SUPPLEMENTARY NOTE

I. Patients and tissue samples

To study gene expression profiles associated with intrahepatic metastases, we collected a total of 107 paired primary HCCs, metastatic HCCs and adjacent non-tumor normal liver tissue samples from 40 patients who were pathologically diagnosed as HCC and underwent hepatectomy at the Liver Cancer Institute, Zhongshan Hospital of Fudan University (former Shanghai Medical University) in China (Supplemental Table 3). Prior to surgery, each patient was examined by computer tomography (CT) of abdomen and chest X-ray, and some patients also were examined by isotope scanning of bone if necessary to determine the extent of intra and extrahepatic metastases.

HCC patients are known to have a poor prognosis because of a high frequency of intrahepatic metastases at the time of diagnosis while they rarely have distant metastases at the time of surgery \(^1,2\), indicating that the liver is the main target organ of HCC metastasis\(^3,4\). There are two forms of intrahepatic metastases, namely, intrahepatic spreads (tumors spreading to other parts of the liver) and tumor thrombus in the branch of portal vein (tumors invading into the portal vein). For easy reference, we referred to intrahepatic spreads as “group P” and tumor portal vein invasion as “group PT”. It has been
demonstrated that the highly metastatic HCC cells mainly invade the adjacent portal vein in animal model systems\textsuperscript{3,5} as well as in patients\textsuperscript{6}. Therefore, we divided our samples into three groups, i.e., P, PT and PN, where the PN referred to as patients who had a single primary HCC lesion without detectable metastasis at the time of surgery. Among the 107 paired samples collected, 81 were from 27 patients who had primary HCC, corresponding adjacent non-tumor liver tissue and metastatic lesions (15 cases as group P and 12 cases as group PT), and 26 samples including corresponding non-tumor liver tissues from 13 PN patients.

To minimize a potential introduction of multicentric tumor samples as the P group, we selected the P samples based on the size difference between the main tumors and their metastatic lesions in which the lesions were always much smaller and satellite-like tumors (P-M) that are nearby the main tumors (P) with a usual distance within or about 1 cm apart, and they were usually from the same segment of the primary lesions. The portal vein thrombi (PT-M) were well separated from their main tumors (PT), as determined by the CT scan to exclude the possibility that they were a part of a continuing growth extension of the main tumors. In addition, we determined whether the main tumors and their metastatic lesions contain the same type of HBV variants by examining the expression of HBx in these paired samples. Among the 40 primary and
metastatic lesions from 20 patients, the expression of a full length HBx sequence can be detected in a total of 12 tumors (6 from P and P-M pairs and 6 from PT and PT-M pairs). Sequence analysis indicates that each pair always contains the same unique HBx variant (data not shown), suggesting that the metastatic lesions were derived from their matched primary tumors.

Tumors and non-tumor tissues were grossly dissected, snap-frozen in liquid nitrogen immediately after removal, and stored at −70°C until use. We confirmed microscopically that tumor tissue samples and their metastases consisted mostly of carcinoma cells and that non-tumor adjacent liver samples did not exhibit any tumor cell invasion. In this study set, the patients were predominantly Chinese males with an average age of 50 ± 9.7 (mean ± SD; range, 36 to 74 years). The size of the primary HCC ranged from 1.3cm to 17.5cm in diameter with a median diameter of 7.2cm, of which 65% (26/40) were >5cm in diameter and remaining were ≤ 5cm in diameter. Thirty-two cases (80%) had co-existing liver cirrhosis. Serologically, all of the 40 patients with an exception of one were HBV-positive, but no one was HCV-positive. However, subsequent analysis of the expression of HBeAg by RT-PCR of the HBV core gene indicates that all of the primary tumors have a detectable expression with varying levels (data not shown). Twenty-seven patients (68%) had an elevated serum concentration of alpha-fetoprotein (AFP) (>20 ng/ml).
II. RNA preparation, cDNA Microarrays and Hybridization

