Identify Metastasis-associated Genes in Hepatocellular Carcinoma through Clonality Delineation for Multinodular Tumor

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ABSTRACT

Disease recurrence and metastasis are frequently observed in many successfully treated localized cancers, including hepatocellular carcinoma in which intrahepatic and extrahepatic recurrence (metastasis) are frequently observed after curative resection. The present study aimed at identifying metastasis-associated genes through delineation of the clonality for multinodular liver cancer. The clonal relationship of 22 tumor foci from six patients was investigated by the genome-wide expression profile via cDNA microarray consisting of 23,000 genes. Tumor molecular properties including p53 protein overexpression and gene mutation, hepatitis B virus integration pattern, and genetic alteration examined by comparative genomic hybridization were compared. Results indicated that gene expression patterns could serve as the molecular fingerprint for clonality identification. Together with the molecular data from p53, hepatitis B virus integration and comparative genomic hybridization profiles, tumor nodules from five patients were confirmed with clonal relationship, and the expression profiles of the primary nodules were compared with their corresponding intrahepatic metastatic nodules. A total of 90 clones were found to be correlated with intrahepatic metastasis by Student’s t test (P < 0.05). With reference to the primary tumor, 63 clones (39 known genes and 24 express sequence tags) were down-regulated whereas 27 clones (14 known genes and 13 express sequence tags) were up-regulated in the metastatic nodules. These metastasis-associated genes may provide clues to reveal patients with increased risk of developing metastasis, and to identify novel therapeutic targets for the treatment of metastasis.

INTRODUCTION

Metastasis is the major cause of cancer morbidity and mortality. Comprehensive analysis of gene expression profiles between a primary tumor and its derived tumors can identify differential genes associated with the metastatic phenotype, which help to elucidate the molecular mechanism of cancer metastasis (1, 2). There are a number of experimental models for the examination of genes responsible for the enhancement or suppression of cancer metastasis (3, 4). However, reliable parameters to define the clonality for multiple nodular HCC have not been established. Conventional discriminations are mostly based on clinical and pathological findings (5–7), which cannot always provide a definitive answer. Commonly used approaches include comparison for the HBV integration site to differentiate between intrahepatic metastasis and independent occurrence (8–10). However, this is only applicable to HCC with HBV integration, whereas tumors related to hepatitis C virus (HCV) or alcohol, which are the common associations in North America (11), cannot be validated. Clonal analysis can be examined based on X chromosome inactivation (12, 13), which can be investigated only in female patients. In East Asia, including Hong Kong, HCC patients are predominantly male; thus, the approach based on X chromosome inactivation does not have a wide implication. DNA fingerprinting (14) and fractional allelic loss (15) analysis have also been used for clonality studies. However, the demanding technique involved combined with the problem of reproducibility and the subjective data analysis all hinder their wide application in clonality examination.

In a recent study, we used the cDNA microarray approach to examine the genome-wide expression profile of over 200 samples consisting of majority HCC and the tumor adjacent liver tissues (16). A consistent expression difference was observed between the tumors and their adjacent nontumor livers. To investigate whether the expression profile can help to elucidate the clonal relationship of the multinodular liver cancers, samples from individual tumor foci were further examined with the conventional molecular genetic approach for tumor properties and genetic aberrations. The expression data of 22 individual tumor foci in six patients with multinodular HCC were compared with the conventional molecular genetic data including p53 protein overexpression and p53 gene mutation, HBV integration, and genetic aberrations investigated by CGH. The data until now had suggested that gene expression profiles were unique for each tumor and could provide sufficient information to delineate clonal relationships. The identity of primary and intrahepatic metastasis tumor nodules was further confirmed by conventional molecular genetic approaches, and the expression profiles of the original and metastatic tumor clones were compared with those of the genes associated with intrahepatic metastases.

MATERIALS AND METHODS

Patients and Samples. Informed consent was obtained from the patients for the collection of liver specimens, and the study protocol was approved by...
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the Ethics Committee of The University of Hong Kong. Between April and July 2000, six patients with multiple nodular liver tumors were treated by surgical resection (Table 1). A total of 22 samples were obtained from individual tumor foci of the surgical specimens. Samples were snap-frozen in liquid nitrogen and stored at −70°C until use. Paralleled tumor specimens were formalin fixed and paraffin embedded for histological examination and immunohistochemical study. Total RNA was extracted with RNeasy kit (Qiagen, Hilden, Germany) and mRNA was isolated from total RNA using Fast-Track (Invitrogen, Carlsbad, CA) or Poly(A)Pure (Ambion, Inc., Austin, TX) mRNA purification kit. DNA was extracted with DNAeasy kit (Qiagen) according to the manufacturer’s instructions.

