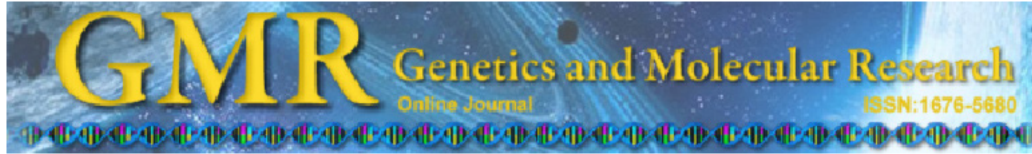

Aberrant gene expression profiles in hepatocellular carcinoma detected by microdissection

Y.M. Wei[1], Y.Y. Li[2], Y.C. Zhang[2] and Y.Q. Nie[2]

[1]Department of Blood Transfusion, Guangzhou First People's Hospital, Guangzhou, China
[2]Department of Gastroenterology and Hepatology, Guangzhou First People's Hospital, Guangzhou, China

Corresponding authors: Y.Y. Li / Y.M. Wei
E-mail: liyyliyy@tom.com / yamingwei@163.com

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ABSTRACT. The aim of this study was to identify genomic aberrations in hepatocellular carcinoma (HCC) by using laser capture microdissection (LCM) combined with microarray analysis. Samples were procured by LCM from HCC and patient-matched normal liver tissue surgically resected from 4 patients. RNA was isolated from the samples and reverse transcribed into cDNA. After 2-cycle linear amplification and 2-color fluorescent labeling, the cRNA was hybridized onto a whole genome microarray. All genes expressed in the normal and HCC samples were counted and analyzed. Differentially expressed genes were identified and the top 10 up and downregulated genes (totally 20 genes) were further evaluated. LCM was able to accurately capture 50-200 cells from HCC and control tissues. The microarray spectrum showed satisfactory detection of HCC-enriched genes. A total of 1361 differentially expressed genes were identified, among which, 607 were upregulated and 754 were downregulated. Among the top 20 up and downregulated genes, 4 genes had not been documented in the literature as being differentially expressed in any tumors. Thus, LCM

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is an effective approach for identifying aberrantly expressed genes in HCC, and may lead to the discovery of biomarkers for diagnostic and therapeutic applications.

Key words: Laser capture microdissection; Hepatocellular carcinoma; Microarray; Gene expression

INTRODUCTION

Hepatocellular carcinoma (HCC) is a leading cause of cancer-related death worldwide with the burden of disease expected to increase in coming years (Venook et al., 2010). The development of HCC is a multi-factorial and multi-step process accompanied by expression alterations in critical growth regulatory genes. Identifying the genes involved in this process may lead to new insights into carcinogenesis and enable genetic diagnosis and treatment. The advent of microarray technology with its capacity to simultaneously monitor the expression of thousands of genes in a small input sample provides a unique opportunity for high-throughput genetic analysis. Oligonucleotide microarray has been commonly used to determine the differences in gene expression in tumors and to identify new candidate genes with diagnostic, prognostic, and therapeutic potential (Alizadeh et al., 2001). However, the cellular heterogeneity of input samples has been a significant barrier in the use of microarray technology, as tumor cells may constitute only a minority of the multiple cell types in the tissues analyzed. Analyzing the complete tumor sample without efficiently separating the tumor cells from unrelated cells confounds the true gene expression profile of the tumors. Therefore, special procedures to isolate tumor cells from heterogeneous tissues are mandatory in microecologic studies. Accomplishing this goal is difficult because tissues consist of large numbers of interacting cell populations from the mesenchymal stroma, blood vessels, and necrotic tissues. This problem has now been overcome by laser capture microdissection (LCM), a technique developed in 1996 (Emmert-Buck et al., 1996). LCM enables the procurement of a subpopulation of cells from a specific microscopic region of a tissue section under direct visualization while eliminating cellular contamination from unrelated cells. Intact RNA from the captured cells can be used as starting material to produce cDNA libraries, generate transcript microarray data, perform differential display, and discover tumor-enriched genes (Espina et al., 2009). Therefore, LCM provides a rapid, reliable, and accurate approach for microanalysis methods. Efficiently coupling LCM with microarray analysis has led to the development of sensitive and quantitative methods for the identification of the genetic profiles of tumors from the lung, prostate, and stomach (Wu et al., 2005). However, this method has not been widely used for documenting differences in gene expression in HCC. Only a few such studies have been reported (Yim et al., 2003; Nagai et al., 2004; Wu et al., 2008, 2010). In this study, we identified genes that are differentially expressed in HCC as compared to normal liver tissue using LCM combined with microarray analysis in order to explore the relationship between the genotype and phenotype of HCC in humans.

