Array-based comparative genomic hybridization reveals recurrent chromosomal aberrations and Jab1 as a potential target for 8q gain in hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is the most common type of liver cancer, and the fourth leading cause of cancer death worldwide (1, 2). Development of HCC is a multiple step event (3). Hepatitis B and C infections are the most important risk factors for HCC (4, 5). While changes in expression and mutations in several oncogenes or tumor suppressor genes have been implicated in HCC development (6), the molecular pathways and genetics of HCC evolution are still poorly defined.

Chromosomal amplifications and deletions are commonly seen in tumors. This allows for the selection of genetic traits that confer a growth advantage for tumor progression, and thereby contributes to tumorigenesis (7, 8). Comparative Genomic Hybridization (CGH) was developed to monitor the DNA copy number changes on a genomic scale at the resolution of ~20 Mb (9). Using traditional and array CGH, frequent DNA copy number gains at 1q, 8q, and 20q, and frequent DNA copy number losses at 1p, 4q, 8p, 13q, 16q, and 17p have been identified in HCC samples (10–14). While some of these regions contain known candidate oncogenes or tumor suppressor genes, for example ZNF217 (20q13) and RB1 (13q14), the relevant genes within other regions remain to be identified.

Array-based CGH is a newly developed technology that allows for high throughput and high resolution (at 1 Mb) screening of genome-wide DNA copy number changes in tumors. This can be achieved using BAC clones or cDNA clones printed on arrays (15, 16). Array CGH has been applied to a number of solid tumors including breast, gastric, kidney and bladder cancers (17–20). It has been shown to be useful in the identification of novel oncogenes and tumor suppressor genes, as well as in differential diagnosis of tumors.

To study the molecular genetics of HCC, we previously reported the global gene expression patterns of over 200 HCC and non-tumor liver samples using microarrays with > 23 000 cDNA clones (21). The study demonstrated the distinct gene expression programs associated with neoplastic and non-neoplastic liver tissue, as well as the heterogeneities of expression profiles among HCC tissues. In addition, we identified 703 unique genes that are highly expressed in HCC (22). These genes therefore represent novel candidate oncogenes, as well as potential diagnostic markers and treatment targets for HCC.

In this paper, we report the study of chromosomal aberrations in HCC using array CGH, and correlation between array CGH and expression arrays with the goal of demonstrating that combining expression arrays and CGH arrays can be a powerful method to identify novel candidate oncogenes and tumor suppressors.

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Materials and methods

Tissue samples, cell lines, DNA and RNA preparation

Samples of tumor and non-tumor liver tissues were collected from liver resections carried out at Stanford University and The University of Hong Kong. Tissue samples were stored in liquid nitrogen at –80 °C until testing.
resected. All sample blocks were revealed by two pathologists to contain >80% tumor cells. Five liver cancer cell lines HepG2, Hep3B, SNU449, SNU475 and SNU423 were purchased from ATCC (Rockville, MD) and maintained in media specified by ATCC plus 10% fetal bovine serum (FBS). Genomic DNA was extracted using the Genomic DNA purification Kit (Qiagen, Valencia, CA). Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA). This study was approved by the Ethics Committee of the University of Hong Kong and the Internal Review Boards of UCSF and Stanford University.

**CGH arrays**

The arrays used in the study were prepared and hybridized as described previously (20). In brief, human 1.14 arrays were obtained from the UCSF Cancer Center Array Core (http://cc.ucsf.edu/microarray/). The arrays consisted of 2433 bacterial artificial chromosome (BAC) clones that covered the human genome at 1.5 Mb resolution. For hybridization, 1 μg of tumor DNA and 1 μg of gender matched reference DNA (isolated from normal donor lymphocytes) was labeled by random priming using Cy3-dCTP and Cy5-dCTP, respectively, using Bioprime Kit (Invitrogen). Unincorporated fluoroscent nucleotides were removed using a Sephadex G-50 column (Amersham, Piscataway, NJ). Sample and reference DNA were mixed with 100 μg Cot-1, precipitated and resuspended in hybridization solution. The hybridization solution was denatured for 10 min at 72°C before being incubated for 1 h at 37°C to allow blocking of repetitive sequences. Hybridization was performed for 48–72 h in a moist chamber on a slow rocking table. The arrays were washed for 10 min in 50% formamide and 2× SSC at 45°C, and 10 min in phosphate buffer at room temperature. Slides were mounted in mounting solutions containing 0.3 μg/ml DAPI. Three single-color intensity images (DAPI, Cy3 and Cy5) were collected for each array using a charge coupled device camera.