Microarray Expression Study. The cDNA microarray slides were printed with 23,075 cDNA clones. Protocols have been established and the details described previously (17, 18). In brief, a fluorescent-labeled (Cy5) cDNA probe was synthesized by reverse transcription using the Superscript II reverse transcription kit (Invitrogen) from each experimental tumor RNA sample to serve as the “test” case. The “reference” mRNA was prepared by a different fluorescent (Cy3) from a pool of mRNAs isolated from different cultured cell lines. This common reference provided an internal standard against which the gene expression of each experimental sample was compared. The two fluorescent-labeled probes were combined, purified, and put onto the arrays. After hybridization, arrays were scanned with a microarray scanner for the fluorescent images. The image files were analyzed with the program GenePix Pro 3.0 (Axon Instruments, Inc., Union City, CA) to quantitate the relative amount of mRNA between the two samples. Data were deposited into the Stanford Microarray Database at: http://genome-www4.stanford.edu/MicroArray/SMD/index.html. Areas of the array with obvious blemishes were flagged. Each fluorescent signal in the array element with intensity 1.5-fold greater than local background was considered as valid data, and those genes with less than 75% valid data points in the sample set were excluded. Genes with expression levels that differed by at least 3-fold from the mean in at least one sample were selected. Hierarchical clustering algorithm (19, 20) was applied to both the genes and the arrays using the Pearson correlation coefficient as the measure of similarity. The results were further analyzed with TreeView (Eisen; http://rana.lbl.gov).

p53 Protein Staining and Gene Sequencing. Immunohistochemistry was performed as described previously (21) with modification. Briefly, antigen retrieval was performed by microwave with sections immersed in citrate buffer. Followed by endogenous peroxidase blocking and biotin blocking reagents (DAKO, Copenhagen, Denmark), antibody DO-7 (DAKO) in 1:100 dilution was applied. Signal was detected by horseradish peroxidase-conjugated secondary antibody and color development with diaminobenzidine (DAB) as the chromogen. Tissue sections were counterstained with hematoxylin.

Direct DNA sequencing was performed for exon 4 to exon 9, which resided with mutational hot-spot and accounted for over 80% of all of the mutations observed (22–24). Primer sets and reaction conditions were adopted from Lehman et al. (25). DNA was amplified by PCR, and direct DNA sequencing was performed with a BigDye sequencing kit (Applied Biosystems, Foster City, CA). Electrophoresis and sequence analysis were performed with ABI PRISM 310 (Applied Biosystems).

HBV Integration by Southern Blot Analysis. DNA was digested overnight with restriction endonuclease (Ref. 10; HindIII or EcoRI, Invitrogen) at 37°C with 10 units per µg of DNA. Fragments were electrophoresed on 1% agarose gel and transferred overnight to Hybond membrane (Amersham Pharmacia Biotech, Piscataway, NJ). The probe was full-length HBV fluorescein-labeled by random prime system (Amersham Pharmacia Biotech). After hybridization, stringency washes, and incubation with anti-fluorescien-horseradish peroxidase conjugate, ECL detection reagent (Amersham Pharmacia Biotech) was used, and a signal was developed by X-Omat AR film (Kodak, Rochester, NY).

CGH. Protocols have been established and details described previously (26, 27). In brief, DNA from tumor and normal reference were labeled with different fluorescent dUTPs by nick translation. Labeled probes were mixed and hybridized onto metaphase cells prepared from the lymphocytes of healthy donors. The slide was counterstained with 4’,6-diamidino-2-phenylindole di-hydrochloride (DAPI) for digital image analysis using the Quips CGH program (Vysis, Downers Grove, IL). The thresholds used for the interpretation of gains and losses of a DNA sequence copy number were defined as the tumor: reference ratio and were >1.25 and <0.75, respectively. The thresholds applied for both the standard and the reverse hybridization methods.