MATERIAL AND METHODS

Tissues samples

Samples and relevant clinical data were obtained from 4 patients with histologically

confirmed HCC (2 males and 2 females, age 39-56 years) undergoing surgical resection in the Department of Gastroenterological Surgery, Guangzhou First People's Hospital, in July 2009. None of them received chemotherapy or radiation therapy before surgery. Fresh cancer tissue and non-cancerous tissue from the same liver were collected from the surgically resected tissues and immediately frozen at -85°C. The study protocols were approved by the ethics committee of Guangzhou First People's Hospital, which is affiliated to Guangzhou Medical University. Written consent was obtained from each patient.

LCM and RNA isolation

Frozen tissues were sectioned at 6 µm with a cryostat section (Thermo Co., USA) at -20°C on polyethylene naphthalate membrane slides. Slides were inverted and mounted onto the microscope stage, where the desired cellular components were excised from the slide by passing the laser through the glass to cut the foil membrane. The sections were allowed to air dry briefly prior to LCM. Gravity facilitated the capture of the dissected cellular components directly into polymerase chain reaction tubes located in a substage tube holder. The dissected cellular components were fixed with RNA stabilization reagent (Qiagen, Germany) for 4 min, and then stained with Mayer's hematoxylin. Pure tumor cell subpopulations and patient-matched non-cancerous populations were microdissected with the use of an LCM system (ASLMD, Leica, Germany) with intensity 70 and speed 8 within 30 min. The paired samples of normal controls from the same sections were also laser captured in the same way. Microdissected cells were stored at -80°C until use. Total RNA extraction was performed using the RNeasy Micro Kit (Qiagen) according to the manufacturer instructions. The extracted RNA was subjected to electrophoresis with the Agilent 2100 Bioanalyzer (Agilent Technologies, USA). The quality of the total RNA was verified using the RNA 6000 Pico Kit with the Bioanalyzer. As recommended by the electrophoresis software of the Bioanalyzer, an index of $A_{260}/A_{280} \geq 1.8$ and RNA integrity number (RIN) ≥ 7 was set as the quality standard.

RNA amplification and transcription

Total RNA was used to generate cDNA with a low RNA input linear amplification kit (PLUS, Two-Color, Agilent) and then transcribed again to cRNA. The cRNA was subjected to 2-cycle linear amplification and was fluorescently labeled with Cy3 for HCC samples and Cy5 for normal samples. An ultraviolet spectrophotometer (ND 1000, NanoDrop, USA) was used to determine the concentration and quality of cRNA. Then, 100 µL labeled cRNA from each sample was hybridized with a whole genome microarray (Agilent human 4*44K) at 65°C for 17 h, according to the manufacturer instructions. The microarray was then washed and scanned with a scanner (Agilent GenePix 4000B). The Cy3 and Cy5 fluorescent intensities of all the genes expressed in the 4 paired samples were analyzed and compared to identify differentially expressed genes. The top 10 up and downregulated genes were further researched with Gene Cards (www.genecards.org, Developed at the Crown Human Genome Center, Department of Molecular Genetics, the Weizmann Institute of Science, Israel) and PubMed.

Statistical analysis

The Agilent Feature Extraction Software (v10.5) was used to normalize the signals of

Cy3 and Cy5. A standard ratio of Cy3/Cy5 ≥ 2 was used as the threshold to identify upregulated genes, and a ratio ≤ 0.5 was used to identify downregulated genes. Cluster 3.0 Gene Cards and PubMed were used to perform literature reviews for evidence of the expression for the 10 most up and downregulated genes that we identified in HCC. Data were examined with the SPSS 13.0 for Windows statistical package (IBM, USA). $P < 0.05$ was considered to be significant.