Total RNA was extracted from each sample using TRIzol Reagent (Invitrogen) according to the manufacturer’s specification. The cDNA microarrays were fabricated at the Advanced Technology Center, NCI. Each array contains 9180 cDNA clones with 7102 “named” genes, 1179 EST clones, and 122 Incyte clones. Preparation of fluorescent cDNA targets by a direct labeling approach and the cDNA microarray hybridization were essentially as previously described. (The detail protocol also can be found at the website: http://nciarray.nci.nih.gov). Briefly, the fluorescent targets were prepared as following: 100 _g of total RNA from non-cancerous liver tissue were labeled with Cy3-conjuagated deoxynucleotides or 200 _g of total RNA from primary HCC or metastasis were labeled with Cy5-conjuagated deoxynucleotides (Amersham) by the oligo dT-primed polymerization using SuperScript II reverse transcriptase (Invitrogen). The targets were then mixed together and added to the microarrays, and then incubated overnight (12-16 hours) at 42°C. Prior to hybridization, each microarray was pre-hybridized at 42°C for at least one hour in pre-hybridization buffer containing 5_ SSC, 0.1% SDS and 1% BSA. The slides were washed at room temperature in each with 2x SSC, 0.1% SDS and 1x SSC and 0.2x SSC for 2 min, respectively, and washed in 0.05x SSC for 1 min. Most
of the tumor tissues, when indicated, were sampled twice using two independent cDNA hybridizations. The Cy3 and Cy5 fluorescent intensities for each clone were determined by the Axon GenePix 4000 scanner, and were analyzed by the GenePix Pro 3.0 software to subtract the background signals. The expression data were then filtered based on their channel intensities, spots size and flag (missing data), and the Cy5/Cy3 ratios were calculated and normalized by median-centering the log-ratio of all genes in each array.

III. Data Analysis and Statistical Analysis

Unsupervised hierarchical clustering analysis was done by the CLUSTER and TREEVIEW software 8 using median centered correlation and complete linkage. We also used the BRB-ArrayTools software (http://linus.nci.nih.gov/BRB-ArrayTools.html), an integrated package for the visualization and statistical analysis of cDNA microarray gene expression data developed by the Biometric Research Branch of the National Cancer Institute, for both unsupervised and supervised analyses. The Class Comparison Tool based on univariate F-tests was used to find genes differentially expressed between predefined clinical groups at a significance level of \( P <0.001 \) or 0.002. The permutation distribution of the F-statistic, based on 2000 random permutations was also used to confirm statistical significance. In comparing primary to metastatic tumors of the same
patient, a paired value t-statistic was used in the same manner. The multi-
ivariate Compound Covariate Predictor (CCP) Tool with a “leave-one-out” cross-
validation test using 2000 random permutations at a significant level of
\( P<0.001 \) was used to classify predefined clinical groups based on their gene
expression profiles. In each cross-validation step one sample is omitted and a
multivariate CCP is created based on the genes that are univariately significant
at the specified level in the training set consisting of the samples not omitted.
This CCP is used to classify the omitted sample and it is then noted whether the
classification is correct or incorrect. This is repeated with all samples excluded
one at a time. The total cross-validated misclassification rate is thereby
determined. The statistical significance of the cross-validated misclassification
rate is determined by repeating the entire cross-validation procedure to data
with the class membership labels randomly permuted 2000 times. Therefore,
the CCP analysis had a built-in training and testing procedures applying to the
testing samples.

The CCP is based on a weighted linear combination of gene expression
variables that are univariately significant in the training set with the weights
being the corresponding t-statistics as described in Radmacher et al. \(^9\). When
the CCP was used to classify paired primary and metastatic tissue, the cross-
validation was performed with one pair at a time omitted and the classification
based on the paired differences in expression for each gene. To deal with the
duplicates, we averaged the log-ratios of expression levels for replicate arrays
of the same specimen prior to any class comparison or class prediction
analyses. This preserves the independence assumption of the statistical
significance tests and preserves the validity of the leave-one-out cross
validation estimates of mis-classification rates. In addition, in comparing
expression profiles of primary tumors with those of metastases, we performed a
paired analysis, pairing by patient. BRB-ArrayTools performed paired leave-one-
out cross validation by omitting one pair at a time and predicting which array of
the omitted pair corresponds to the primary and which to the metastasis.