RESULTS

Gene Expression Profile. We applied a cDNA microarray with 23,075 cDNA clones to study the gene expression patterns in 103 liver cancer samples from 81 patients. The tumors and genes were grouped on the basis of their similarity in expression pattern by hierarchical clustering algorithm (19, 20). A total of 7,830 cDNA clones were shown to have significant variation (genes with at least 3-fold of expression difference in at least one array and 75% valid data points; Fig. 1A). The characteristic expression relationship of the 22 tumor foci from the six patients (Table 2) with multinodular liver tumors is highlighted in Fig. 1B.

The gene expression patterns of the two tumor foci from patient M1 were very different, and they are separated into different major subbranches in the dendrogram. The correlation coefficient of the overall gene expression patterns between T1 and T2 was 0.28. In patient M2, T1 (tumor nodule 1, as assigned according to gross size and proximity to the main tumor mass) to T6 demonstrated similar gene expression profiles and they were clustered into one terminal branch. Correlation coefficient ranged from 0.45 to 0.89. Tumor nodule T7 of patient M2 showed a distinct pattern of gene expression and it segregated into another major branch different from T1 to T6 of the same patient, with an overall very low correlation, around 0.1 to 0.2 with other nodules from patient M2. The two tumor foci from patient M3 showed similar gene expression patterns, residing in one terminal branch with a correlation of 0.52. Similarly, in patient M4, T1 to T5 clustered into one terminal branch with an overall correlation coefficient ranging from 0.79 to 0.85. Expression patterns of T2 and T3 from patient M5 were more similar. They were clustered into one terminal branch with a correlation of 0.80. Whereas T1 of the same patient M5 was less tightly linked to T2 and T3, they nonetheless resided closely together in the same major branch of the dendrogram with a correlation of 0.51. Correspondingly, in patient M6, T2 and T4 showed a more similar expression pattern with a correlation of 0.51. Tumor nodule T1 of the same patient M6 resided in the same subbranch with the correlation of 0.30 for T2 and 0.28 for T4. These data suggest a complex gene expression program between the multiple tumor nodules from the same patients.

As a control, we investigated whether sampling the same nodule

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**Table 1** Clinical patient details

<table>
<thead>
<tr>
<th>Patient&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Sex/Age</th>
<th>TNM stage</th>
<th>HBsAg&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Edmonson grade</th>
<th>Venous invasion</th>
<th>Survival (mo)</th>
<th>Current status</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>F/66</td>
<td>II</td>
<td></td>
<td>2</td>
<td>absent</td>
<td>14.9</td>
<td>Alive, disease free</td>
</tr>
<tr>
<td>M2</td>
<td>F/54</td>
<td>III</td>
<td></td>
<td>3</td>
<td>present</td>
<td>0.2</td>
<td>Decased</td>
</tr>
<tr>
<td>M3</td>
<td>F/43</td>
<td>IVA</td>
<td></td>
<td>3</td>
<td>present</td>
<td>5.1</td>
<td>Decased</td>
</tr>
<tr>
<td>M4</td>
<td>M/66</td>
<td>III</td>
<td></td>
<td>3</td>
<td>present</td>
<td>13.1</td>
<td>Alive, disease free</td>
</tr>
<tr>
<td>M5</td>
<td>M/13</td>
<td>IVA</td>
<td></td>
<td>3</td>
<td>present</td>
<td>6.2</td>
<td>Decased</td>
</tr>
<tr>
<td>M6</td>
<td>M/70</td>
<td>II</td>
<td></td>
<td>2</td>
<td>absent</td>
<td>8.5</td>
<td>Decased</td>
</tr>
</tbody>
</table>

<sup>a</sup> Patients M1 to M5 had primary liver tumors, and M6 had recurrent HCC after primary HCC resection and TOCE treatment for recurrence.

<sup>b</sup> HBsAg, hepatitis B surface antigen; TOCE, transarterial oily chemoembolization.
from the same tumor multiple times would also show variation similar to that seen from the multiple nodular tumors which were the discrete tumor nodules. Replicated samples, a total of 10, were taken from four patients from different regions of a single tumor nodule for comparison. All of the replicate samples from an individual were clustered into one terminal branch of the dendrogram with high correlation. (Fig. 1B). This demonstrates that each tumor nodule has its own distinct gene expression patterns, and the differences that we observed in the expression profiles in multiple nodular tumors are not attributable to a sampling artifact.