RESULTS

Microdissection and RNA quality control

HCC and normal control tissues were microdissected with LCM. Pure subpopulations with 50-200 target cells were successfully captured without contamination (Figure 1). The quality of RNA extracted from all samples reached the standard index of $A_{260}/A_{280} \geq 1.8$ and RIN ≥ 7 indicating no contamination from protein or DNA (Table 1). Clear RNA bands upon electrophoresis indicated high purity of RNA without degradation (Figure 2). The A_{260}/A_{280} ratio (between 2.0 and 2.1) and the rate of fluorescence incorporation (>8 pmol/µg) of cRNA indicated satisfactory purity (Table 2). The high detection rates of the genes are shown in Table 3, the uniformity of fluorescence intensity is shown in Figure 3, and the coincident trend of gene expression is shown via scatter plots in Figure 4. All products reached quality standards.

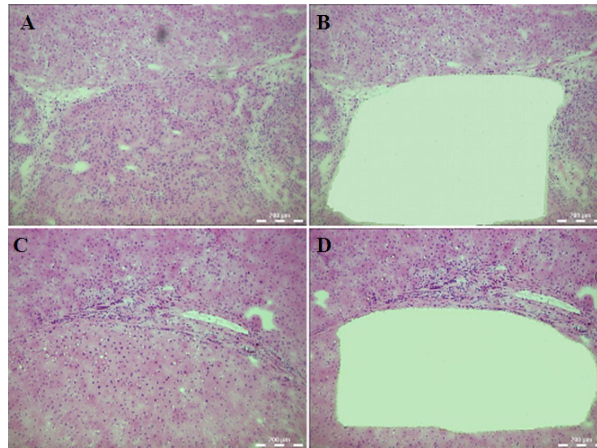

Figure 1. Microdissection of HCC and normal tissues with LCM (HE x 40). **A.** HCC (before LCM); **B.** HCC (after LCM); **C.** Normal control (before LCM) **D.** Normal control (after LCM).

Table 1. Quality control of RNA extractions.

Code	Concentration (ng/uL)	A_{260}/A_{280}	RIN
T1	18.00	1.92	9.50
N1	10.00	1.97	8.20
T2	24.00	2.01	7.80
N2	16.00	1.93	8.00
T3	23.00	1.98	8.30
N3	25.00	1.90	8.20
T4	28.00	1.95	8.40
N4	17.00	1.94	8.80

A = absorbance; T = HCC; N = normal; RIN = RNA integrity number.

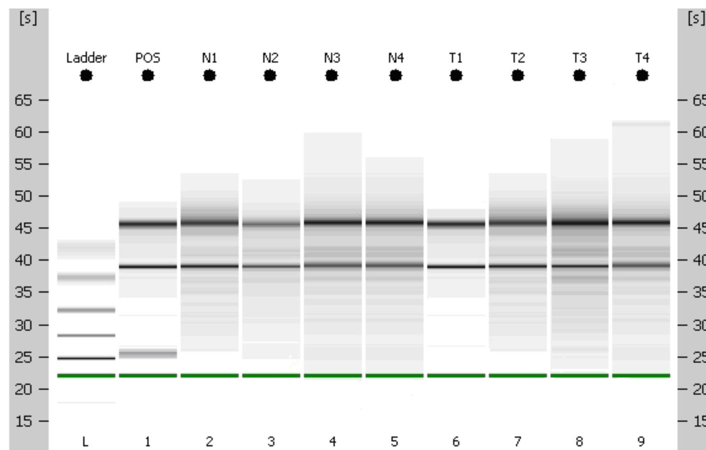

Figure 2. Electropherogram for RNA quality control. Pos = positive control; T = HCC; N = normal control.

Table 2. Results of cRNA amplification and fluorescence labeled.

