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*BRIEF ARTICLES*

# Genomic-wide analysis of lymphatic metastasis-associated genes in human hepatocellular carcinoma

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## Abstract

**AIM:** To identify the genes related to lymph node metastasis in human hepatocellular carcinoma (HCC), 32 HCC patients with or without lymph node metastasis were investigated by high-throughput microarray comprising 886 genes.

**METHODS:** The samples of cancerous and non-cancerous paired tissue were taken from 32 patients with HCC who underwent hepatectomy with lymph node dissection. Total RNA was extracted from the cells obtained by means of laser microdissection (LCM) and was amplified by the T7-based amplification system. Then, the amplified samples were applied in the cDNA microarray comprising of 886 genes.

**RESULTS:** The results demonstrated that 25 up-regulated genes such as cell membrane receptor, intracellular signaling and cell adhesion related genes, and 48 down-regulated genes such as intracellular signaling and cell cycle regulator-related genes, were correlated with lymph node metastasis in HCC. Amongst them were included some interesting genes, such as *MET, EPHA2, CCND1, MMP2, MMP13, CASP3, CDH1*, and *PTPN2*. Expression of 16 genes (*MET, CCND1, CCND2, VEGF, KRT18, RFC4, BIRC5, CDC6, MMP2, BCL2A1, CDH1, VIM, PDGFRA, PTPN2, SLC25A5* and *DSP*) were further confirmed by real-time quantitative reverse transcriptional polymerase chain reaction (RT-PCR).

**CONCLUSION:** Tumor metastasis is an important biological characteristic, which involves multiple genetic changes and cumulation. This genome-wide information contributes to an improved understanding of molecular alterations during lymph node metastasis in HCC. It may help clinicians to predict metastasis of lymph nodes and assist researchers in identifying novel therapeutic targets for metastatic HCC patients.



**Key words:** Hepatocellular carcinoma; Lymphatic metastasis-associated genes; cDNA microarray; Expression profiling

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## INTRODUCTION

Hepatocellular carcinoma (HCC), endemic to sub-Saharan African and Asian with a rising incidence in Western countries, is one of the most common fatal malignancies in the world[1-3]. This is due to different risk factors. Chronic hepatitis B virus (HBV), hepatitis C virus (HCV) infection[4] and exposure to the carcinogen aflatoxin[5] are important risk factors for African and Asian populations. HCC has a poor prognosis, with a 5-year survival of less than 3% in inoperable cases. The high mortality associated with this disease is mainly attributed to its high tendency to metastasize. In fact, local lymph node and blood metastases could occur at an early stage, which may be the key factors related to its recurrence and poor prognosis[5]. Thus, a better understanding of the molecular mechanism of metastasis can improve prevention and treatment of HCC.

Metastatic spread of tumor cells is a process involving multiple steps. To metastasize, tumor cells need to detach from the primary tumor mass, migrate to a distant secondary site, and rapidly expand in the new environment. The whole process requires activation and deactivation of multiple specific genes[6,7]. The present challenge is to identify the crucial genes controlling the metastasis and determine the regulatory mechanism of these genes. Hence, global analysis of expression profiles of a large number of genes in clinical HCC specimens is an essential step to clarify the detailed mechanism and discover potential biomarkers of lymphatic metastasis in HCC. Microarray techniques, which have been developed since the early 1990s, provide a platform where one can measure the expression levels of tens of thousands of genes in a sample simultaneously[8-10], and it is now possible to uncover the complete picture of lymphatic metastasis of HCC. In the current study, we analyzed gene expression profiles in a total of 32 HCC patients using cDNA microarray technology and their relation to pathological features based on lymph node metastasis staging. Validating the cellular functions of these genes will help to identify the key or candidate genes/pathways responsible for lymph node metastasis, which might be used as diagnostic markers and therapeutic targets for lymph node metastasis.

## MATERIALS AND METHODS

### *Patient material*

The Institutional Review Board on Medical Ethics, Zhejiang Provincial People Hospital (China), approved the method of tissue collection. The present study was based on 32 patients who underwent hepatectomy for sporadic HCC without preoperative radio- or chemotherapy in the Surgery Department, Zhejiang Provincial People Hospital. All of the samples were immediately frozen in liquid nitrogen, and stored at -80℃ until use. A total of 32 HCC samples from 15 lymph node-negative and 17 lymph node-positive cases were used (Table 1).