**p53 Protein Expression and Mutation.** To investigate the molecular genetics of these multiple nodular HCC samples, we studied the expression of p53 in these samples by immunohistochemistry. Tissue sections from tumor foci of patients M1, M2, and M4 all showed a negative staining result for p53 protein. And all of the tumor foci from patient M3 showed a positive signal of p53. In patient M5, a heterogeneous staining pattern was observed (Fig. 2A). For MST1, some of the tumor areas had less than 10% cells positive, whereas some of the tumor patches had more than 50% of cells positive. For MST2 and T3, a homogeneous staining pattern was observed, and the majority of tumor cells showed overexpression of p53 protein. In patient M6, a similar heterogeneity pattern was observed. The majority of tumor cells in M6T2 were negative, although a rare positive signal could be observed in some of them (Fig. 2B). In M6T1, the majority of tumor cells showed overexpression of the p53 protein.

The p53 gene sequence was further analyzed in those patients who had exhibited p53 protein overexpression to confirm the presence of gene mutation. Direct DNA sequencing was performed for exon 4 to exon 9, in which >80% of all mutations were observed (22, 24). Both of the tumor foci T1 and T2 from patient M3 showed a mutation at exon 6, amino acid 213. In patient M5, all three of the tumors contained a single mutation at exon 7, amino acid 249 (Fig. 2A). For patient M6, all three of the tumors exhibited a mutation at exon 5, amino acids 160 and 161 (Fig. 2B).

**HBV Integration Pattern.** To further confirm the clonality of those multiple tumor nodules with distinct gene expression profiles and heterogeneous p53 overexpression patterns, we applied Southern blot analysis for the HBV integration site. As the HBV integration is generally an early event during tumorigenesis, tumors arising from the same clone generally have the same integration pattern. The two tumor nodules, T1 and T2, of patient M1 had different HBV integration patterns (Fig. 3), which indicated that T1 and T2 were formed from two distinct clones. Tumor nodules T1 and T2 from patient M3 showed identical integration patterns. Patient M6 also demonstrated the same HBV integration pattern in both T1 and T2. This suggests that tumors from M3 and M6, respectively, were clonally related.

**CGH.** Using CGH, we further explored the genetic alterations among the multiple tumor nodules (Fig. 4; Table 3). The CGH profiles of the two tumor foci T1 and T2 in patient M1 were basically distinct. T1 to T6 from patient M2 shared similar genetic aberrations that were different from T7. Patient M3 showed similar structural changes in both of the tumor foci, with T1 showing additional genetic aberrations compared with the common ones observed in T2. T1 to T5 in patient M4 showed a common structural change. T2 and T3 from patient M5 showed additional genetic aberrations when compared with T1. In patient M6, common genetic aberrations were observed in both T1 and T2, whereas T1 exhibited additional chromosomal changes.

**Determination and Delineation for the Clonality of Multinodular Liver Cancer Samples.** Through the above analysis, we were able to determine the clonality of these multinodular liver cancer
samples. It also helped to determine the primary tumor nodules versus metastatic nodules.

For patient M1, the two nodules T1 and T2 showed distinct gene expression patterns (Fig. 1B). The HBV integration site was different (Fig. 3) and the genetic composition of the two nodules was discrete by CGH (Fig. 4). In addition, the two nodules were separated by a physical distance of 7 cm, which clinically would be classified as a multicentric occurrence (7) or multicentric HCC (6). All of the data obtained supported the observation from the gene expression profile, which suggested that the two tumor nodules were independent clones.

For patient M2, the gene expression profiles of T1 to T6 were very similar (Fig. 1B). T7, however, segregated to a different major branch, and was likely to be different in clonality. Clinically, T1 to T6 fit the definition for unicentric HCC (6). They appeared as a solitary tumor mass surrounded by contiguously smaller nodules located within 1 cm of each other. However, T7 was an isolated nodule away from the