Code	Fluorescence	A_{260}/A_{280}	cRNA concentration (ng/uL)	Fluorescence concentration (pmol/uL)
T1	Cy3	2.03	212.70	14.30
N1	Cy5	2.07	97.60	5.00
T2	Cy3	2.08	201.00	11.70
N2	Cy5	2.10	276.70	22.80
T3	Cy3	2.05	257.40	16.70
N3	Cy5	2.03	182.70	11.10
T4	Cy3	2.05	201.70	12.80
N4	Cy5	2.03	159.10	13.20

A = absorbance; T = HCC; N = normal.

Table 3. Results of microarray.

Code	Samples (Cy3)	Samples (Cy5)	CV	Detection (%)
1	T1	N1	6.45	72.12
2	T2	N2	8.27	74.91
3	T3	N3	4.68	69.65
4	T4	N4	5.99	70.30

CV = coefficient of variation.

Differentially expressed genes

The expression of a total of 1361 genes was detected to be significantly altered ($P < 0.05$) in the 4 pairs of HCC and control samples. Among these, 607 were upregulated and 754 were downregulated. The differentially expressed genes were ranked and classified according to their signal log ratio. The 20 genes showing the greatest changes in expression, i.e. the top 10 upregulated and downregulated genes were researched through literature searches via the Gene Cards and PubMed databases. Among them, 5 genes were previously shown to be related to HCC and 7 were validated as being associated with other tumors. The remaining 4 genes had not been reported as differentially expressed genes in any tumors (Tables 4 and 5).

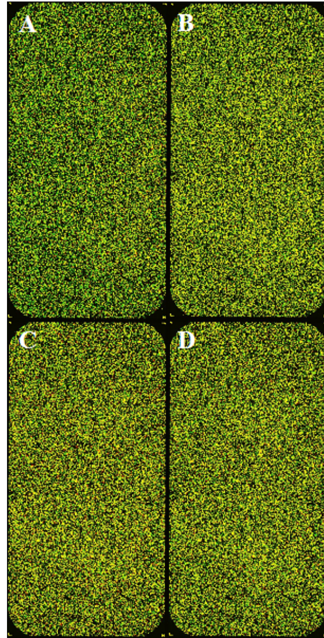

Figure 3. Two-color fluorescent (Cy3, Cy5) labeled cRNA hybridization. **A.** HCC 1 and normal 1; **B.** HCC 2 and normal 2; **C.** HCC 3 and normal 3; **D.** HCC 4 and normal 4.

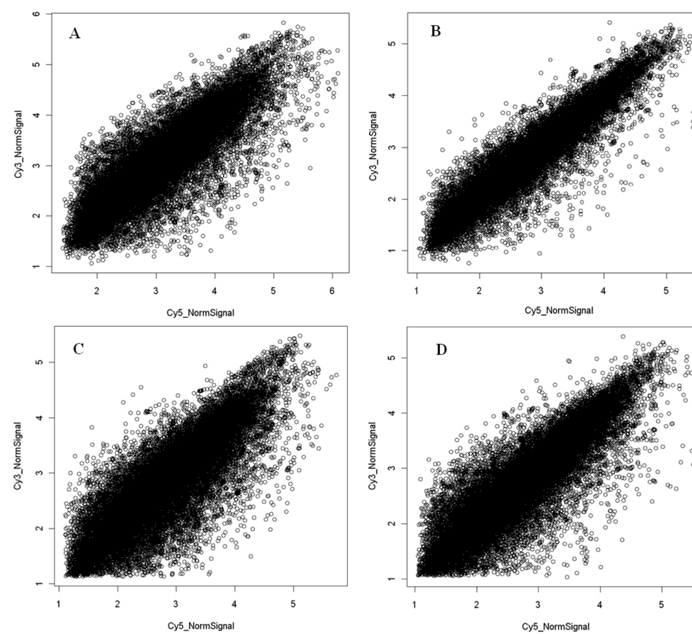

Figure 4. Scatter diagrams of differential gene profiles of HCC and normal control **A.** HCC 1 and normal 1; **B.** HCC 2 and normal 2; **C.** HCC 3 and normal 3; **D.** HCC 4 and normal 4.

Table 4. Top ten upregulated genes in HCC.