**Table 1 Clincal data of patients with hepatocellular carcinoma**

| Case | Sex | Age | Hepatitis virus | Differentiated grade | TNM score |
|---|---|---|---|---|---|
| 1 | M | 54 | HBV | WD | T1N0M0 |
| 2 | M | 60 | HCV | WD | T1N0M0 |
| 3 | F | 61 | HBV | WD | T2N0M0 |
| 4 | M | 62 | HBV | WD | T2N0M0 |
| 5 | M | 58 | HBV | WD | T1N0M0 |
| 6 | F | 56 | HCV | MD | T3N0M0 |
| 7 | F | 44 | HBV | WD | T2N0M0 |
| 8 | M | 49 | HCV | WD | T1N0M0 |
| 9 | M | 58 | HBV | WD | T2N0M0 |
| 10 | M | 67 | HCV | PD | T3N0M0 |
| 11 | M | 69 | HBV | WD | T2N0M0 |
| 12 | F | 63 | HCV | WD | T1N0M0 |
| 13 | M | 48 | HCV | MD | T2N0M0 |
| 14 | F | 63 | HBV | WD | T1N0M0 |
| 15 | M | 49 | HCV | MD | T1N0M0 |
| 16 | F | 51 | HBV | PD | T3N1M0 |
| 17 | M | 65 | HCV | MD | T3N1M0 |
| 18 | F | 58 | HBV | PD | T4N1M1 |
| 19 | M | 60 | HBV | MD | T2N1M0 |
| 20 | F | 56 | HCV | PD | T3N1M1 |
| 21 | M | 42 | HCV | PD | T3N1M0 |
| 22 | M | 55 | HBV | PD | T4N1M1 |
| 23 | M | 66 | HBV | MD | T3N1M0 |
| 24 | F | 70 | HCV | WD | T2N1M0 |
| 25 | M | 58 | HBV | PD | T4N1M1 |
| 26 | M | 53 | HCV | PD | T3N1M0 |
| 27 | M | 61 | HBV | PD | T4N1M0 |
| 28 | F | 65 | HBV | MD | T3N1M0 |
| 29 | M | 59 | HCV | MD | T3N1M1 |
| 30 | M | 50 | HBV | PD | T3N1M0 |
| 31 | F | 63 | HCV | PD | T4N1M1 |
| 32 | M | 66 | HCV | PD | T3N1M0 |

M: Male; F: Female; HBV: Hepatitis B virus infection; HCV: Hepatitis C virus infection; WD: Well differentiated HCC; MD: Moderately differentiated HCC; PD: Poorly differentiated HCC.

### *Laser microdissection*

The 8 μm-thick sections of frozen tissue were continuously cut at -20℃ and stained with H&E. Under microscopic observation, parts of cancer cell nests in the invasive and intraductal components were microdissected using the LM100 laser capture microdissection system (Arcturus Engineering, Mountain View, CA, USA). We used a 15 μm-diameter beam to capture the tumor cells and corresponding noncancerous liver tissues, respectively. The cell nests were transferred to the laser microdissection (LCM) transfer film (CapSure TF-100S transfer film carrier, 5 mm-diameter optical-grade transparent plastic; Arcturus Engineering).

### *RNA preparation and T7-based RNA amplification*

Total RNA was isolated from the dissected specimens using Trizol reagent (Gibco BRL) and a modified acidic guanidinium phenol-chloroform method, following the manufactures recommendations. Total RNA was treated with DNase I for removal of genomic DNA. mRNA was purified using a poly(A) purification kit (Oligotex, Qiagen ) according to the manufactures instructions. The quality of mRNA was assessed by OD 260/280 ratios and the contamination of genomic DNA was checked using the PCR method. cDNA was synthesized with T7-oligo (dT) primer (Ambion) and Superscript Ⅱ enzyme (Gibco BRL) following

the instruction manual. cDNA was purified by cDNA clean-up column (DNA clear™ kit, Ambion). cRNA was generated by T7 MEGAscript™ kit (MEGAscript *in vitro* Transcription Kit, Ambion, Austin, TX, USA) following the manufactures recommendations. Column purification of cRNA was performed with RNeasy kit (Qiagen) according to the manufactures protocol. The concentration and quality of cRNA were analyzed by GeneQuant pro RNA/DNA Calculator (Amersham Pharmacia Biotech, Buckinghamshire, England).

### Microarray hybridization and scanning

Human Cancer Chip version 4.0 (IntelliGene, TaKaRa) was used for these studies. This array was spotted with 886 cDNA fragments of human genes, which are composed of 588 kinds of human identified genes related to cancer and 298 cDNA fragments prescreened by differential display methods between cancer tissue and normal tissues, on a glass slide. Three μg of cRNA from the tumor and the matched normal tissue were respectively labeled with Cy3-dUTP and Cy5-dUTP (Amersham Pharmacia Biotech, Buckinghamshire, England) using a labeling kit (RNA Fluorescence Labeling Core kit, TaKaRa), following the manufactures instructions. Labeled probe was purified by centrifugation in a spin column (Centrisep, Princeton Separations, Adelphia, NJ). Two separate probes were combined, and then, 2 μL of 5 × competitor containing CotI (Gibco BRL), poly dA (Amersham Pharmaca Biotech), and tRNA (TaKaRa) were added. After addition of 50 μL of 100% ethanol and 2 μL of 3 mmol/L sodium acetate (pH 5.2), the mixture was cooled at -80℃ for 30 min, followed by centrifugation at 15000 rpm for 10 min. For final probe preparation, the pellet was washed in 500 μL of 70% ethanol twice, and eluted in 10 μL hybridization buffer (6 × SSC, 0.2% SDS, 5 × Denhardt's solution, 0.1 mg/mL salmon sperm solution). The probes were denatured by heating for 2 min at 95℃, cooled at room temperature, and centrifuged at 15000 rpm for 10 min (20-26℃). Supernatants were placed on the array and covered with a 22 mm × 22 mm glass coverslip. The coverslip was sealed with a glue, and the probes were incubated overnight at 65℃ for 16 h in a custom-made slide chamber with humidity maintained by underlying moist papers. After hybridization, the slides were washed in 2 × SSC with 0.1% SDS, 1 × SSC, and 0.05 × SSC, sequentially for 1 min each, and then spin dried. Hybridized arrays were scanned using a confocal laser-scanning microscope (Affymetrix 428 array scanner, Santa Clara, CA). Image analysis and quantification were performed with ImaGene 4.2 software (BioDiscovery) as per the manufactures instructions.

