Distinct Chromosomal Bias of Gene Expression Signatures in the Progression of Hepatocellular Carcinoma

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ABSTRACT

To identify the chromosomal aberrations associated with the progression of liver cancer, we applied expression imbalance map analysis to gene expression data from 31 hepatocellular carcinomas and 19 noncancerous tissues. Expression imbalance map analysis, which detects mRNA expression imbalance correlated with chromosomal regions, showed that expression gains of 1q21-23 (74%), 3q13-21 (48%), 12q23-24 (41%), 17q12-21 (48%), 17q25 (25%), and 20q11 (22%) and losses of 4q13 (48%), 8p12-21 (32%), 13q14 (32%), and 17p13 (29%) were significantly associated with hepatocellular carcinoma. Most regions with altered expression identified by expression imbalance map were also identified in previous reports using comparative genomic hybridization. We demonstrated chromosomal copy number gain in 1q21-23 and loss in 17p13 by genomic quantitative PCR, suggesting that gene expression profiles reflect chromosomal alterations. Furthermore, expression imbalance map analysis revealed that more poorly differentiated hepatocellular carcinoma contain more chromosomal alterations, which are accumulated in a stepwise manner in the course of hepatocellular carcinoma progression: expression imbalance of 1q, 8p, 8q, and 17p occur as early events in hepatocarcinogenesis, and 12q, 17q25, and 20q occur as late events. In particular, expression gain of 17q12-21 and loss of 4q were seen to accumulate constantly through the dedifferentiation process. Our data suggest that gene expression profiles are subject to chromosomal bias and that expression imbalance map can correlate gene expression to gene loci with high resolution and sensitivity.

INTRODUCTION

Hepatocellular carcinoma develops with dedifferentiation after liver injury by chronic hepatitis viral infection. In multistep hepatocarcinogenesis, several molecular events accumulate in a fashion that parallels the clinical progression of liver cancer (1, 2). Some studies have tried to identify the genes altered in dedifferentiation of hepatocellular carcinoma using samples with a nodule-in-nodule appearance (2, 3). Still, little is known about the genes that play a pivotal role during the course of liver cancer progression.

Genomic amplification of oncogenes and inactivation of tumor suppressor genes are frequently associated with cancer progression. Comparative genomic hybridization (CGH) has contributed to our basic cancer understanding and diagnosis of cancer (4) but can only detect genomic alterations >20 Mb. This low resolution makes it difficult for CGH to identify genes differentially expressed in carcinogenesis. To detect amplification events involving small genomic regions, high-resolution analysis of DNA copy number variation using cDNA microarrays has been used (5–7). However, because this technology only analyzes genomic DNA, supplementary experiments are required to confirm the expression of candidate genes in tumorigenesis. Microarrays have also been used to obtain comprehensive measurements of genome-wide expression profiles. Using such techniques, classification of cancer specimens or identification of gene sets for carcinogenesis and cancer progression have been reported by many researchers (2, 8–11).

Information about genome dosage and transcriptome may be obtained through microarray technologies if gene expression and gene localization data are integrated. In our study of oligodendroglioma, we showed that biological differences between genetic subsets of oligodendroglioma are reflected in their gene expression profiles and that genomic copy number alteration consistently accompanies perturbation of the transcriptome (12). Similarly, Virtaneva et al. (13) demonstrated an association between trisomy 8 and overexpression of genes on chromosome 8 in acute myeloid leukemia, and Tay et al. (11) performed CGH and expression microarray using gastric cancer specimens integrating CGH data with the microarray results.

Recently, based on mRNA expression, we have reported a method for constructing a transcriptome map (14). The expression imbalance map is a visualization method for detecting expression imbalance in regions of chromosomes. It extends beyond simple spatial mapping of microarray expression profiles on chromosomal locations to profile genomic losses and gains at a much higher resolution than CGH. In the expression imbalance map, signal noise can be reduced using a moving average compared with conventional methods, and users can determine thresholds. Furthermore, the expression imbalance map not only detects the expression imbalance frequency across all cases but also detects individual differences between cancer specimens. In addition, the expression imbalance map allows observation of both expression imbalance and the expression of each gene simultaneously. We previously applied the expression imbalance map to gene expression data derived from squamous cell lung carcinoma and detected, as with regional signal images, several novel and many known loci with frequent genomic losses or gains on various chromosomes (14).