**CGH array data analysis**

The UCSF SPOT software (23) (http://gainlab.ucsf.edu/Downloads.html) was used to automatically segment the spots based on the DAPI images, perform local background correction and calculate various measurement parameters, including log2 ratios of the total integrated Cy3 and Cy5 intensities for each spot. A second custom program SPROC (http://gainlab.ucsf.edu/Downloads.html) was used to associate clone identities and a mapping information file with each spot, so that the data could be plotted relative to the position of the BACs in the human genome. Chromosomal aberrations were classified as a gain when the normalized log2 Cy3/Cy5 ratio was >0.225 and as a loss when the ratio was <-0.225. This number was determined as 3-fold of the average SD of normal versus normal array CGH hybridization. Steep copy number changes with the graph showing a peak rather than a plateau, and a minimal normalized log2 Cy3/Cy5 ratio of 0.9 and higher were classified as amplifications. Likewise, log2(Cy3/Cy5) ratio of ≥0.8 and lower were classified as homozygous deletions. Multiple gains, losses and amplifications were counted as separate events. The threshold of gain or loss of an entire chromosome arm was defined as the median log2 ratio of >0.225 or <-0.225 for all clones on the chromosome arm. Correlation between DNA copy number changes and clinical parameters was calculated using significant analysis of microarray (SAM) analysis (24).

**Real-time RT–PCR**

Quantitative RT–PCR was performed as described (25). In brief, total RNA was further digested by DNase I (Ambion, Austin, TX) to remove any genomic DNA contamination. Human 18s rRNA primer and probe reagents (Applied Biosystems, Foster City, CA) were used as the normalization control in subsequent quantitative analysis. Quantification was performed using the ABI Prism® 7000HT Sequence Detection System via a two-step non-multiplexed Taqman® 5′ → 3′ exonuclease assay, according to the relative standard method. Transcript quantification was performed in triplicate for each sample and reported relative to RNA. The primers and probe used for Jab1 in this study were obtained from ABI assay on demand system (Applied Biosystems).

**Quantitative microsatellite analysis**

Quantitative microsatellite analysis (QuMa) was performed to measure Jab1 DNA copy numbers. The microsatellite markers used for the reference pool included D11S1315, D12S1699, D31S1283, D31S1286, D21S1904 and D25S185. According to our recent and other published CGH analysis of HCC samples and cell lines, these markers are located at chromosomal regions that rarely show alterations (10–13). All primer pairs were tested for PCR efficiency individually and all demonstrated >90% efficiency. Microsatellite within the Jab1 intron was identified using the UCSC genomic DNA database. All primer sequences used in the study are available in Supplemental Table I. QuMa was performed as described (26). Assays were carried out using the software supplied with the ABI 7900 (Applied Biosystems). Copy numbers of three blood samples from healthy donors were averaged and normalized to two, which was subsequently used to normalize the copy numbers of HCC samples.

