DEGS

DAVID online tool was used to elucidate GO and KEGG pathways in the enriched co-DEGs. The results demonstrated that for biological process (BP), the up-regulated genes were mainly enriched in transition of mitotic cell cycle and regulation of cell migration, whereas the down-regulated ones are categorized in regulation of apoptosis; for molecular function (MF), the up-regulated genes were dominantly enriched on protein kinase binding and enzyme binding; for cell component (CC), the up-regulated genes were mainly associated to nucleoplasm; and for KEGG pathway, the up-regulated genes were mainly involved in cell cycle relevant cascades (Tables 1 and 2).

3.3. PPI network and hub genes selection

String web tool and Cytoscape were applied to verify the protein interaction network among the co-DEGs and two important modules were constructed during this process (Fig. 2). We selected 21 genes with high connective degree (> 10), and the genes attributed to the two modules as the hub genes.

3.4. Heatmap, GO and clinical analysis of hub genes

Two heatmaps of hub genes in GSE27635 and GSE28248 were drawn by pheatmap package in R (Fig. 3A). GO analysis was accomplished using Cytoscape plugin BiNGO, and the results showed that the hub genes were mainly enriched in regulation of cell cycle, cell death, cell apoptosis, interphase of mitotic cell cycle for biological process (BP); protein binding, kinase binding, enzyme binding, protein dimerization activity, cyclin-dependent protein kinase regulator activity for molecular function (MF); and nuclear part, intracellular organelle, macromolecular complex for cellular component (CC) (Fig. 3B). Among the hub genes, six with higher fold changes (mean $|\log FC| > 2$ in two datasets) were recollected to subsequently explore their impact on HCC patients' survival. The Kaplan Meier plots indicates that high expression of CDC25 A, CDK1, HMMR, MYBL2, TOP2A leaded to worse overall survival, whereas up-regulation of ESR1 represented better life expectancy (Fig. 4A). Moreover, boxplots were also made by GEPIA based on TCGA database to compare different mRNA level of the six genes between tumor and peritumor tissue. Consistent with the survival analysis, five genes except ESR1 showed significantly higher expression in HCC patients (Fig. 4B).