Gene symbol	Unigene code	Ratio	Name and description
TACSTD1[a]	Hs.692	23.2529	Tumor-associated calcium signal transducer 1
TTK[a]	Hs.169840	27.3729	TTK protein kinase
NEK2[b]	Hs.153704	32.6949	NIMA serine/threonine related kinase 2
CTNND2[b]	Hs.314543	28.4809	Catenin (cadherin-associated protein), delta 2 (neural plakophilin-related arm-repeat protein)
CENPA[b]	Hs.1594	23.7030	Centromere protein A, 17 kDa
CCR6[b]	Hs.46468	21.3423	Chemokine (C-C motif) receptor 6
CDCA7[b]	Hs.470654	19.1093	Cell division cycle associated 7
CDCA1[b]	Hs.234545	17.2919	Cell division cycle associated 1
DEPDC1B[c]	Hs.482233	24.6761	DEP domain containing 1B
ASPM[c]	Hs.121028	22.0722	Asp (abnormal spindle)-like, microcephaly associated (*Drosophila*)

[a]Documented HCC differential gene; [b]documented tumor differential gene unrelated to HCC; [c]unclassified tumor differential gene.

Table 5. Top ten downregulated genes in HCC.

Gene symbol	Unigene code	Ratio	Name and description
CYP1A2[a]	Hs.1361	0.0148	Cytochrome P450, family 1, subfamily A, polypeptide 2
OIT3[a]	Hs.8366	0.0197	Oncoprotein induced transcript 3
VIPR1[a]	Hs.348500	0.0198	Vasoactive intestinal peptide receptor 1
CYP26A1[b]	Hs.150595	0.0098	Cytochrome P450, family 26, subfamily A, polypeptide 1
CYP2E1[b]	Hs.12907	0.0125	Cytochrome P450, family 2, subfamily E, polypeptide 1
MME[b]	Hs.307734	0.0158	Membrane metallo-endopeptidase (neutral endopeptidase, enkephalinase, CALLA, CD10)
NAT2[b]	Hs.2	0.0161	N-acetyltransferase 2 (arylamine N-acetyltransferase)
SRD5A2[b]	Hs.458345	0.0165	Steroid-5-alpha-reductase, alpha polypeptide 2 (3-oxo-5 alpha-steroid delta 4-dehydrogenase alpha 2)
FCN2[c]	Hs.54517	0.0097	Ficolin (collagen/fibrinogen domain containing lectin) 2 (hucolin)
BBOX1[c]	Hs.144845	0.0191	Butyrobetaine (gamma), 2-oxoglutarate dioxygenase 1

[a]Documented HCC differential gene; [b]documented tumor differential gene unrelated to HCC; [c]unclassified tumor differential gene.

DISCUSSION

LMD is a powerful technology for isolating specific cells or cell groups for further analysis. It eliminates the problems of cell-type heterogeneity within tissue samples by providing homogeneous cell populations for analysis. The technique allows highly specific comparisons of cancer cells with normal cells from the same tissue sample. Microarray analysis offers a new avenue to understand the biological diversity of cells (Alizadeh et al., 2001). Most previous gene expression studies have made use of whole tumor tissues. Although this data is comprehensive, it is limited by the fact that the tumor samples were contaminated with unrelated cells (Okabe et al., 2001). Therefore, special procedures to isolate cancer cells from heterogeneous tissues are necessary. LCM enables the isolation of pure cell populations from a native tissue environment. The percentage of cell contamination with LCM is estimated to be less than 0.3% (Nakamura et al., 2004). In the past, the amount of RNA procured from cancer cells isolated by LCM was not sufficient for hybridization with a cDNA microarray. Recent advances in linear RNA amplification have provided the key to resolve this problem. This technique not only provides sufficient RNA for further analyses but also preserves overall genetic information (Aoyagi et al., 2003).