In the present study, we have focused on chromosomal bias in transcripts during multistep hepatocarcinogenesis. We applied the expression imbalance map method to gene expression data of hepatocellular carcinoma and observed a stepwise change of expression along at defined chromosomal loci in liver cancer progression. Genomic quantitative PCR (qPCR) analysis confirmed that expression imbalance map data correlated with genomic aberrations. The novel regions identified by the expression imbalance map may play pivotal roles during the course of dedifferentiation of hepatocellular carcinoma and likely contain some candidate genes responsible for liver cancer progression.

MATERIALS AND METHODS

Patients and Tissue Samples. Thirty-one patients with hepatocellular carcinoma undergoing hepatectomy in the Hepato-Biliary-Pancreatic Surgery Division, Department of Surgery, Graduate School of Medicine, University of

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Tokyo, or the Department of Surgery, Saitama Cancer Center, were included in this study with informed consent. Among the 31 patients with hepatocellular carcinoma, 10 were positive for hepatitis B surface antigen and 21 for hepatitis C virus antibody. GeneChip analysis was performed using 50 samples, including 31 hepatocellular carcinomas and 19 surrounding noncancerous tissues. Clinical factors and tumor status based on histologic findings of resected specimens are summarized in Table 1.

The surgical specimens were immediately cut into small pieces after resection, snap frozen in liquid nitrogen and stored at −80°C.

**RNA Extraction and Oligonucleotide Microarray.** Total RNA was isolated from frozen tissue using IsoGene (Nippon Gene, Tokyo, Japan), according to the manufacturer’s protocol. Experimental procedures for GeneChip were performed according to GeneChip Expression Analysis Technical Manual (Affymetrix, Santa Clara, CA), using 15 μg of total RNA. Samples were analyzed on U95A array chips (Affymetrix).

**Normalization and Filtering of Intensity of Gene Expression.** Before analysis, we normalized and filtered the raw data. A quantile normalization procedure was used for the probe intensity distribution across different chips. The average of expression level intensity, the average difference, was scaled to 100, and for each expression data set, where the average difference values lay outside the range (1 to 10,000), the values was reset to a minimum 1 and to a maximum of 10,000. Genes where the coefficient of variation through all of the samples was <0.3 were excluded from analysis. Subsequently, all values were log-transformed for further analysis.

**Expression Imbalance Map.** The expression imbalance map was applied to gene expression data of hepatocellular carcinoma and noncancerous tissues obtained by microarray. Gene locus information was obtained from the web sites for Genes On Specimen Map (Homo sapiens build 33). The basic concept and method of expression imbalance map are described elsewhere (14). Briefly, gene expression levels of cancer specimens were compared with the average of noncancerous tissues, and the regions in which the numbers of up-regulated or down-regulated genes were significantly concentrated were mapped on the chromosomal region and constituted an expression imbalance map.

**RESULTS**

**Expression Imbalance Map for Detecting Expression Imbalance Regions.** The expression imbalance map was applied to 31 hepatocellular carcinomas, comparing their expression data to the average of expression across 19 noncancerous tissues (Fig. 1A). Various criteria may be used to define expression imbalance regions. When expression imbalance regions were defined as those with E values >2 and a region of >3.0Mb, compared with the background liver, expression gains on chromosome arms of hepatocellular carcinoma were observed on 1q21-23 (74%), 8q13-21 (48%), 12q23-24 (41%), 17q12-21 (48%), 17q25 (25%), and 20q11 (22%) and expression losses were detected on 4q13 (48%), 8p12-21 (32%), 13q14 (32%), and 17p13 (29%; Fig. 1, B and C). To provide a comparison between our data and previously reported observations of chromosomal alterations of hepatocellular carcinoma obtained by CGH, chromosomal arms with expression gain or loss are summarized in Table 2. Most regions with altered expression, as determined by the expression imbalance map, were also indicated in previous reports using CGH. Notable exceptions are a gain of 12q, which has never been reported previously, and a loss of 16q, which has been demonstrated in many previous reports, but was not identified in our analysis.

Gene list of the imbalanced region is shown with their median and log 10 P.