**Tissue microarrays and immunohistochemistry**

Archival tissue samples of hepatic tumors were obtained from the Universities of Bonn and Cologne, Germany (1995–2004) and consisted of human partial and total hepatectomy as well as liver biopsies. A tissue-microarray (TMA) was constructed from paraffin embedded formalin fixed tissue of hepatic tumors and tumor-like lesions as well as normal and cirrhotic liver tissue (n = 200) using a manual Tissue Puncher/Arrayer (Beecher Instruments, Silver Spring, MD), as previously described (27). Amongst the samples there were 119 cases of hepatocellular carcinoma (n = 119), Duplicate 1 mm cores were obtained for all tumor samples except for dysplastic nodules (single 0.5 mm core). Unstained 5-μm sections were cut from the paraffin block and deparaffinized by routine techniques. The TMAs were placed in 200 ml of target retrieval solution, pH 6.0, for 20 min at 100°C. After cooling for 20 min, slides were quenched with 3% H2O2 for 5 min and incubated with anti-Jab1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA; 1:750) using the Dako Autostainer, and developed using EnVision Kit (Dako, Carpinetaria, CA). Scoring of tumor cells was determined positive if >50% of tumor cells stained in a nuclear pattern.

**Cell assays**

The human HCC cell line Hep3B was maintained in DMEM plus 10% FCS. For Jab1 knock-down experiments, siRNA against Jab1 was generated as described previously (28) and transfected into Hep3B cells using Oligofectamine (Invitrogen), according to the manufacturer’s instructions. Cells were harvested 48 h post-transfection. Western blots were used to analyze the expression of Jab1 as described previously (29) and the WST-1 assay (Roche, Indianapolis, IN) was used to analyze the cell growth rate, according to the manufacturer’s instructions. For Jab1 over-expression experiments, HA-Jab1/pcDNA3.1 was kindly provided by Dr S.J.Kim of NCI. pcDNA3.1 and HA-Jab1/pcDNA3.1 were transfected into Hep3B cells and stable cell lines were selected using G-418. A total of 50 000 cells were seeded in 96-well plates and grown in normal serum (10%) or low serum (0.5%) media. Cells were harvested every 2 days for 7 days, and viable cells were counted by trypan blue exclusion assay.

**Results**

**Array-based CGH in hepatocellular carcinoma**

We applied BAC array-based CGH to study chromosomal DNA copy number variations in 44 HCCs and 5 HCC cell lines. The raw data are available in Supplemental Table 2. The resulting CGH maps of a representative HCC sample are shown in Figure 1.

Using array-based CGH we found that an average of 673 clones were gained or lost in HCC DNA samples, representing 27.7% of the genome. This includes 321 clones representing 13.2% of the entire genome gained and 352 clones representing 14.5% of the genome lost.

Interestingly, we found that recurrent high level genomic DNA amplifications and homozygous deletions are relatively rare in HCC. Among 2433 BAC clones printed on the arrays only 17 BAC clones (0.7% of total clones) showed amplification (defined as log2 ratio >0.9) in at least three HCC samples and 22 BAC clones (0.8% of total clones) showed homozygous deletion (defined log2 ratio <=-0.8) in at least three HCC samples. The information of most frequently amplified or deleted clones are summarized in Supplemental Table 3.

In the HCC samples (Figure 2). The percentage of chromosomal arm gains and losses are listed in Table I. In addition, we have also listed the percentage of DNA copy number gain and loss separately for 44 HCCs and 5 liver cancer cell lines.
separately in Supplemental Table 3. Most common DNA copy number variations included gains of 1q (59%), 6p (25%), 8q (39%) and 20q (31%), and losses of 4q (43%), 8p (41%), 13q (37%), 16q (43%) and 17p (49%). Importantly, 46 of the 49 HCC samples assayed in this study harbored at least one of these common DNA copy number gains or losses. The information of most frequently gained or lost clones are summarized in Supplemental Table 4.

Next, we correlated the array CGH data with clinicopathological features of HCC, including tumor size, tumor stage, venous invasion, p53 immunohistochemistry and beta-catenin mutation status. Overall, we did not find any BAC clones that revealed statistically significant association with these genetic and clinical parameters.

Correlation of array CGH with gene expression array data at 8q region

In our previous study using cDNA microarrays, we characterized gene expression patterns of 82 HCC and 74 non-tumor liver samples, and identified 703 genes that were highly expressed in HCC (22). Microarray data were obtained from 82 HCC samples, of which array CGH data were analyzed for 44 samples. Using both sets of data obtained for the 44 HCC samples, gene expression was quantitatively correlated with corresponding DNA copy number variations on a case by case basis.