The combination of LCM with microarray analysis has been successfully used to identify the molecular subtypes of many different tumors (Wu et al., 2005). Several animal and human studies of HCC have been published that report the differential expression of genes in

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HCC as compared to same tissues with other liver disease. Nagai et al. identified 72 upregulated and 57 downregulated genes in liver-cirrhosis nodules as compared to HCC tissue from a single patient. Among them, the expression of 31 upregulated and 7 downregulated genes were positive both in HCC and liver-cirrhosis nodules (Nagai et al., 2004). Wu et al. showed that the NS5A protein downregulated the expression of the mitotic spindle protein ASPM and induced an aberrant mitotic cell cycle, which was associated with chromosome instability and subsequently HCC (Wu et al., 2008). Wu et al. studied human osteopontin positive and negative HCC tissues and found some tumor-related genes, such as *SMR3B*, *MUC7*, *EPHA5*, *SPP1*, and *CLDN10* to have significantly higher expression in osteopontin positive HCC than in osteopontin negative HCC (Wu et al., 2010). Yim et al. identified 855 differentially expressed genes in HCC and hepatic adenomas using a transgenic rat model. In total 855 genes, 71 were positive expressed in both HCC and hepatic adenomas, and among 71 positive genes, 28 of them were upregulated in both tissues and 41 were downregulated in both tissues (Yim et al., 2003).

In this study, we generated new data that adds to our understanding of HCC and complements the data from the studies described above. We successfully identified 1361 HCC-enriched genes with more than 95% accuracy. In order to further understand our results, we performed literature searches for the top 10 up and downregulated genes using the Gene Cards and PubMed databases. Of these 20 genes, 16 had a known association with HCC (Reubi et al., 2000; Okabe et al., 2001; Kim et al., 2004; Chen et al., 2006) or other tumors (Hein, 2002; Tomonaga et al., 2003; Agundez, 2004; Lu et al., 2005; Osthus et al., 2005; Hayward and Fry, 2006; Bethke et al., 2007). Therefore, our results are consistent with the literature. Of the 16 genes associated with HCC or other tumors, the following genes were the most interesting: 1) *TACSTD1* (tumor-associated calcium signal transducer 1): this gene encodes the epithelial cell adhesion molecule EpCAM, which is expressed in the hepatic stem cells of embryos. *TACSTD1* shows the most significantly altered level of gene expression in adult cirrhosis and HCC (Lu et al., 2005), 2) *NAT2* (N-acetyltransferase 2): this gene encodes an important enzyme involved in the biotransformation of carcinogens and exhibits genetic polymorphisms. *NAT2* acetylator genotypes are important modifiers of human cancer susceptibility (Hein, 2002), 3) *NEK2* (NIMA-related serine/threonine kinase): this gene contributes to chromosome instability and is abnormally expressed in a wide variety of human cancers (Reubi et al., 2000). P450 family, including CYP1A2[a], CYP26A1[b] and CYP2E1[b] downregulated in these HCC tissues, have been reported to be related to the susceptibility of many types of cancers (Agundez, 2004; Bethke et al., 2007).

The remaining 4 differentially expressed genes, which were not previously reported in the literature to be associated with carcinogenesis, were of particular interest. The discovery of these 4 genes is novel and that these genes may correspond to the different histological phenotypes of HCC. If so, they may be useful as candidate genes for the diagnosis and treatment of HCC. The *ASPM* (abnormal spindle-like microcephaly) gene that encodes a mitotic spindle protein is widely expressed in fetal and proliferating adult tissues (Kouprina et al., 2005; Wu et al., 2008). The *SCFCN2* (Ficolin 2) gene encodes ficolin-2 (L-ficolin), which regulates innate immunity (Munthe-Fog et al., 2007). *BBOX1* (butyrobetaine 2-oxoglutarate dioxygenase 1) encodes an enzyme responsible for the biosynthesis of l-carnitine, a key molecule in fatty acid metabolism. Its activity has been detected in the kidney, liver, and brain (Rigault et al., 2006). The *DEPDC1B* (DEP domain containing 1B) gene encodes the receptor-like protein-

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tyrosine-phosphatase (PTP), which may contribute to the inhibition of cell growth (Ostman et al., 1994). These findings suggest that genes associated with tissue proliferation, metabolism, and immune response might play roles in the development of HCC.

In this study, we extracted RNA from microdissected human HCC and patient-matched normal liver cells for the examination of their gene expression profiles. We demonstrated that this strategy is a rapid and efficient way to identify the transcriptional signature of HCC. Further studies are needed to determine the potential value of the genes identified here as novel biomarkers for diagnostic and therapeutic applications.

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