On the basis of the statistical probability, P, of rank sum, the differential level E value was defined as follows: $E = \frac{\log_{10} P}{\log_{10} \Phi}$. The chromosomal regions with expression gains or loss were defined according to both E value and range of alteration of gene expression.

**Clustering Hepatocellular Carcinoma Samples Using Chromosomal Regions with Expression Imbalance.** A heat map was constructed using expression imbalance map-positive chromosomal regions as binary variables. Hepatocellular carcinoma samples were arranged in order of the total number of positive regions, and the samples with less than two positive regions were defined as group1, three or four positive regions as group 2, and more than five positive regions as group3. Next, considering the length of positive regions, expression imbalance in each 100-kb region was calculated, and the sum of the length of positive chromosomal change in each sample was displayed graphically as the intensity of red or blue. Thirty-one tumor samples were arranged in accordance to tumor differentiation grade, and the total length of transcriptome alterations in each sample were compared by ANOVA.

**DNA Extraction and qPCR.** Genomic DNA was extracted from frozen tissue, using the QIAamp DNA Mini kit (Qiagen, Valencia, CA), according to the manufacturer’s instructions. PCR amplification was performed using Taq Polymerase and 20 ng of sample DNA. Oligonucleotide primers were designed to amplify genomic DNA fragments near the chromosomal locus of 1q21-23, encoding CCT3 (145 bp), PSMB4 (115 bp), 12q23-24, encoding CAKAP4 (141 bp), UNG (140 bp), 17p13, encoding ASGR1 (136 bp), Dapk2p5666H073 (168 bp), and β-actin (132 bp) as a control reference. The primers used were as follows: CCT3, 5’-TACACGATGCTGATGAGCCA AAC-3’ and 5’-GCATCTTGCTGAGCGCAAC-3’; PSMB4, 5’-TGGCTGGTTTCC GCAACTC-3’ and 5’-ACCATCTGCGAGACATG-3’; CAKAP4, 5’-GT GCAGTTTGAAGCCAC-3’ and 5’-CTGAGTGGTCCGACACTTC-3’; UNG, 5’-GTGCTTAAAAACGCTCTTG-3’ and 5’-GGCCAGATTCTG- GAAGCTAA-3’. Expression levels of tumor samples were compared with the average expression in each 100-kb region was calculated, and the sum of the length of positive chromosomal change in each sample was displayed graphically as the intensity of red or blue. Thirty-one tumor samples were arranged in accordance to tumor differentiation grade, and the total length of transcriptome alterations in each sample were compared by ANOVA.

**DNA Extraction and qPCR.** Genomic DNA was extracted from fresh-frozen tissue, using the QIAamp DNA Mini kit (Qiagen, Valencia, CA), according to the manufacturer’s instructions. PCR amplification was performed using Taq Polymerase and 20 ng of sample DNA. Oligonucleotide primers were designed to amplify genomic DNA fragments near the chromosomal locus of 1q21-23, encoding CCT3 (145 bp), PSMB4 (115 bp), 12q23-24, encoding CAKAP4 (141 bp), UNG (140 bp), 17p13, encoding ASGR1 (136 bp), Dapk2p5666H073 (168 bp), and β-actin (132 bp) as a control reference. The primers used were as follows: CCT3, 5’-TACACGATGCTGATGAGCCA AAC-3’ and 5’-GCATCTTGCTGAGCGCAAC-3’; PSMB4, 5’-TGGCTGGTTTCC GCAACTC-3’ and 5’-ACCATCTGCGAGACATG-3’; CAKAP4, 5’-GT GCAGTTTGAAGCCAC-3’ and 5’-CTGAGTGGTCCGACACTTC-3’; UNG, 5’-GTGCTTAAAAACGCTCTTG-3’ and 5’-GGCCAGATTCTG- GAAGCTAA-3’. Expression levels of tumor samples were compared with the average expression in each 100-kb region was calculated, and the sum of the length of positive chromosomal change in each sample was displayed graphically as the intensity of red or blue. Thirty-one tumor samples were arranged in accordance to tumor differentiation grade, and the total length of transcriptome alterations in each sample were compared by ANOVA.