Table 2: Clonality details for the multinodular liver tumors

<table>
<thead>
<tr>
<th>Patient</th>
<th>Tumor nodule</th>
<th>Gross appearance</th>
<th>Size (cm)</th>
<th>Separation distance</th>
<th>Array p53</th>
<th>Mutation, exon 4–9/a.a.</th>
<th>HBV integration</th>
<th>Histology</th>
<th>Derived clonal relationship</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>T1</td>
<td>Nodular</td>
<td>2</td>
<td>7</td>
<td>Different branch</td>
<td>1 and 2 = 0.28</td>
<td>Negative</td>
<td>Different site</td>
<td>HCC</td>
</tr>
<tr>
<td>T2</td>
<td></td>
<td></td>
<td>2</td>
<td></td>
<td>Negative</td>
<td></td>
<td></td>
<td></td>
<td>Multicentric occurrence</td>
</tr>
<tr>
<td>M2</td>
<td>T1</td>
<td>Massive</td>
<td>3.5</td>
<td>0</td>
<td>T1 to 6, same branch</td>
<td>1 and 2 = 0.58</td>
<td>Negative</td>
<td></td>
<td>HCC</td>
</tr>
<tr>
<td>T2</td>
<td></td>
<td></td>
<td>3</td>
<td></td>
<td>2 and 3 = 0.45</td>
<td>Negative</td>
<td></td>
<td>HCC</td>
<td>Same clone</td>
</tr>
<tr>
<td>T3</td>
<td></td>
<td></td>
<td>2.5</td>
<td>0</td>
<td>T7: far away</td>
<td>3 and 4 = 0.52</td>
<td>Negative</td>
<td>Mixed*</td>
<td>Intrahepatic metastasis</td>
</tr>
<tr>
<td>T4</td>
<td></td>
<td></td>
<td>3</td>
<td></td>
<td>4 and 5 = 0.69</td>
<td>Negative</td>
<td></td>
<td>HCC</td>
<td></td>
</tr>
<tr>
<td>T5</td>
<td></td>
<td></td>
<td>2.5</td>
<td>0</td>
<td>5 and 6 = 0.89</td>
<td>Negative</td>
<td></td>
<td>HCC</td>
<td>T7</td>
</tr>
<tr>
<td>T6</td>
<td></td>
<td></td>
<td>2</td>
<td>0</td>
<td>6 and 7 = 0.18</td>
<td>Negative</td>
<td></td>
<td>HCC</td>
<td>Separate clone</td>
</tr>
<tr>
<td>T7</td>
<td></td>
<td></td>
<td>1</td>
<td>5</td>
<td>1 and 7 = 0.15</td>
<td>Negative</td>
<td></td>
<td>adenoCA Multi-centric occurrence</td>
<td></td>
</tr>
</tbody>
</table>

For each patient, tumor nodule 1 (T1) was the largest tumor foci on gross morphology and T2 was the tumor next to it, and so forth.


b. Separation distance: calculated with reference to the major tumor mass. 0 cm = immediately adjacent with the main tumor mass but grossly separated foci.

c. a.a., amino acid; adenoCA, adenocarcinoma.

d. Mixed: presence of both HCC and adenocarcinoma cell types.

Fig. 2. p53 protein overexpression and p53 gene mutation analysis. In patient M5, different patterns of positive signals were observed in the tumor nodules (A). T1 was heterogeneous with respect to the p53 protein expression. Some areas of the tumor cells had less than 10% of the population showing protein accumulation, whereas some had more than 50%. T2 (and T3) were similar in that the majority of tumor cells showed overexpression. Sequence analysis supported the observation that the mutant sequence had already emerged in T1. This population was further selected and expanded in T2, which had a larger proportion of cells demonstrating mutant sequence at exon 7, amino acid 249. Similar observation for the p53 aberration in patient M6 (B). T2 was heterogeneous in p53 protein overexpression with positive signal shown only rarely. Sequence analysis demonstrated that mutation had developed at exon 5, amino acid 160–161, in the background of wild-type sequence. The p53 aberration was further accumulated in T1, with the majority of tumor cells overexpressing the protein and exhibiting predominantly the mutant sequence.
main tumor mass. The histological parallel sections of T1 to T2 and T4 to T6 were all confirmed to be HCC. Interestingly, T3 was reported to have a mixture of tumor cell types, HCC and adenocarcinoma, in the same section. The expression profile by microarray and genetic changes by CGH of T3 were indistinguishable from the other closely surrounded nodules, which suggested that the HCC trait overwhelmed and masked the other tumor cell components. However, T7 was histologically confirmed to be adenocarcinoma without an intermix of HCC components. T7 was clearly demonstrated to be distinct with its gene expression by microarray and chromosomal aberrations by CGH. The data obtained suggested that T1 to T2, and T4 to T6, were clonally linked. T1 revealed the fundamental genetic aberrations common to all of the other tumor nodules and, therefore, was regarded as the primary tumor. T7 was an independent tumor clone.