To generate a prediction model to classify HCC with metastasis potential,
we first randomly selected 10 PN samples and 10 PT samples both as training
and testing set. A total of 20 new HCC samples were then included as a new
testing set for a further independent validation. For independent validation
based on the gene list derived from cross-validated CCP analysis, we used the
following linear combination: $L = \sum t_i (x_i - m_i)$, where $t_i = t$-value for gene $i$ in
the classifier, $x_i = \text{log-ratio of gene } i \text{ in the new sample to be classified, and } m_i =$
midpoint between PN and PT groups for gene $i$ (see supplemental Table 1) to
compute new HCC samples. However, this approach does not have a cross-
validation step.
To further validate the predictive value, we performed additional CCP analysis with leave-one-out cross validation using all of the 40 patients. Moreover, using all of the 40 patients, we applied three additional class prediction algorithms, including the Nearest Neighbor Predictor, the Nearest Centroid Predictor and the Support Vector Machine Predictor. The Nearest Neighbor Predictor was based on determining which expression profile in the training set was most similar to the expression profile of the specimen whose class is to be predicted. Euclidean distance was used as the distance metric. The Nearest Centroid Predictor used a centroid vector derived from a gene list that is univariately significantly differentially expressed between the two classes at a p value of 0.001 to predict the test samples. The distance of the expression profile for the test sample to each of the two centroids was measured and the test samples were predicted to belong to the class corresponding to the nearest centroid. The Support Vector Machine Predictor used linear kernel functions, in which the predictor was a linear function of the log-ratios that best separated the data subject to penalty costs on the number of specimens misclassified. We used one penalty cost and one cost of misclassifying a specimen of class 1 relative to misclassifying a specimen of class 2.
All of the class prediction methods used the same cross validation approach and the cross-validated misclassification rates were computed. Using leave-one-out cross validation to estimate the mis-classification rate, the genes included in the classifier were re-determined for each leave-one-out training set. They were determined based on univariate t-test statistical significance at the 0.001 significant level. Consequently, the number of genes and the identity of the genes vary for each training set. For purposes of describing a predictor to be used for new specimens, we developed a compound covariate classifier based on the complete data set. For more details, see the BRB-ArrayTools Users Guide at http://linus.nci.nih.gov/BRB-ArrayTools.html. The Kaplan-Meier Survival analysis was used to compare patient survival, using an Excel-based WinSTAT software (http://www.winstat.com). The statistical $P$ value was generated by the Cox-Mantel log-rank test when PN was compared to P or PT.

IV. Semi-quantitative PT-PCR and Western blotting

Total RNA was reverse-transcribed with SUPERSCRIPT™ II RNase H Reverse Transcriptase and Random hexamers (Invitrogen Inc.). PCR was done with 26 cycles (94°C, 30 sec; 53°C, 30 sec; 72°C, 1 min) followed by an extra cycle at 72°C for 10 min using the following primers: OPN sense 5’-GACTGAACGACTCTGATGATGTA-3’; OPN antisense 5’-CTGGGACACGGGGATGG-
3'; and HotStarTaq Master Mix (QIAGEN). QuantumRNA™ 18S (Ambion) was used as an internal standard. Densitometry was used to quantify the amount of OPN, which was normalized by the 18S product. Western blot analysis was done essential as described 7. Briefly, protein lysates from CCL13, SK-Hep-1 and Hep3B cells were prepared in RIPA buffer (50 mM Tris-HCl, pH 7.4/150 mM NaCl/1% Triton X-100/1% deoxycholate/1.0% SDS/1% aprotinin), separated on 10% SDS-PAGE, transferred to an Immobilin-P membrane (Millipore, Bedford, MA), probed with a rat monoclonal anti-OPN antibody (Chemicon International), and visualized by the ECL-based assay (Amersham).

V. Cell lines and In vitro invasion assay

Seven cell lines (SK-Hep-1, Hep3B, HuH1, HuH4, HuH7, SMMC7721 and MHCC97) derived from human hepatocellular carcinoma with different metastatic potentials and one non-transformed liver cell line, CCL13 (Chang liver cells), were used to determine the functional association of OPN with metastatic potential using an in vitro invasion assay10-12. SK-Hep-1, Hep3B and CCL13 cells were obtained from American Type Culture Collection (www.atcc.com). These cells were routinely maintained at 37°C in a humidified
atmosphere of 5% CO\textsubscript{2} in EMEM (GIBCO) medium supplemented with 10% fetal bovine serum (FBS), 1_ nonessential amino acids, 1_ sodium pyruvate, 2 mM glutamine and penicillin/streptomycin. SMMC7721 and MHCC97 cells were maintained in RPMI1640 medium containing 10% FBS. HuH1, HuH4 and HuH7 cells were maintained in DMEM (GIBCO) medium containing 10% FBS.