**Comparison with existing CGH data sets for hepatocellular carci-
Fig. 1. Expression imbalance map for detecting expression imbalance regions in hepatocellular carcinoma. A. Regions of under- and overexpression in hepatocellular carcinoma were visualized on the left side and the right side, respectively, as gray regional signals. Each column represents tissue samples. The lighter areas correspond to higher probability of an expression imbalance region. The expression imbalance map enables the user to identify many more genes by referring to the more expanded area with lower luminance. This figure shows the expression imbalance at an E value \( E_{H11022}^2 \).

B. Expression imbalance region at an E value \( E_{H11022}^2 \) and a range of expression gain \( \geq 3.0 \) Mb. The length assigned to each chromosome is proportional to the number of Locus IDs on the chromosome.

C. Expression imbalance region at an E value \( E_{H11022}^2 \) and a range of expression loss \( \geq 3.0 \) Mb.

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noma suggests that gene expression profiles reflect chromosomal alterations and that the expression imbalance map is a useful tool for estimating chromosomal change from expression data.

**Classification of Hepatocellular Carcinomas by Chromosomal Bias of Gene Expression.** Expression imbalance map analysis showed that genes with expression change are derived from particular chromosomal regions with altered copy number. To identify possible association between these chromosomal alterations and liver cancer progression, we investigated the relationship between expression imbalance and tumor differentiation grade (Fig. 2).

First, we counted the expression-positive regions by expression imbalance map and arranged the samples according to the sum of positive regions. This resulted in the identification of 9 samples that constitute the first group and contain less than two positive regions, 10 samples in the second group with three or four positive regions, and 12 samples in the third group with more than five positive regions. Interestingly, all of the samples from the first group were well differentiated, and all of the poorly differentiated samples were concentrated in the third group (Fig. 2A), demonstrating a distinct trend for poorly differentiated grade tumors to contain more chromosomal aberrations.

Summing the number of 100-kb regions with expression imbalance, tumor differentiation grade and total length of chromosomal change were significantly correlated: the mean length of expression changes were 9.7 ± 5.6, 28.3 ± 13.0, and 45.2 ± 7.9 Mb in well differentiated, moderately differentiated, and poorly differentiated, respectively (P < 0.00001 by ANOVA; Fig. 2B). Some chromosomal regions showed consistent relationships with tumor progression. For example, expression gains of chromosome arms were observed on 12q23-24 and 20q11 with differentiation from well differentiated to moderately differentiated, whereas an expression gain of 17q25 was significantly associated with differentiation from well differentiated and moderately differentiated to poorly differentiated (P < 0.05 by Student-Newman-Keuls test). Notably, a gain of 17q12-21 and loss of 4q13 were found in each step of tumor dedifferentiation (Fig. 2C).

These results suggest that expression alterations of 1q21-23, 8p12-21, 8q13-21, and 17p13 occur as early events in hepatocarcinogenesis and 4q13, 12q23-24, 17q12-21, 17q25, and 20q11 as later events. Thus, liver cancer progresses with stepwise chromosomal expression change.

**Expression Imbalance Map Was Correlated with Genomic Aberrations on 1q21-23 and 17p13 by Genomic qPCR.** Many genomic aberrant regions in hepatocellular carcinoma detected by the expression imbalance map in this study are the same as those identified by CGH in previous studies (Table 2). To investigate the relationship between genomic and transcriptional aberrations, genome dosage in 1q21-23, 12q23-24, and 17p13 regions was confirmed using genomic qPCR for 27 hepatocellular carcinoma and the corresponding noncancerous liver samples (15).

qPCR was performed using 27 noncancerous liver samples. Average copy numbers for the loci of CCT3 and PSMB4 genes were 1.28 ± 0.37 and 0.90 ± 0.22, respectively. The genome dosage of hepatocellular carcinoma tended to increase in accordance with tumor differentiation grade (Fig. 3, A and B). In particular, consistent with the expression imbalance map data, genome dosage, shown as relative quantification, was significantly increased in moderately differentiated and poorly differentiated compared with well differentiated, both in CCT3 and PSMB4. To clarify the relationship between genome dosage and the probability of expression imbalance in the 1q21-23 region, the relative quantification between samples with E values < 3 was compared with those samples with E values > 3. As shown in the top right section of Fig. 3, the relative quantification of CCT3 and PSMB4 increased significantly in the higher E value group (P < 0.05, Mann-Whitney U test; Fig. 3, A and B).