For patient M3, T1 and T2 were clustered into one terminal branch with a high correlation coefficient of gene expression, suggestive of clonal linkage. Tumor cells from both nodules overexpressed p53 protein with identical mutation point. The HBV integration patterns were also identical. Common genetic aberrations were observed by CGH analysis with T2 showing the basic chromosomal changes. T1 exhibited additional alterations and, thus, should be the derived metastatic tumor node as defined by the genetic approach on cancer progression. The gross size of T1 (14 cm) was much bigger than that of T2 (5.5 cm). The difference in tumor size could be explained if the additional genetic changes had conferred it with growth advantage. Nonetheless, the two nodules were situated immediately adjacent and could be clinically classified as unicentric HCC. All of the data agreed well with the gene expression profiles: the two nodules were derived from the same clone and T1 was the metastatic nodule.

All of the tumor nodules from patient M4 showed similar expression profiles. They clustered in one terminal branch of the dendrogram with high values of correlation coefficient, which indicated intrahepatic spread from the original tumor clone. Chromosomal changes as examined by CGH were also similar and supported the clinical observation of clonal relationship. All of the tumor nodules revealed the fundamental genetic alterations specific to this patient, and subtle differences were observed among different nodules. Thus, for the designation of primary tumor, the conventional approach was used, which referred the major tumor mass T1 as the primary.

In patient M5, expression profiles of T2 and T3 were tightly linked and showed some distinct differences from T1. As shown in the dendrogram and by the correlation coefficient, T2 and T3 should be clonal with closer linkage to each other than to T1. The p53 expression pattern corresponded well with the overall gene expression difference, in which the majority of tumor cells in T2 and T3 were positive, and only a proportion of tumor cells in T1 were positively stained for the p53 protein (Fig. 2A). However, all of the nodules showed an identical point mutation of the p53 gene at exon 7, amino acid 249. Consistent with the p53 protein overexpression pattern, the mutant sequence in T1 barely emerged, whereas a predominant mutant sequence was observed in T2. The p53 data here indicated that this gene mutation and protein accumulation had conferred growth and/or metastasis advantage to the tumor cells, and such property was selected for and expanded during tumor progression. In addition, common chromosomal aberrations were observed with T1 demonstrating the fundamental changes, whereas T2 and T3 showed additional alterations. Grossly, T1 was the major tumor mass, whereas T2 and T3 were smaller surrounding nodules. Expression profiles supported other genetic data and clinical observation that all three nodules arose from the same clone, and T2 and T3 were metastatic clones derived from the T1 tumor nodule.

Patient M6 presented a similar situation. The expression profiles of T2 and T4 were tightly linked suggesting definite clonal linkage, whereas T1 was not as tightly clustered but still resided on the same subbranch in the dendrogram. The p53 protein expression pattern coincided with the global gene expression profile. T2 and T4 were similar in the way that only rare tumor cells showed a positive signal, but T1 clearly demonstrated a positive expression for p53 (Fig. 2B). Identical gene mutations were observed in all three of the tumor foci. Coherent with the p53 protein expression pattern, the mutant sequence in T2 and T4 had just emerged, whereas a predominant mutant sequence was observed in T1. Similar with the situation in patient M5, the p53 aberration in patient M6 accumulated during tumor progression from T2 to T1. The genetic alterations as revealed by CGH indicated that both T1 and T4 had extra changes in addition to those observed in T2. The similar global gene expression patterns together with p53 aberration, HBV integration site, and common chromosomal aberrations, all implicated that the nodules had clonal relationship and that T1 and T4 were the metastatic tumor and that T2 was the primary.

Differential Genes between Primary and Metastatic Tumor.
Having established the clonal relationship of the multiple nodules and the delineation for the primary and metastatic tumors by the above investigations, the next step was to identify genes differentially expressed between the original and their derived clones. The expression profiles of the primary (T1, unless otherwise indicated that it was not the primary, as described in the following) and their metastatic nodules with the longest physical distance demonstrated with more genetic aberrations were compared. For patient M1, the two tumor foci were independent clones and thus were not included in this part of the study. For patient M2, T1 (primary) was compared with T6 (metastasis) because they indicated clonal linkage and the latter was the furthest away from T1. Similarly, M4T1 was compared with M4T5; and M5T1 was compared with M5T3. For patient M3, T2 was revealed as primary, and T1 would be the metastasis foci. In patient M6, T2 was the primary whereas T1 was the metastasis tumor foci.