As the first step of our analysis, we decided to focus on chromosomal 8q gain, one of the most commonly gained (39%) regions in HCC. We reasoned that because of the high

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Fig. 1. Representative array-based CGH profile from one HCC tumor DNA. The profile shows copy number changes relative to normal, sex matched DNA ordered by chromosome. Green dots represent clones demonstrating chromosomal gains (log2 ratio > 0.225), white dots demonstrate clones demonstrating chromosomal losses (log2 ratio < -0.225). Open dots represent excluded data point due to large SD (> 0.3) between three duplicated spots printed on the arrays. Note that this sample shows the following DNA copy number variations: +1q, +2, +4p, +5p, +7, +15q, +17q, +20q, +Xq, -4q, -6q, -11q, -13q and -17p. In addition, it also has amplification at 11q13.

Fig. 2. Overall frequency of DNA copy number alterations by array-based CGH. Frequency analysis measured as a fraction of cases gained or lost over all the BAC clones on the arrays. Data presented are ordered by chromosomal map position of the clones. Lower black bars represent losses or deletions, and the upper grey bars represent gains or amplifications. The dashed lines represent the boundary between each chromosome.

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frequency of 8q gain this region is likely to harbor oncogenes important for HCC development. In addition, gain of 8q has been shown to be associated with large tumor size, and may be important during HCC progression (32,33).

Among the 703 genes that are highly expressed in HCC, 48 mapped to 8q. For each of these 48 genes, correlation between the gene expression levels and DNA copy number (estimated using the closest BAC clone or average of the two closest BAC clones within 2 Mb of each gene) was calculated in the 44 HCC samples. The correlation ranged from $r = 0.32$ in the case of FLJ20421 to 0.61 in the case of PGCP (Supplemental Table 5).

To further search for genes that may be candidate targets for 8q gain in HCC, the genes that showed statistically significant correlation between DNA copy number and RNA expression (in this case, we used cutoff $r > 0.35$) were then each examined in detail for known biological function, according to currently available information from the medical literature. This analysis brought our attention to the cDNA clone corresponding to human Jab1. Jab1 is also known as CSN5 (COP9 signalosome subunit 5), and has been reported to bind and promote the degradation of p27Kip1. High expression of Jab1 has been associated with low levels of p27Kip1 and poor prognosis in multiple tumor types, indicating that Jab1 may play an important role in tumor progression. Because of the high correlation between Jab1 expression and DNA copy number gain ($r = 0.5$) and its possible function in tumorigenesis, we decided to further characterize Jab1 in HCC development.

**Jab1 DNA copy number correlation with Jab1 expression in HCC samples**

Jab1 is located at the 68.0 Mb position at chromosome 8q according to the UCSC July 2003 freeze. Data from two BAC clones on CGH arrays, RP11-258B14, located at 69.3 Mb and CTD-2169M23, located at 69.7 Mb, were used to estimate the DNA copy number variations for Jab1. There was a statistically significant correlation between Jab1 expression (from microarray data) and its DNA copy number gain ($r = 0.50$ and $P < 0.01$) (Figure 3A).

To further demonstrate the DNA copy number gains of the Jab1 locus in HCC, real-time PCR-based QuMA was performed using CA repeats within the intron of Jab1 as a probe. QuMA has been shown to be a reliable and sensitive method for detecting single copy number changes at individual gene loci in tumor samples (26,34,35). QuMA was performed in 38 HCC samples. Of these, 23 HCCs (60%) showed at least one copy gain (copy number $\geq 3$) and 6 HCCs (16%) showed high level of amplification at the Jab1 locus (copy number $> 5$). We found a strong correlation ($r = 0.75$) between QuMA and CGH results (Figure 3B). Jab1 DNA copy number gains revealed by QuMA also showed statistically significant correlation with Jab1 expression levels ($r = 0.46$, $P < 0.05$) in the 38 HCC samples that were tested (Figure 3C). The results therefore demonstrate that the DNA copy number gain at the Jab1 locus may be an important factor in regulating its high expression in HCC.