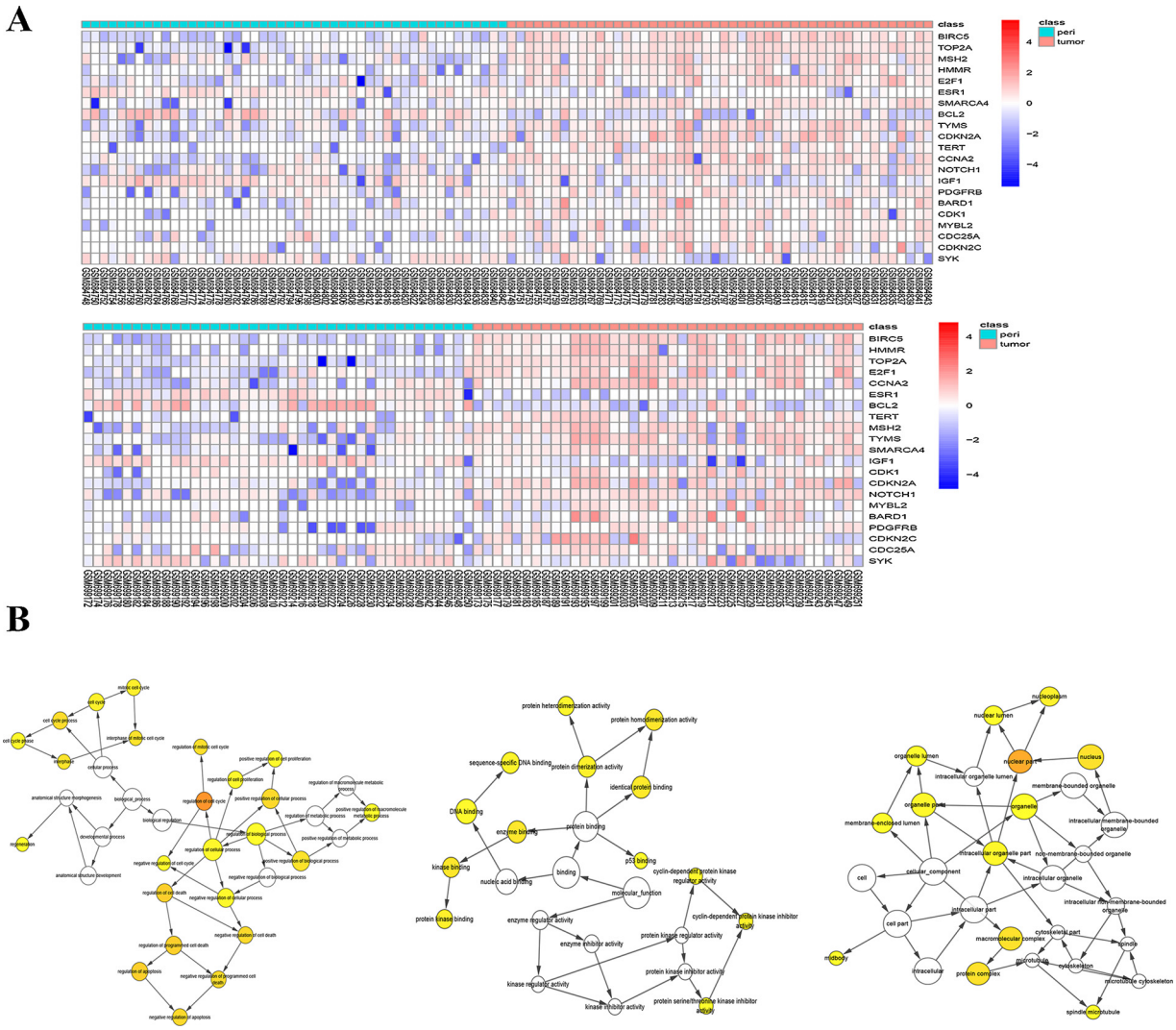

Fig. 3. Heatmap, GO function analysis of hub genes. (A) Up panel and down panel indicated heatmaps of hub genes expression in GSE27635 and GSE28248 respectively. (B) The GO function analysis by the use of Cytoscape, from left to right indicated BP, MF, and CC, respectively; the node size represented the number of genes enriched in the category, while the node color represented the statistical significance (yellow indicated $p < 0.05$ and deeper color was paralleled with smaller p value).

3.5. Screening of metastasis-related genes and followed experimental validation

To identify the potential metastasis-relevant genes in HCC, we compared gene profiling between patients with or without bone metastases in GSE27635, and patients with or without lymphatic metastases in GSE28248. A total of 9 and 23 differentially expressed genes that might be partially attributed to bone or lymphatic metastases respectively were isolated (Table 3). Intriguingly, only one gene PDGFRA was showed to be dominantly appeared in both groups which has been reported correlated with cell migration in HCC cell line [24]. In a forward step, we combined these 32 genes and overlapped them with the previously discovered co-DEGs to detect the genes with both activity in tumor progression and invasion. In this process, four genes were screened out as HCK, PTHLH, NQO1, PLA2G2A (Table 3), among which, NQO1 and PTHLH were up-regulated whereas HCK and PLA2G2A were down-regulated according to the PPI analysis. Therefore, we hypothesized NQO1 and PTHLH might have the potential to positively mediate HCC metastasis. To examine this hypothesis, wound healing and transwell assay were sequentially implemented. The results suggested that both si-RNAs targeting NQO1 or PTHLH induced attenuated ability of cell invasion and migration in HEP3B cell line (Fig. 5).

4. Discussion

HCC is a highly heterogeneous carcinoma with poor prognosis. Since most HCC patients are diagnosed at an advanced stage, for which curative therapies are lacking. It is imperative to seek promising strategies against the disease. To date, the rapid progress of high throughout technique and public gene database make it possible to screen out more disease-related gene based on numerous data using microarrays, analyze them from an integral perspective and thus find out potential novel targets for both early diagnosis and therapy.

In the present study, two data sets GSE27635 and GSE28248 comprising 48 and 40 pairs of tissue, respectively, were downloaded from GEO database. A total of 48 cancer-related genes were obtained from both two datasets, including 31 up-regulated genes and 17 down-regulated genes. For a more in-depth understanding of function regarding these genes, we performed analysis to identified enriched GO function and KEGG pathway.