Genomic loss was also detectable in 17p13 region. Consistent with the expression imbalance map data, relative quantification was significantly decreased in samples with E values > 2, compared with those with E values < 2, both in ASGR1 and DKFZP566H073 (Fig. 3, C and D), whereas genomic amplification on 12q23-24 was not observed by qPCR (data not shown).

Through these results, we demonstrated that genes identified by expression imbalance map had altered genome dosage with statistical significance, and alterations of their mRNA expression levels may reflect a gain or loss of genomic copy number at the locus from 147.4 to 158.1 Mb on 1q21-23 and from 2.5 to 7.7 Mb on 17p13. On the other hand, genome dosage of chromosome 12q23-24, which has never detected in the previous CGH, has no change between hepatocellular carcinoma and chronic liver disease in DNA using qPCR.

**Search for Candidate Genes Responsible for Hepatocarcinogenesis and Liver Cancer Progression.** To identify candidate genes responsible for hepatocarcinogenesis and liver cancer progression, we further investigated the transcription of each of 55 genes in the 1q21-23 region (147.4–158.1 Mb; Fig. 4). We found four related genes that were highly expressed in hepatocellular carcinoma compared with noncancerous tissues, HAX1 (1q22), SHC1 (1q21), CKS1B (1q21), and CCT3 (1q23). Similarly, two growth-related genes were identified on the other nine regions selected by expression imbalance map: A1TF (17q11-12) and TK1 (17q23-25) had increased expression compared with noncancerous tissues.

**DISCUSSION**

Using CGH, many investigators have already reported various chromosomal regions of hepatocellular carcinoma that undergo cytogenetic change (16–28). Consistent with the previous data, the expression imbalance map identified an increase in gene expression on chromosomes 1q, 8q, 17q, and 20q and a decrease on 4q, 8p, 13q, and 17p. To validate the relationship between expression imbalance and...
genomic imbalance, we confirmed genomic aberration on chromosomes 1q21-23 and 17p13 using genomic qPCR. Specifically, all underexpressed regions 4q, 8p, 13q, and 17p have been identified by comprehensive allelotyping study (29–32), indicating that the regions with expression loss detected by expression imbalance map may be the result of loss of heterozygosity. In addition to the regions detected in previous CGH studies, we identified an expression gain in expression imbalance map at 12q23-24. The fact that 12q23-24 was identified only by the expression imbalance map and was not detected by both conventional methods and our data using qPCR suggests that there is no amplification of genome dosage but, rather, a transcriptional regulation because nine genes on 12q23-24 contain MAZ, SP1, and E2F binding sites in their upstream beyond expectation (data not shown). Thus, by using a new method without selective threshold processing in conjunction with a distribution-based algorithm, the expression imbalance map was able to detect regionally over- or underexpressed genes from gene expression data of oligonucleotide arrays with higher resolution than had been achieved previously (14).

Furthermore, the expression imbalance map is based on microarray data and can, therefore, offer mRNA expression and expression imbalance information simultaneously.

Despite morphologic change, comprehensive expression profiling without expression imbalance map analysis fails to distinguish well differentiated from moderately differentiated. In this study, using the expression imbalance map, we focused the gene alteration of differentiation grade of hepatocellular carcinoma. Expression gains of 1q21-23 and 8q13-21 and losses of 8p12-21 and 17p13 were observed in carcinogenesis from chronic liver disease to well differentiated, whereas up-regulation of 12q23-24, 17q12-21, 17q25, and 20q11 and a down-regulation of 4q13 were found only in moderately differentiated and poorly differentiated, although others reported that loss of 4q region occurred in various steps, e.g., in the step from adenoma to well differentiated (33) or from well differentiated to moderately differentiated (33).