The expression profiles of M2T1, M3T2, M4T1, M5T1, and M6T2 were considered as one group representing the primaries. M2T6, M3T1, M4T5, M5T3, and M6T1 were considered as another group representing the metastatic tumors. Student’s t test was used to select for genes that could differentiate between these two groups with a cutoff value at 0.05; 90 representative clones passed the criterion (Fig. 5). A total of 63 clones were down-regulated in the metastatic tumors compared with the primary tumors, where 39 clones represented 35 known genes; and 24 clones were ESTs with limited information for their biological function (Table 4). A total of 27 clones were up-regulated in the metastatic tumor, in which 14 clones were known genes and 13 clones were ESTs.
DISCUSSION

Examination of the HBV integration site is the conventional method to confirm HCC clonality. Southern blotting was commonly used (6, 9) but was labor intensive, time consuming, and applicable only to tumors with HBV infection. Cloning and PCR of the integrated HBV DNA could examine archive tissue in paraffin (28). However, it encountered a similar restriction in applicability. Molecular cytogenetic studies by CGH had also been used to determine tumor clonality (29). It is noteworthy that assessment of HBV integration and chromosomal content involved complicated protocols and procedures. However, expression profiles examined by microarray involved standard labeling, hybridization, and a data analysis approach that were researcher-independent. Microarray technique had the potential of standardization and automation (including array manufacture, probe synthesis, hybridization, and signal detection), suggesting a wider application compared with conventional investigation approaches to confirm the clonal relationship of separate tumor foci.

Comparison of the expression profiles from multiple specimens taken from an individual patient could indicate the unique character-

Fig. 4. CGH profiles. In A, in patient M1, the chromosomal alterations in T1 were distinctly different from T2. In patient M2, common genetic changes were observed from both tumor foci at chromosomes 1, 4, 8, 11, 13, and 17; and T6 revealed additional alterations at chromosomes 6, 12, and 22. In patient M3, similar genetic changes were observed from both tumor foci at chromosomes 2, 3, 4, 5p, 8, 10, 12, 17, and 22; and T1 exhibited additional aberrations at chromosomes 1, 5q, 11, 14, 16, 17, 18, 20, and 21. In B, in patient M4, comparable alterations were observed at chromosomes 1, 5, 8, 16, 17, 19, 20, and 22; and T5 exhibited extra aberrations at chromosome 4. In patient M5, similar genetic changes were observed at chromosomes 1, 5, 6pter, 15, 16, 17p, 19, and 21; and T3 exhibited extra alterations at chromosome 4, 6p, 8, 13, 17q, 18, 19, and 22. In patient M6, similar genetic alterations were observed from both tumor foci at chromosomes 1, 4, 6q, 7, 8p, 16, and 17; and additional genetic aberrations of T1 were observed at chromosomes 8q, 9, and 20; additional genetic aberrations of T4 were observed at chromosomes 6p, 7, 8q, 11, and 16.
Fig. 4. Continued
Tumors from individual patients were compared for their differential similarities and differences. The lineage of the multiple nodules had been elucidated through the analysis of CGH data. The present study indicated that the genome-wide expression data contributed substantial information to the molecular characterization and clonality of HCC. Poor prognosis is frequently associated with a high recurrence rate and metastasis. Discrimination for independent multicentric occurrence or intrahepatic metastasis would, therefore, be essential for multiple nodular tumors and in patients with recurrence after curative surgery. Verification in clonality will affect prognosis and disease management. Conventional discriminations are mostly based on clinical and pathological findings, which cannot always provide a definitive answer. Using the present method, we confirmed that four patients demonstrated unicentric HCC with intrahepatic metastatic lesions (M3, T1–2; M4, T1–5; M5, T1–3; M6, T1, 2, and 4). One patient (M2) had intrahepatic metastasis (T1, 2, 4–6) as well as an independent tumor clone (M2, T7). Another patient (M1) had multicentric liver tumor (T1 and T2 were independent clones). The present approach can provide reliable parameters to define the clonality of multiple nodular liver cancer, which has not been established before.

The lineage of the multiple nodules had been elucidated through expression and genetic data. A number of metastasis-associated genes have been identified through gene expression profiling. The next crucial step is to identify the key gene/pathway responsible for metastasis. Although a large number of genes are associated with metastasis, some of the changes are believed to be the secondary events; the expression changes as a result of metastasis rather than as an initiator of the metastasis event. Furthermore, as the present data are based on the study on intrahepatic metastasis, further investigation and validation on extrahepatic metastasis are necessary to consolidate the clinical significance.