### Data processing

Each spot was defined by manual positioning of a grid of circles over the array image. For each fluorescent image, the average pixel intensity within each circle was determined, and a local background outside of 3 pixel buffer range from the circle was computed for each spot. Net signal intensity was determined by subtraction of this local background from the average intensity of each spot. Signal intensities between the two fluorescent images were normalized by the intensities of the housekeeping genes provided on the arrays. The fluorescence intensities of Cy5 (non-tumor) and Cy3 (tumor) for each target spot were adjusted so that the mean Cy3:Cy5 ratios of 32 housekeeping gene spots were equal to one. Because data derived from low signal intensities are less reliable, we first determined cutoff values for signal intensities on each slide so that all of the filtered genes had greater S:N (signal to noise) ratios of Cy3 or Cy5 than three, and we excluded genes for further analysis when both Cy3 and Cy5 dyes gave signal intensities lower than the cutoff. To estimate the range of expression ratio within which the expression change could be considered as fluctuation in noncancerous cells, we compared expression profiles of noncancerous cells from 6 patients. Because 90% of expression ratios in noncancerous cells fell within the range of 1.726 and 0.503, we categorized genes into three groups according to their expression ratios (Cy3:Cy5): up-regulated (ratio: 2.0); down-regulated (ratio: 0.5); and unchanged expression (ratios between 0.5 and 2.0); provided that signal counts of T (Cy3) and R (Cy5) were > 500. Genes with Cy3:Cy5 ratios > 2.0 or < 0.5 in more than 75% of the cases examined were defined as commonly up- or down-regulated genes, respectively.

### Real-time reverse transcription-PCR

LightCycler (Roche Diagnostics) technology was applied to confirm the data which were obtained by cDNA microarray. The primer sequences of 16 genes were obtained from the GDB Human Genome Database (http://www.gdb.org/gdb/). We used the same RNA from the dissected cells in microarray analysis. First-strand cDNA was obtained by reverse transcription using a commercially available kit (first strand synthesis kit, Amersham). For each PCR, 2 μL (20 ng) first strand cDNA template, 50 pmol of each primer, 2.4 μL (3 mmol/L) $MgCl_2$, and 2 μL 10 × SYBR Green I (Roche Laboratories) were mixed in 20 μL of PCR mixture. The running protocol has been programmed based on the following three steps. In the first step, initial denaturation, reaction mixture was incubated for 10 min at 95℃. In the second step, DNA was amplified for 45 cycles at 95℃ for 10 s, specific annealing temperature (the primer sequences dependent) for 0-10 s, and elongation at 72℃ for some seconds (amplicon [bp]/25 s). Finally, the temperature was raised gradually (0.2℃/s) from the annealing temperature to 95℃ for the melting curve analysis. Twelve μL of PCR products were visualized by electrophoresis on 2% agarose gel stained with ethidium bromide. The amount of gene expression was normalized to the amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using Human GAPDH kit (GmbH Heidelberg, Heidelberg, Germany). We carried out qRT-PCR analysis in triplicate for each cDNA sample and used median values in three experiments. Up- and down-regulation were defined as the median value > 2.0 and < 0.5, respectively.

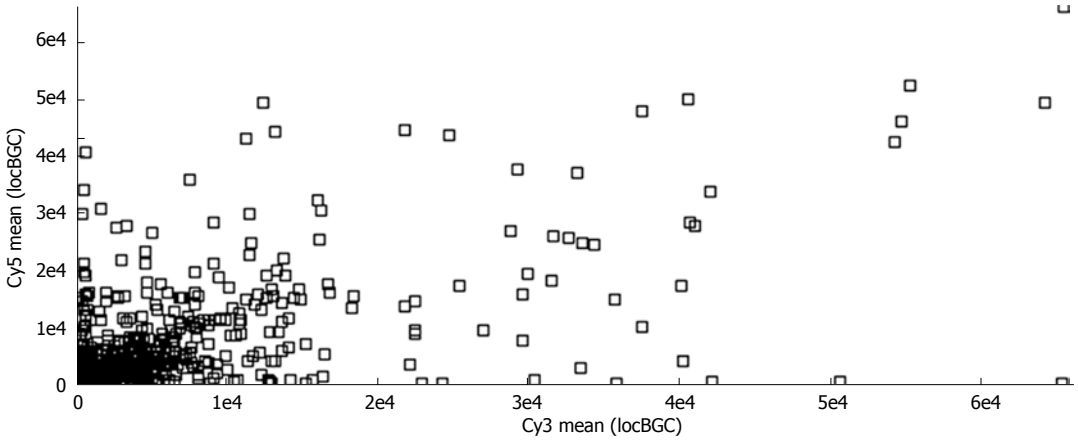


**Figure 1 Scatter plots of cDNA microarray analysis.** Primary carcinoma cells (Cy3-labeled) and normal cells (Cy5-labeled) from case 20 are labeled and hybridized to the cDNA microarray.

### *Statistical analysis*

A statistical analysis among mean values was performed on the association of lymph node metastasis with expression levels by applying non-parametric Kruskal-Wallis and Mann-Whitney *U* tests. Statistical significance was defined as a *P*-value < 0.05. Differential expression between the groups of lymph node metastasis and the group of non-lymph metastasis was considered significant, where *P* value < 0.05.