The results demonstrated that the up-regulated genes were mainly involved in transition of mitotic cell cycle, regulation of cell migration (BP), protein kinase binding, enzyme binding (MF), nucleoplasm (CC) and cell cycle relevant cascades (KEGG pathway), while the down-regulated genes were mainly enriched in regulation of apoptosis and

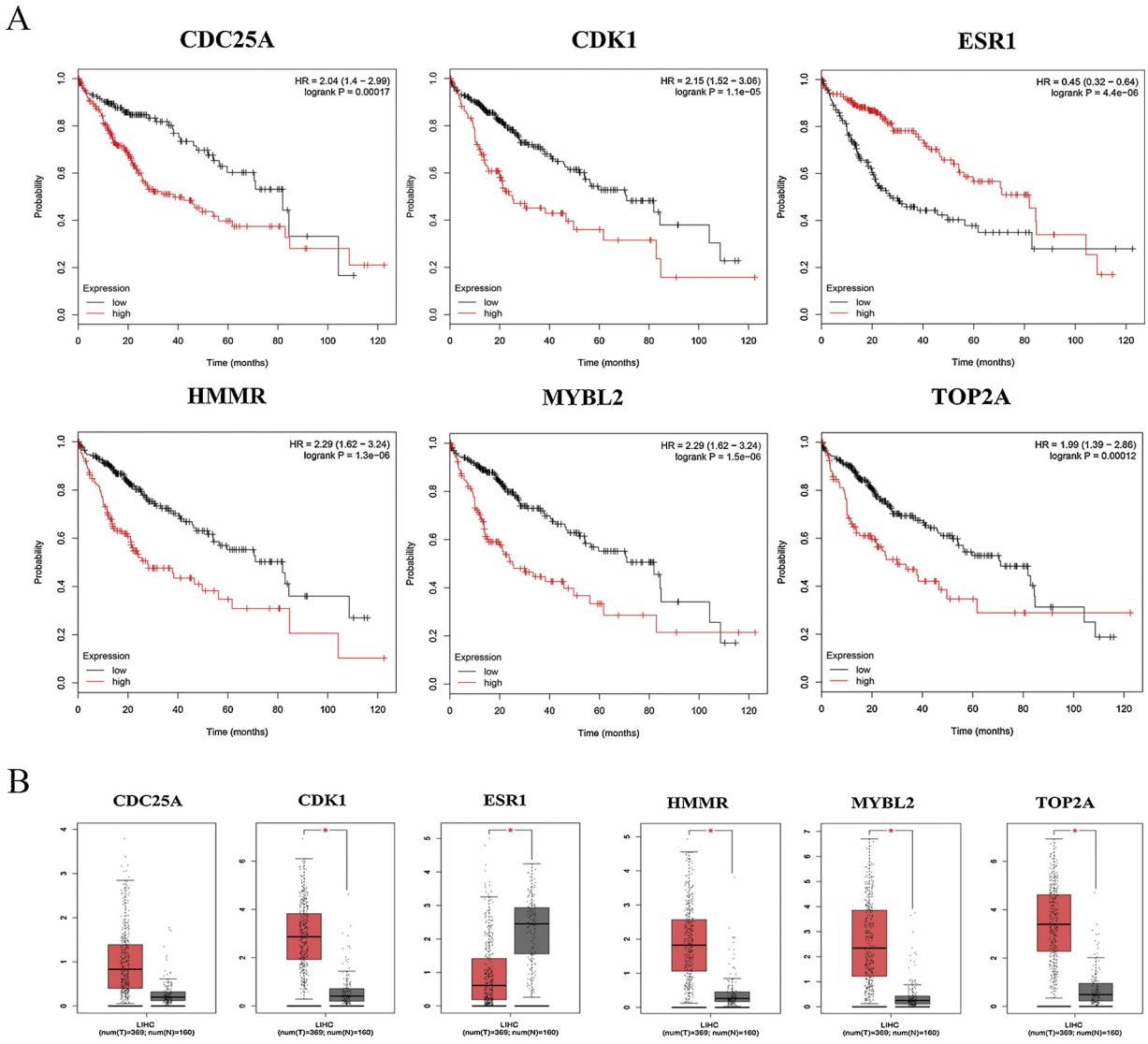

Fig. 4. Clinical analysis of hub genes. (A) Overall survival analysis of CDC25 A, CDK1, HMMR, MYBL2, and TOP2A. (B) Different expression pattern of CDC25 A, CDK1, HMMR, MYBL2, and TOP2A between tumor and peri-tumor based on TCGA.