Fig. 2. Chromosomal bias of gene expression in liver cancer progression. A. Of 10 chromosomal regions selected by the expression imbalance map, the positive chromosomal regions were counted, and three groups were defined according to the sum of positive regions. Each column represents tissue samples and each row chromosomal regions. Relative expression levels in each chromosomal region are shown in red (high levels) and blue (low levels). B. Chromosomal alteration in each tumor differentiation grade. Total amount of chromosomal alteration in 10 positive chromosomal regions by the expression imbalance map is increased as liver cancer develops. Y axis represents the total length of positive regions by the expression imbalance map (×100 kb). C. This analysis was carried out using 10 chromosomal regions selected by the expression imbalance map. Chromosomal change associated with each 100 kb is scored, and samples arranged according to the tumor differentiation grade. Each column represents tissue samples and each row chromosomal regions. Relative expression levels in each chromosomal region are shown in red (high) and blue (low). Statistical significance is considered as P < 0.05 by Mann-Whitney U test and shown on the left (+, statistical significance between well differentiated (WD) and moderately differentiated (MD); **, statistical significance in each differentiation grade of liver cancer; +, statistical significance between WD and MD + poorly differentiated (PD); ++, statistical significance between WD+MD and PD). Statistical significance is considered as P < 0.05.

6 Unpublished observations.
differentiated and poorly differentiated (34). These observations suggest that, including 17q12-21, which exhibits an imbalance of expression in each differentiation grade, chromosomal aberration of 4q, 12q, 17q12-21, 17q25, and 20q may play pivotal role in liver cancer progression. Our results further suggest that 1q, 8p, and 17p may be associated with initiation of hepatocarcinogenesis. Besides the chromosomal regions concerning in liver cancer progression described above, our data showed 13q14 region was concentrated on moderately differentiated and poorly differentiated, although there has no statistical significance ($P = 0.1652$), consistent with the previous reports by CGH or allelotype study (26, 34). In addition, Wilkens et al. (33) demonstrated that gains of 1q, 8q, and 16p and losses of 4q, 8p, and 17p were found in hepatocarcinogenesis, which were compatible to our data except for 4q and 16p. These results demonstrate a stepwise expression change in chromosomal loci correlating with hepatocellular carcinoma progression.

On 1q21-23, we focused on four growth-related genes: HAX-1, SHC1, CKS1B, and CCT3. HAX-1 and SHC1 have been demonstrated to induce activation of tyrosine kinases (35, 36), whereas CKS1B and CCT3 may accelerate the cell cycle (37, 38). These expression gains of tyrosine kinase and cell cycle-related genes are consistent with the less differentiated, rapidly growing, liver cancer. Apart from 1q21-23, several genes that may be associated with liver cancer progression are clustered on 17q12-21 and 17q25. AATF, which interferes with the

![Fig. 3. Genomic imbalance of chromosome by qPCR. Genomic imbalance of chromosome 1q21-23 verified by qPCR applied to detecting copy number for liver cancer, according to differentiation grade. Amplification plots were obtained for CCT3 and PSMB4, both of which were part of a highly expressed region identified by the expression imbalance map. Data are expressed as relative quantification (RQ) defined in Materials and Methods and are the mean ± SD of three determinations per experiment from three separate experiments. The broken line indicates the average of genome dosage of nondiseased area of livers (1.28 for CCT3 and 0.90 for PSMB4). Genome dosage was compared between the two groups, an E value is <3, and >3 in the expression imbalance map, using the Mann-Whitney U test (shown in the top right side). (*, statistical significance was considered as $P < 0.05$). A in CCT3 and B in PSMB4. Genome dosage was also compared between the two groups, an E value is <2 and >2 in expression imbalance map in 17p13 region. C in ASGR1 and D in DNFZP566H073. (**, $P < 0.001$ and ***; $P < 0.0001$). Samples m3, m4, p2, and p4 were not determined because of lack of frozen specimens.)
induction of cell apoptosis through MAP3K/DLK (39), and TK1, a marker for the development for breast cancer (40), were also upregulated in liver cancer.

We focused on expression change with chromosomal bias using the expression imbalance map, a recently developed method for the detection of mRNA expression imbalance regions. Comparison of expression imbalance map data with previous analysis of chromosomal imbalance identified by CGH and validation using qPCR indicate that gene expression profiles reflect chromosomal alteration. Furthermore, the expression imbalance map provides a direct measure of mRNA expression gain or loss that eliminates the need for confirmation of transcription levels of each gene. This method has the advantage that chromosomal bias and gene expression can be observed simultaneously with ease and reliability.

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