## RESULTS

Quality analysis of total RNA after LCM and cRNA after T7-based amplification was carried out. About 20 slides were prepared in every sample, and the target cells were captured with at least approximately 1000 cells per slide. Consequently, we captured a total of approximately 25000-30000 tumor and normal cells for RNA extractions, respectively. The quality of total RNA extracted after LCM was assessed by A260/A280 and electrophoresis. To be considered for microarray analysis, the RNA samples needed to pass the quality control criteria, namely integrity of 28S and 18S, and A260/A280 greater than 2.0. Products of cDNA synthesis and cRNA were also checked by A260/A280 and electrophoresis. Results showed that A260/A280 of all the RNA samples met the quality control criteria for sample preparation. Clear image appearance of 28S and 18S of ribosomal RNA was seen under the electropherogram for each total RNA sample, which had to be considered as intact or without degradation. RNA was subjected to two rounds of T7-based RNA amplification after removal of DNA contamination by RNase-free DNase I treatment as described in Methods. All RNA was successfully amplified an estimated 250-fold by using T7 RNA polymerase. cDNA synthesis and cRNA showed satisfactory quality control criteria, which was 1.5 kb < cDNA < 5.0 kb; 1.0 kb < cRNA < 4.5 kb); and A260/A280 ratio of cDNA and cRNA greater than 2, respectively.

### *Identification of expressed genes associated with lymph node metastasis*

After reverse transcription, each cDNA probe was labeled with Cy3- or Cy5-conjugated dyes and hybridized to microarray cDNAs with 886 genes. We evaluated the expression profiles comparing the cancer cells and the corresponding normal cells in each case. A representative scatter plot of microarray analysis between the metastatic carcinoma cells and non-cancerous tissue in case 20 is shown in Figure 1. Up-, down-regulated and unchanged genes indicated by red, green and blue spots respectively are shown in Figure 2. We first arranged the relative expression of each gene (Cy3/Cy5 intensity ratio) into one of four categories: up-regulated (ratio: > 2.0), down-regulated (ratio: < 0.5), unchanged (ratio: between 0.5 and 2.0), and not expressed (or slight expression but under the cutoff level for detection).

To identify the genes related to lymph node metastasis, 32 cases were divided into two groups: a metastatic group in which lymph node metastasis was positive in 17 patients (No. 16-32) and a non-metastatic group in which lymph node metastasis was negative in 15 patients (No. 1-15) (Table 1). When comparing gene expression profiles between two groups, there were 25 genes that were commonly up-regulated and expressed more than 1.87-fold in the lymphatic metastasis groups compared with those in the negative groups. On the other hand, 48 down-regulated expressed genes were significantly correlated with the lymphatic metastasis groups. Tables 2 and 3 show the list of these differentially expressed genes and their category based on GO (Gene Ontology) system and TreeView. The up-regulated genes were associated with cell adhesion molecules, cell membrane receptors, intracellular signaling related genes, etc. The up-regulated genes included interesting genes, such as *MET*, *EPHA2*, *CCND1*, *MMP2* and *MMP13*. The down-regulated genes were mostly cell adhesion molecules, cell cycle regulators and intracellular signaling molecules. The down-regulated genes included *CASP3*, *CDH1*, and *PTPN2*.

### *Gene expression confirmation by real-time RT-PCR*

To investigate the reliability of cDNA microarray data, real-time quantitative RT-PCR was performed for measuring the expression levels of 16 genes (*MET*, *CCND1*, *CCND2*, *VEGF*, *KRT18*, *RFC4*, *BIRC5*, *CDC6*,

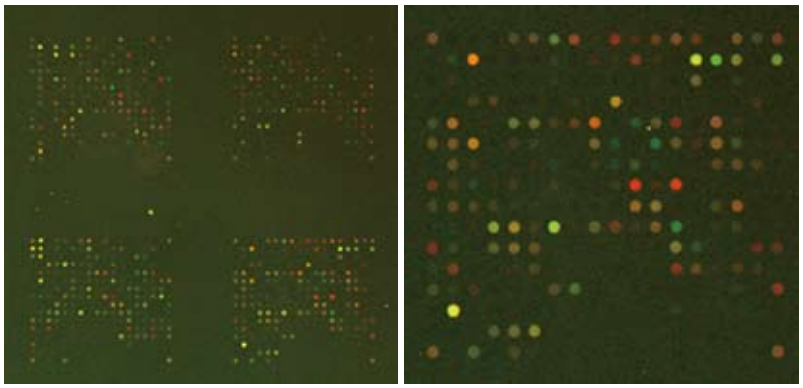

**Figure 2 A representative of cDNA microarray expression pattern obtained from case 20.** Up-, down-regulated and unchanged genes are indicated by red, green and blue spots, respectively.