For the invasion assay, we used the BD BioCoat\textsuperscript{TM} Matrigel\textsuperscript{TM} Invasion Chamber (BD Biosciences) in which the upper and bottom chambers were separated by a membrane (with 8 µm pores) coated with matrigel, and followed the manufacture’s instruction. Briefly, a total of 5×10\textsuperscript{4}-2×10\textsuperscript{5} cells were seeded in each of the upper chambers (24 well chambers) in 0.5 ml of serum-free medium, and the bottom chambers contain appropriate medium with 5% FBS as chemoattractants. Invasion was carried out by incubating cells in the absence or presence of either recombinant murine OPN (2 µg/ml) (R&D Systems) or a well-documented neutralizing antibody against OPN (3 µg/ml) (R&D Systems) for 20 hours. An identical chamber with the same amount of seeded cells but containing no matrigel was used as a control to calculate the percentage of invasion for each cell type so that the values represent a relative potential for invasiveness of each cell line. After removing the non-invading cells (upper surface of the membrane), cells at the bottom side of the membranes were fixed and stained with Diff-Quik stain (Allegiance). The number of cells invading
through the Matrigel™ membrane (Matrigel chamber) or just migrating through the membrane (control chamber) was counted under light microscope with a 40x magnification. To minimize bias, at least 10 randomly selected fields of triplicate membranes for each condition were counted. Data were an average of triplicate determinants for each condition. Experiments were repeated at least three times. Data is expressed as the percent invasion through the Matrigel Matrix and membrane relative to the migration through the control membrane. A Student t-test was used to determine any statistical significance before or after addition of OPN, or antibody of OPN to each cell line. The groups with significant p values (<0.05) are indicated.

VI. In vivo spontaneous metastasis assay

We used a well-established pulmonary metastasis nude mice model system to examine the role of OPN on metastatic potential of HCC cells. HCCLM3 cells, which were derived from an in vivo selection of MHCC97 cells for spontaneous pulmonary metastasis when injected subcutaneously into nude mice, were used for this study. Male athymic BALB/c nu/nu mice at an age of 4-6 weeks were obtained from Shanghai Institute of Materia Medica, Chinese Academy of Science and maintained in specific pathogen-free condition. The study protocol on mice was approved by the Shanghai Medical Experimental Animal Care
Commission. A total of $5 \times 10^6$ HCCLM3 cells were mixed with anti-OPN neutralizing antibody (9 µg per mouse; R&D Systems) (anti-OPN group) or a control mouse IgG (control group), and then were injected subcutaneously into the left upper flank region of each mouse. Each experimental group contained 10 mice. One week later, each mouse from the anti-OPN group was injected via tail vein with another 9 µg of anti-OPN neutralizing antibody. Thirty-five days later, mice were euthanized and lungs were collected for the process of histopathology. A total of 100 serial sections were made for every half of the lung tissue blocks, and the first of every decade of sections were analyzed under the microscope for the presence of the tumor cell clusters by two pathologists. Analyses were done with a blinded manner. The metastases were classified into four grades based on the number of tumor cells present at the maximal section for each metastatic lesion: grade I, tumor cells $\leq 20$; grade II, tumor cells 20-50; grade III, tumor cells 50-100; and grade IV, tumor cells $>100$.

VII. Tissue histology analysis

Tumor histology following surgery was initially evaluated by the pathologists in the Pathology Department at Zhongshan Hospital. Some of the Paraffin-embedded tissue blocks were available to immunohistochemistry (IHC) analysis.
These include 13 HCC cases that were also used in the microarray study, and 16 new HCC cases and 8 normal livers from healthy donors that were from archived databases. These blocks were then subjected to serial sections with a thickness of 5 µm mounted on electrically charged glass slides. Slides were subjected to hematoxylin and eosin (H&E) staining and read by two pathologists independently. Tumors were graded according to Edmonson’s criteria\textsuperscript{14}. The IHC analysis of OPN was performed by a contractor with a blinded fashion. For IHC, slides were deparafinized and processed for immunostaining as described\textsuperscript{15}. Briefly, slides were incubated in microwave oven for 15 min in 1X citrate buffer for antigen retrieval and then quenched with 3% hydrogen peroxide to block the endogenous peroxidase activity for 20 min. Following incubation with 10% donkey serum to block the non-specific binding, the sections were incubated over night at 4°C with a rat monoclonal anti-OPN antibody at 1:1500 dilution (Chemicon International). For the negative control, slides were incubated in the absence of primary antibody. Biotinylated secondary antibodies and streptavidin peroxidase complex (ABC Elite kit, Vector Labs) were used. Chromogenic development was obtained by the immersion of sections in 3-3' di-aminobenzidine (DAB) solution (0.25 mg per ml with 3% hydrogen peroxide). The slides were counter-stained with Harris= Hematoxylin and de-hydrated with alcohol to Xylene, and mounted with Permount (Sigma).
VIII. References