Table 3
The potential metastasis relevant genes.

Bone metastasis	Lymphatic metastasis	Shared genes	Union set overlapped with DEGs
AR, CSF2 MST1R, PTHLH GAS7, MCF2 LCN2, PDGFRA L1CAM	KIT, PLA2G2A IFNGR1, SPP1 CXCL8, IGF2 NQO1, ABCB1 E2F5, FLT1 HCK, TDGF1 VEGFA, MECOM FGF3, FOSL2 GLI1, WNT1 PDGFRA, TYRO3 MCAM, FGF6 SRC	PDGFRA	HCK, PTHLH, NQO1, PLA2G2A

regulation of programmed cell death (BP). These findings are highly consistent with reported studies indicating the pivotal roles of cell cycle transition, apoptosis, cell migration and nuclear components during the progression of HCC [25,26]. Moreover, the clinical application of TKI (tyrosine kinase inhibitor) also implies the importance of protein kinase binding [4], which is supportive with our current results. Further PPI analysis was carried out to construct a protein interaction network which might show a holistic map of these important genes. Among this process, 21 genes with higher connectivity were selected as hub genes. GO analysis of hub genes using Cytoscape was consistent with results from differential genes indicating hub genes mainly focused on regulation of cell cycle, cell death, cell apoptosis, interphase of mitotic cell cycle (BP), kinase binding, enzyme binding, protein dimerization activity, cyclin-dependent protein kinase regulator activity (MF), and nuclear part, intracellular organelle, macromolecular complex (CC).

Among the 21 hub genes, six genes were recollected for the survival analysis due to their higher expression pattern in both datasets. Kaplan-Meier plots suggested CDC25 A, CDK1, HMMR, MYBL2, TOP2A were associated with worse overall survival whereas ESR1 manifested an opposite trend. The expression distinction analysis between tumor and peri-tumor based on TCGA database also indicated higher levels of CDC25 A, CDK1, HMMR, MYBL2, TOP2A and a lower level of ESR1 in tumor tissues, which was in accordance with the PPI network results (red: up-regulated, blue: down-regulated). Previous studies have provided abundant evidence about the function of the six genes in cancer development. For instance, CDC25 A is known as an oncogene to assist both G1/S and G2/M progression in various types of cancers including HCC [27]; similarly, CDK1 is a member of Ser/Thr protein kinase