**Table 2 Up-regulated genes correlated with lymphatic metastasis**

| Gene name | Symbol[a] | Accession[b] | Fold change[c] | pN1:pN0[d] |
|---|---|---|---|---|
| Cell adhesion proteins | | | | |
| CD58 antigen, (lymphocyte function-assocoated antigens) | CD58 | NM_001779 | 7.75 | 2.28 |
| Integrin αM | ITGAM | NM_000632 | 5.04 | 2.06 |
| Integrin β5 | ITGB5 | NM_002213 | 3.98 | 1.87 |
| Opioid-binding protein/cell adhesion molecule-like | OPCML | NM_002545 | | |
| Cell membrane receptor | | | | |
| CD86 antigen, (CD28 antigen ligand 2, B7-2 antigen) | CD86 | NM_006889 | 6.76 | 2.33 |
| v-jun sarcoma virus 17 oncogene homolog (avian) | JUN | NM_002228 | 7.43 | 2.27 |
| Met proto-oncogene (hepatocyte growth factor receptor) | MET | NM_000245 | 10.11 | 3.46 |
| EphA2 | EPHA2 | NM_004431 | 8.26 | 2.13 |
| Epidermal growth factor receptor [avian erythroblastic leukemia viral (v-erb-b) oncogene homolog] | EGFR | NM_005228 | 8.35 | 2.62 |
| Cell death regulator | | | | |
| BCL2/adenovirus E1B 19kDa interacing protein 3 | BNIP3 | NM_004052 | 3.88 | 2.29 |
| Sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 4D | SEMA4D | NM_006378 | 5.71 | 3.64 |
| Intracellular signaling | | | | |
| Rho GDP dissociation inhibitor γ | ARHGDIG | NM_001175 | 5.37 | 1.97 |
| Ras-related C3 botulinum toxin substrate 3 (rho family, small GTP binding protein Rac3) | RAC3 | AK054993 | 4.63 | 2.07 |
| Insulin-like growth factor binding protein 3 | IGFBP3 | M35878 | 5.39 | 2.15 |
| Coagulation factor Ⅱ (thrombin) receptor | F2R | NM_001992 | 6.17 | 2.43 |
| Growth/differentiation factor | | | | |
| Vascular endothelial growth factor C | VEGFC | NM_005429 | 5.68 | 2.47 |
| Cell cycle regulator | | | | |
| Cyclin D1 (PRAD1: parathyroid adenomatosis 1) | CCND1 | NM_053056 | 11.56 | 4.31 |
| Cyclin-dependent kinase 4 | CDK4 | NM_000075 | 8.73 | 3.59 |
| Others | | | | |
| Tissue inhibitor of metalloproteinase 3 (Sorsby fundus dystrophy, pseudoinflammatory) | TIMP3 | NM_000362 | 3.95 | 1.89 |
| Ubiquitin-conjugating enzyme E2A (RAD6 homolog) | UBE2A | NM_003336 | 4.38 | 1.95 |
| v-yes-1 Yamaguchi sarcoma viral oncogene homolog 1 | YES1 | NM_005433 | 5.66 | 2.37 |
| P450(cytochrome) oxidoreductase | POR | AF258341 | 4.74 | 1.99 |
| Matrix metalloproteinase 13 | MMP13 | NM_00247 | 5.67 | 2.45 |
| Matrix metalloproteinase 2 (gelatinase A, 72kD gelatinase, 72kD type Ⅳ collagenase) | MMP2 | NM_004530 | 7.13 | 3.36 |

[a]Symbol in LocusLink database; [b]GeneBank accession number; [c]Fold change, ratio of mean expression values in lymph node metastasis cases (cancer cells *vs* non-cancerous cells); [d]pN1:pN0, ratio of mean expression values (lymph node positive cases to lymph node negative cases).

*MMP2*, *BCL2A1*, *CDH1*, *VIM*, *PDGFRA*, *PTPN2*, *SLC25A5* and *DSP*). One representative case (case 8) is shown in Figure 3. We used cDNA synthesized from 32 pair samples without amplification as template for real-time quantitative reverse transcription PCR. The results demonstrated that the samples obtained by means of T7-based amplification well reflected the status of the original RNA in a proportional manner.

## DISCUSSION

The application of high-throughput cDNA microarray permits simultaneous analysis of genome-wide expression of thousands of genes in a sample and to investigate the correlation between clinicopathological phenotypes and gene expression status[8-10]. This technology is a powerful tool for screening genes,