Primarily, special attention has been paid to the genes that were down-regulated in the metastatic tumors because they could be metastasis-suppressor genes (1, 2), responsible for regulating the growth of disseminated cancer cells. This may lead to the identification of novel therapeutic targets for the treatment of metastatic disease. The present study has identified 63 clones, with 35 known genes that exhibited a lower expression level in metastatic tumors compared with the primaries. One of these genes, the breast-cancer metastasis suppressor 1 (BRMS1), has been shown to possess the functional capability of decreasing the metastatic potential of breast cancer cells that have been transplanted with the gene (30). This would be very important in the investigation of its functional role in liver cancer cell lines and other cancer types, and to explore whether this gene, BRMS1, is the common key gene as “metastasis-suppressor.” At least two other genes may have a key participation: the CD53 and the EMP3 (Fig. 5A). CD53 is a member of the transmembrane 4 superfamily (TM4SF), which is expressed in a number of different cell types and may be involved in transmembrane signal transduction, regulation of cell proliferation, and differentiation (31). EMP3 encodes for transmembrane protein and could be responsible for cell growth, differentiation, and apoptosis (32). Both of these genes, CD53 and EMP3, are membrane proteins that consist of four transmembrane domains, and possess an important feature of KAI1, a recently dis-

<table>
<thead>
<tr>
<th>Patient</th>
<th>Tumor node</th>
<th>Differential chromosomal changes</th>
<th>Common alterations in the same patient</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>T1</td>
<td>+1p31.2, +2q23.3, +4p, +4q12.1-q27, +5p, +7q11.23-qter, −16, −18q</td>
<td>+1q31-qter, −6q22.3-qter, −8p-tel, −17p, −19p, −22q</td>
</tr>
<tr>
<td>M2</td>
<td>T1, 2, 3, 4, 6</td>
<td>−4q21.1-q26, −13p</td>
<td>+1q21.1-qter, −8p, −17p, +17q21-qter</td>
</tr>
<tr>
<td>M3</td>
<td>T1</td>
<td>+1p31.2-q13.1, +4p12-q13.3, +q21-q23.3, −1q21-21.2, +14q11.2-q21, −14q24.1-qter, −16q</td>
<td>+1q23-qter, +5p, +6p-tel-p21.1, −15q, −16q, −19p, −21p</td>
</tr>
<tr>
<td>M4</td>
<td>T1, 2, 3, 5</td>
<td>+1q21.1-q41, +5p</td>
<td>+1q23-qter, +5p, +6p-tel-p21.1, −15q, −16q, −19p, −21p</td>
</tr>
<tr>
<td>M5</td>
<td>T1</td>
<td>+4q28-q32, +13q14.1-qter</td>
<td>+1q23-qter, +5p, +6p-tel-p21.1, −15q, −16q, −19p, −21p</td>
</tr>
<tr>
<td>M6</td>
<td>T1</td>
<td>+6q22.3-qter, +8q21.1-qter, −9p, +20q</td>
<td>+1q21-qter, −4q, −6q11-q22.1, +7q31.1-qter, −8p, −16q, −17p</td>
</tr>
</tbody>
</table>

* M2, T1 to T4, and T6 to T7 were examined.
covered metastasis-related gene the down-regulation of which had been associated with lymph node or distant metastases in prostate cancer (33), pancreatic cancer (34), and colon cancer (35). Reduced expression of KAI1 has also been observed in liver cancer (36). As the protein structure of both CD53 and EMP3 shows a high resemblance to that of KAI1, it is crucial to further investigate their functional role in liver cancer progression to consolidate their role in metastasis.

In summary, the present study demonstrated that expression profiles could provide a reliable method to delineate the clonal relationship of multiple nodules of liver cancer. Clonality verification is essential because prognosis and disease management are different for independent multicentric occurrence and intrahepatic metastasis. The same approach can be applied to recurrent disease and distant metastasis in various cancer types to elucidate the clonal lineage. Most importantly, metastasis-associated genes can be identified through the present approach. Identification of candidate genes can help to improve the ability to distinguish between unambiguously malignant lesions and indolent lesions, and to assist in the differentiation of tumors that are more likely to metastasize (1). Through elucidation of the molecular pathways of cancer progression, these intrahepatic
metastasis-associated genes will ultimately be targeted not only for the improvement of patient management but, more importantly, for the treatment of metastasis diseases.

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