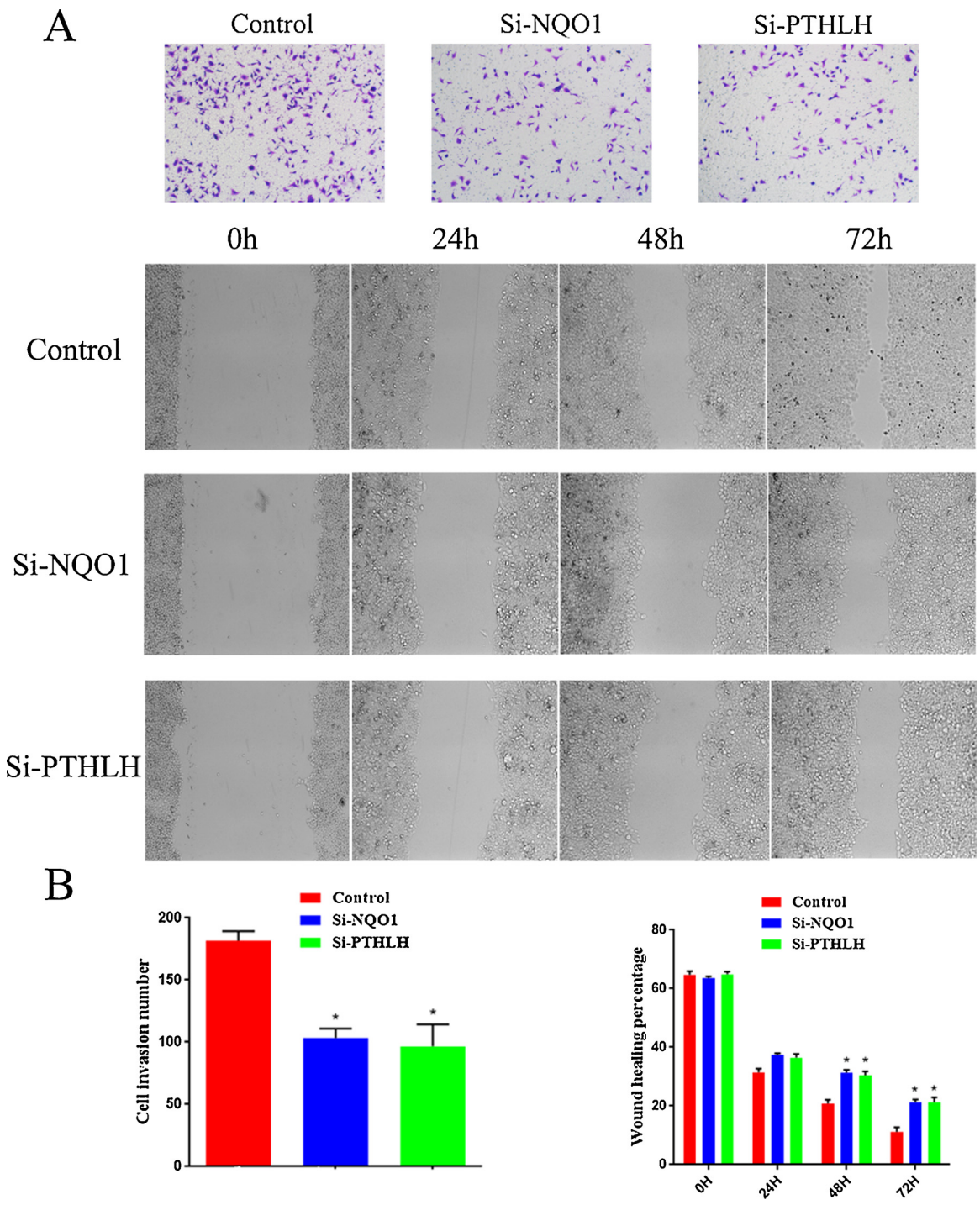

Fig. 5. Wound healing and transwell assay in HEP3B cell line. (A) Wound healing and Transwell assay demonstrated suppressed ability of cell migration and invasion by knocking down of NQO1 and PTHLH. (B) Relative invasive cell numbers and wound healing area according to the assays. *, $p < 0.05$.

essential for cell cycle transition, and its inhibitor can enhance the efficiency of sorafenib in PDX tumor models [28,29]; extracellular HMMR forms a complex with CD44 which activates intracellular signaling pathways and regulates tumor cell survival, proliferation and migration [30]; MYBL2 together with E2F1-3 regulates the genes related to G2/M phase such as CDC2, CyclinA, and CyclinB1 (31); ESR1 over-expression induces cell apoptosis via binding with SP1 in Hep3B cell line [32]. Of note, although HMMR has been reported to exert pro-tumor effects in various kinds of cancers, its exact function or mechanism mediating HCC progression remains poorly understood and worth in-depth study.

Analysis of differentially expressed genes between different metastasis statuses in two datasets was also developed. A total of 32 genes were found that might partially contribute to the metastasis of HCC, while PDGFRA was the only one gene altered in both bone and lymphatic metastasis groups. The results are consistent with previous studies that PDGFRA was positively correlated with microvessel growth and capable of mediating cell migration and invasion in HCC cell lines [24]. Further overlap analysis determined four genes including HCK, PTHLH, NQO1, PLA2G2A that might contribute to both tumor metastasis and progression. Since our results confirmed that PTHLH and NQO1 had higher expression in tumor tissues, and prior studies demonstrating their ability in enhancing cell invasion in other cancers

[33,34], hence, we reasoned that PTHLH and NQO1 might be more preferable positively relating to cell migration and invasion in HCC. The validation experiments also confirmed our hypothesis that knocking-down of PTHLH and NQO1 in HCC cells greatly reduces migration ability.

In summary, our present work by analyzing multiple datasets from GEO database coupled with experimental validation identifies the dominant genes and their interaction network during the progression and metastasis of HCC. Some of these genes have never been reported to affect the progression of HCC and may serve as potential targets for HCC therapy. However, due to the low quantity of gene probes in our chosen datasets, the number of discovered DEGs was strikingly limited. More genes and non-coding RNAs should be detected to enrich the network for a more comprehensive and integrate understanding of HCC development.

Acknowledgments

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