**Table 3 Down-regulated genes correlated with lymphatic metastasis**

| Gene name | Symbol[a] | Accession[b] | Fold change[c] | pN1:pN0[d] |
|---|---|---|---|---|
| Cell adhesion proteins | | | | |
| Desmoplakin (DPI, DPII) | DSP | NM_004415 | -14.22 | 0.24 |
| Protocadherin gamma subfamily C, 3 | PCDHGC3 | NM_002588 | -7.88 | 0.41 |
| Integrin, beta 4 | ITGB4 | NM_000213 | -13.39 | 0.26 |
| Integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor) | ITGA3 | NM_002204 | -6.24 | 0.38 |
| Catenin (cadherin-associated protein), alpha 1 (102kD) | CTNNA1 | NM_001903 | -8.53 | 0.37 |
| Cadherin 1, type 1, E-cadherin (epithelial) | CDH1 | NM_004360 | -15.85 | 0.25 |
| Cell cycle regulator | | | | |
| Cell division cycle 25B | CDC25B | NM_021784 | -6.90 | 0.46 |
| Cyclin-dependent kinase 5 | CDK5 | NM_004935 | -5.51 | 0.48 |
| Cyclin D2 | CCND2 | NM_001759 | -6.29 | 0.43 |
| Microtubule-associated protein, RP/EB family, member 1 | MAPRE1 | NM_012325 | -13.16 | 0.29 |
| Protein phosphatase 1D | PPM1D | NM_003620 | -11.77 | 0.39 |
| Ataxia telangiectasia and Rad3 related | ATR | NM_001184 | -7.86 | 0.41 |
| Protein phosphatase 2, regulatory subunit B (B56), alpha isoform | PPP2R5A | NM_006243 | -3.89 | 0.49 |
| Intracellular signaling | | | | |
| Mitogen-activated protein kinase 14 | MAPK14 | NM_001315 | -5.67 | 0.43 |
| Mitogen-activated protein kinase 7 | MAPK7 | NM_002749 | -7.15 | 0.39 |
| Mitogen-activated protein kinase 4 | MAPK4 | NM_002747 | -3.83 | 0.32 |
| Small inducible cytokine B subfamily (Cys-X-Cys motif), member 13 (B-cell chemoattractant) | SCYB13 | NM_006419 | -4.58 | 0.46 |
| Signal transducer and activator of transcription 5B | STAT5B | NM_012448 | -8.13 | 0.37 |
| Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 1 | SERPINB1 | NM_030666 | -4.47 | 0.48 |
| Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 2 | SERPINB2 | NM_002575 | -5.72 | 0.41 |
| SH3-domain binding protein 2 | SH3BP2 | AB000462 | -3.29 | 0.52 |
| G protein-coupled receptor kinase 6 | GPRK6 | NM_002082 | -14.12 | 0.29 |
| GTP-binding protein ragB | RAGB | NM_016656 | -6.77 | 0.46 |
| Protein tyrosine phosphatase, receptor type, F | PTPRF | NM_002840 | -7.21 | 0.34 |
| Cell membrane receptor | | | | |
| EphB6 | EPHB6 | NM_004445 | -12.88 | 0.38 |
| Kangai 1 (suppression of tumorigenicity 6, prostate; CD82 antigen (R2 leukocyte antigen, antigen detected by monoclonal and antibody IA4) | KAI1 | NM_002231 | -2.24 | 0.67 |
| Small inducible cytokine subfamily A (Cys-Cys), member 25 | SCYA25 | NM_005624 | -3.76 | 0.72 |
| Monoglyceride lipase | MGLL | NM_007283 | -4.66 | 0.43 |
| Interferon (alpha, beta and omega) receptor 1 | IFNAR1 | NM_000629 | -2.39 | 0.81 |
| Insulin-like growth factor binding protein 6 | IGFBP6 | NM_002178 | -3.53 | 0.62 |
| Metabolic enzyme | | | | |
| Serine protease inhibitor, Kunitz type, 2 | SPINT2 | NM_021102 | -5.44 | 0.46 |
| Protein phosphatase 2, regulatory subunit B (B56), alpha isoform | PPP2R5A | NM_006243 | -7.12 | 0.37 |
| Protein tyrosine phosphatase, non-receptor type 2 | PTPN2 | NM_002828 | -19.73 | 0.23 |
| Deoxyribonuclease I-like 3 | DNASE1L3 | NM_004944 | -8.23 | 0.29 |
| Cathepsin L | CTSL | NM_001912 | -4.39 | 0.37 |
| Cell death regulator | | | | |
| Caspase 3, apoptosis-related cysteine protease | CASP3 | NM_004346 | -14.57 | 0.29 |
| Programmed cell death 10 | PDCD10 | NM_007217 | -7.19 | 0.36 |
| DNA damage response | | | | |
| Ataxia telangiectasia and Rad3 related | ATR | NM_001184 | -4.78 | 0.48 |
| X-ray repair complementing defective repair in Chinese hamster cells 5 (double-strand-break rejoining; Ku autoantigen, 80kD) | XRCC5 | NM_021141 | -2.59 | 0.73 |
| Mouse double min 2 | MDM2 | NM_002392 | -6.37 | 0.51 |
| Growth/differentiation factor | | | | |
| Bone morphogenetic protein 5 | BMP5 | NM_021073 | -2.78 | 0.53 |
| Keratin 4 | KRT4 | NM_002272 | -3.12 | 0.62 |
| Keratin 13 | KRT13 | NM_002274 | -4.24 | 0.48 |
| Connective tissue growth factor | CTGF | NM_001901 | -5.51 | 0.42 |
| Others | | | | |
| Heat shock 70kDa protein 4 | HSPA4 | AB023420 | -3.19 | 0.56 |
| Retinoid X receptor, α | RXRA | NM_002957 | -2.89 | 0.77 |
| Ubiquitin-activating enzyme E1-like | UBE1L | NM_003335 | -2.95 | 0.53 |
| Solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 5 | SLC25A5 | NM_001152 | -6.18 | 0.46 |

[a]Symbol in LocusLink database; [b]GeneBank accession number; [c]Fold change, ratio of mean expression values in lymph node metastasis cases (cancer cells *vs* non-cancerous cells); [d]pN1:pN0, ratio of mean expression values (lymph node positive cases to lymph node negative cases).

the expression of which can be correlated with pathological phenotypes of various tumors[11,12]. Analysis of gene expression profiles not only has disclosed specific patterns that may reflect prognosis and drug sensitivity of tumor cells but has also revealed the identity of genes involved in malignant transformation,

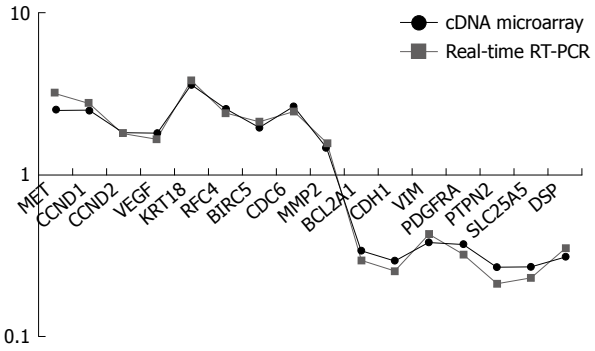


**Figure 3 Relative abundance of the mRNA levels of 16 genes in one representative case (case 8), determined by cDNA microarray and real-time RT-PCR, respectively.**

progression, and metastasis of tumors[13-16]. However, the existence of bulky surrounding cells produces much interferential noise information because of interstitial effects[17]. Therefore, selection of cancer cells using LCM is of indispensable value in combination with the cDNA microarray. This study clearly demonstrated that an analysis of global gene expression profiles can be performed with RNA samples obtained from primary HCC tissues by using LCM, T7-based RNA amplification, and cDNA microarray. It is thus possible to focus directly on the gene expression profile of an individual cell population consisting of tumor tissue.

Lymph node metastasis is one of the most important prognostic factors in HCC patients[18,19]. This is a highly selective sequential step involving multiple genes, multiple signals pathways and regulatory mechanisms during the process, which favors the survival of a subpopulation of metastatic cells preexisting within the primary tumor mass to produce clinically relevant metastases[20-23]. The metastatic cells exhibit a complex phenotype that is regulated by transient or permanent alterations in various genes at mRNA level. In the present study of HCC patients, we compared gene expression in lymph node metastasis and in those without lymph node metastasis by using cDNA microarray analysis. We found that 25 up-regulated and 48 down-regulated genes were correlated with lymph node metastasis of HCC. The up-regulated genes included cell adhesion related genes (*CD58*, *ITGAM*, *ITGB5*, *OPCML*), cell membrane receptor (*CD86*, *JUN*, *MET*, *EPHA2*, *EGFR*), cell death regulator (*BNIP3*, *SEMA4D*), intracellular signaling related genes (*ARHGDIG*, *RAC3*, *IGFBP3*, *F2R*), growth/differentiation factor (*VEGFC*), cell cycle regulator (*CCND1*, *CDK4*), and other genes (*TIMP3*, *UBE2A*, *YES1*, *POR*, *MMP13*, *MMP2*). The down-regulated genes included cell adhesion related genes (*DSP*, *PCDHGC3*, *ITGB4*, *ITGA3*, *CTNNA1*, *CDH1*), cell cycle regulator (*CDC25B*, *CDK5*, *CCND2*, *MAPRE1*, *PPM1D*, *ATR*, *PPP2R5A*), intracellular signaling related genes (*MAPK14*, *MAPK7*, *MAPK4*, *SCYB13*, *STAT5B*, *SERPINB1*, *SERPINB2*, *SH3BP2*, *GPRK6*, *RAGB*, *PTPRF*), cell membrane receptor (*EPHB6*, *KAI1*, *SCYA25*, *MGLL*, *IFNAR1*, *IGFBP6*), metabolic enzyme (*SPINT2*, *PPP2R5A*, *PTPN2*, *DNASE1L3*, *CTSL*), cell death regulator (*CASP3*, *PDCD10*), DNA damage response related genes (*ATR*, *XRCC5*, *MDM2*), growth/differentiation factor (*BMP5*, *KRT4*, *KRT13*, *CTGF*), and other genes (*HSPA4*, *RXRA*, *UBE1L*, *SLC25A5*).

Amongst these genes are some which are well documented in the literature as being involved in the malignant potential of some types of carcinomas. CCND1, one of the cell cycle regulators, is synthesized in the G1 phase, mixed with CDK2, CDK4 and CDK5, and related closely with cell cycle control[24,25]. Our results showed that CCND1 is highly expressed in HCC with lymphatic metastasis, which may be a useful marker that relates with the poor prognosis of HCC, and overexpression of CCND1 in HCC without lymphatic metastasis may be used as useful information for treatment measures after operation[26,27]. CDK4, an important member of the CDKs protein family, was correlated positively with CCND1 as reported by some documents[24-26]. Our results showed that CDK4 was remarkably up-regulated in HCC patients with lymph node metastasis, and was consistently correlated with that of CCND1[28]. EphA2, one member of the tyrosine activating enzyme acceptor Eph family, can be used as a ligand of ephrins connecting with cells and involved in cell interaction[29]. Our study demonstrated that up-regulation of EphA2 was related to lymph node metastasis of HCC, however, the mechanism remains in need of further studies. It has been proven that the formation of new blood vessels plays a role in the growth and metastasis of solid tumors, and various growth factors secreted from tumor cells determine the pace of the progression process[30]. It is documented that VEGF is up-regulated in most solid tumors, whereas there is few or none in the normal tissues, and its expression level is positively related with microvessel density, invasion, metastasis and prognosis of tumors[31]. Present results showed that the expression levels of VEGF were related closely to the clinical stage of HCC, its expression levels increased gradually with the increase of TNM, there were significant differences in expression levels between HCC patients with and those without lymph node metastasis ($P < 0.05$), and its expression levels were a useful marker of the recurrence, the distant metastasis and the poor prognosis of HCC (data not shown). The extracellular matrix (ECM) is the first barrier during the process of invasion and metastasis of tumor cells[32]. Tumor cells and their neighbor interstitial cells, such as the endothelial cell, the macrophage and so on, may produce a great quantity of proteinase for degradation of ECM, and help tumor cells to migrate easily. Of these proteinases, the matrix metalloproteinase (MMP) is an important one. MMPs can degrade the stroma collage, which favors the action of tumor cells to shake off the yoke of ECM and to migrate. This is the biochemical basis of the circumambience invasion of tumor cells[33,34]. It was found that MMP13 and MMP2 expressions in HCC with lymph node metastasis were significantly higher than that of those without lymph node metastasis, and MMP2 in the lymph

node metastasis group was 3.12-fold of those without metastasis. Lymph node metastasis of HCC may be related to the "thundering" activity of the growth factor signaling pathway[35]. It has been proven that the signaling pathway of tyrosine kinase receptor and G-protein connective receptor are the most important two of all pathways[36]. EGFR, containing the sequence of tyrosine kinase, was always up-regulated in HCC with lymph node metatasis patients. It has been reported that the signaling pathway mediated by all growth factors for cell proliferation purposes was always dependent on the tyrosine kinase receptor signaling pathway, and tyrosine kinase was kept activated in many tumors[37].

It has been reported that down-regulation of desmoplakin (DSP, member of the cadherin family) is correlated with tumor invasion and metastasis[38]. Our results showed that DSP was suppressed in the lymph node metastasis group compared with the non-metastasis group. Tumor cells always secrete some new adhesion molecules when removed from the primary lesion and grown again in another position, whereas CTNNA 1 was down-regulated in our results, suggesting its role was maybe different from that of the primary lesion[39,40]. E-cadherin (a cell membrane superficial molecule mediating adhesion among normal cells) was down-regulated in lymph node metastasis HCC patients. Ephrin, ligand of tyrosine kinase receptor, was also down-regulated in the metastasis group. It was reported that the combination of ephrin-B2 ligand and EphB4 was related to the occurrence and metastasis of some solid tumors[41,42]. KAI1, a new gene found recently, was down-regulated in many lymphatic metastasis tumors, and this gene is associated with the motion and metastasis of tumor cells[43]. Protein tyrosine phosphatase, which has an action contrary to that of tyrosine kinase, takes part in signal regulation, energy metabolism, cell proliferation and promoting MHCI expression mediated by many hormones, such as insulin and epidermal growth factor, and others[44,45]. The decrease of tyrosine phosphatase activity may reduce the MHCI expression of cells superficially so that tumor cells escape the inspection of the immunological system[44]. PTPN2 and PTPRF were down-regulated in the lymph node metastasis group, suggesting that the tyrosine phosphatase was associated with invasion and metastasis of tumor cells[44-46].

Our study demonstrated that lymph node metastasis comes from the result of the structural and functional abnormality of cellular and extracellular multigenes, since many genes and signaling pathways play key roles during the metastasis of tumor cells by dominating the cell proliferation, differentiation and death. Potential metastatic biological behavior of tumor cells was characteristically the release of cell-cell adhesion, the abnormality of cell cycle regulator and cell signal pathways, which suggest that the invasive character of tumor cells is determined by cellular interaction with the extracellular environment rather than the proliferative potential. The abnormality of apoptosis and proliferation ability may occur owing to loss of control of the cell cycle and the obstruction of cell signal molecules transmitting communication, which may be is one of the mechanisms of accelerating tumor invasion and metastasis[47,48]. Although we were able to extract some genes related to lymph node metastasis in HCC, further examination is necessary of other genes as well as the interaction with stromal tissues. Since these genes are thought to affect each other, it is important to further analyze each gene in detail to elucidate the mechanism of lymph node metastasis in HCC.

## COMMENTS

### *Background*

Hepatocellular carcinoma (HCC), endemic to sub-Saharan Africa and Asia with a rising incidence in Western countries, is one of the most common fatal malignancies in the world. The high mortality associated with this disease is mainly attributed to its high tendency to metastasize. In fact, local lymph node and blood metastases could occur at an early stage, which may be the key factors relating to its recurrence and poor prognosis. Thus, a better understanding of the molecular mechanism of metastasis can improve prevention and treatment of HCC.

### *Research frontiers*

The purpose of this study is to identify the genes related to lymph node metastasis in human hepatocellular carcinoma (HCC). Thirty two HCC patients with or without lymph node metastases were investigated by high-throughput microarray comprising 886 genes. The results demonstrated that 25 up-regulated genes such as cell membrane receptor, intracellular signaling and cell adhesion related genes, and 48 down-regulated genes such as intracellular signaling and cell cycle regulator-related genes, were correlated with lymph node metastasis in HCC.

### *Innovations and breakthroughs*

The application of high-throughput cDNA microarray permits analysis of genome-wide expression of thousands of genes in a sample and thus to investigate the correlation between clinicopathological phenotypes and gene expression status. This study clearly demonstrated that an analysis of global gene expression profiles can be performed with RNA samples obtained from primary HCC tissues by using LCM, T7-based RNA amplification, and cDNA microarray. Thus, it is possible to focus directly on the gene expression profile of an individual cell population consisting of tumor tissue.

### *Applications*

This genome-wide information contributes to an improved understanding of molecular alterations during lymph node metastasis in HCC. It may help clinicians to predict metastasis of lymph nodes and assist researchers in identifying novel therapeutic targets for metastatic HCC patients.

### *Terminology*

DNA microarray is a high-throughput and powerful technology used in molecular biology and in biomedicine areas. It consists of an arrayed series of thousands of microscopic spots of DNA oligonucleotides. A short section of a gene or other DNA element can be used as a probe to hybridize a cDNA or cRNA sample (called target) under high-stringency conditions. Probe-target hybridization is usually detected and quantified by fluorescence-based detection of fluorophore-labeled targets to determine relative abundance of nucleic acid sequences in the target.

### *Peer review*

The authors claimed that the expression of distinct sets of genes was either enhanced or decreased in hepatocellular carcinoma tissues with lymph node metastasis, compared with those without any lymph node metastasis. Based on the clinical information described in this article, the patients with lymph node metastasis are in more advanced stages (T3, T4, or M1), compared with those without metastasis. Thus, their observed changes in gene expression may arise from the local tumor growth or distant metastasis. The authors should exclude this possibility.

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