**Table I.** Frequency of chromosomal arm gains or losses in 49 HCC samples and cell lines assayed by BAC array-based CGH

| Chromosome | Gain (%) | Loss (%) | Chromosome | Gain (%) | Loss (%) |
|------------|----------|----------|------------|----------|----------|
| 1p         | 2.0      | 10.2     | 11p        | 2.0      | 10.2     |
| 1q         | **59.2** | 0.0      | 11q        | 4.1      | 16.3     |
| 2p         | 10.2     | 2.0      | 12p        | 2.0      | 8.2      |
| 2q         | 10.2     | 2.0      | 12q        | 6.1      | 0.0      |
| 3p         | 4.1      | 4.1      | 13q        | 2.0      | **36.7** |
| 3q         | 4.1      | 4.1      | 14q        | 2.0      | 20.4     |
| 4p         | 10.2     | 16.3     | 15q        | 4.1      | 12.2     |
| 4q         | 0.0      | **42.9** | 16p        | 6.1      | 20.4     |
| 5p         | 10.2     | 2.0      | 16q        | 4.1      | **42.9** |
| 5q         | 6.1      | 6.1      | 17p        | 2.0      | **49.0** |
| 6p         | 24.5     | 0.0      | 17q        | 22.4     | 0.0      |
| 6q         | 4.1      | 20.4     | 18p        | 4.1      | 8.2      |
| 7p         | 16.3     | 2.0      | 18q        | 2.0      | 18.4     |
| 7q         | 16.3     | 0.0      | 19p        | 2.0      | 8.2      |
| 8p         | 2.0      | **40.8** | 19q        | 12.2     | 6.1      |
| 8q         | **38.8** | 2.0      | 20p        | 18.4     | 4.1      |
| 9p         | 0.0      | 20.4     | 20q        | **30.6** | 0.0      |
| 9q         | 2.0      | 6.1      | 21q        | 0.0      | 10.2     |
| 10p        | 14.3     | 4.1      | 22q        | 4.1      | 8.2      |
| 10q        | 10.2     | 18.4     | Xp         | 8.2      | 12.2     |
|            |          |          | Xq         | 18.4     | 4.1      |

Frequent chromosomal gains or losses (>24%) are given in bold.
Jab1 RNA expression in non-tumor liver and HCC samples

From microarray studies of 82 HCCs and 74 non-tumor liver tissues, we found that Jab1 was highly expressed in HCC ($P = 3.7 \times 10^{-11}$, and an average 1.67-fold increased expression in HCC) (Figure 4A). This difference was highly significant even after we adjusted for multiple hypothesis testing in microarrays analyzed using Bonferroni correction ($P = 1.8 \times 10^{-7}$). Among the 56 patients who had both HCC and adjacent non-tumor samples analyzed by cDNA microarray, the median ratio for Jab1 expression in HCC versus non-tumor liver was 1.51.

To validate this observation, total RNA was extracted from an independent set of 28 HCC and 28 adjacent HCC samples (none of them were used in the microarray study). The expression level of Jab1 was quantified using Taqman real-time quantitative RT–PCR. The expression level of Jab1 was significantly higher in HCC than adjacent non-tumor liver tissues. This was consistent with the microarray study ($P < 0.001$) (Figure 4B). The medium ratio of paired HCC/non-tumor liver was 4.2. A total of 19 out of 28 pairs (67.9%) showed Jab1 expression, at least twice as high in HCCs than in non-tumor hepatic tissue. In conclusion, the data demonstrate that Jab1 is highly over-expressed in a subset of HCC samples.

Jab1 expression in HCC by immunohistochemistry

To study Jab1 expression at protein levels, we performed immunohistochemical staining of Jab1 on tissue microarrays in 119 HCC samples. Immunostaining in a nuclear pattern was seen in 39.5% of HCCs. Weak cytoplasmic staining or complete negative results were seen in 60.5% of HCCs (Figure 5B and D). Weak nuclear staining of reactive bile ducts served as an internal control in normal and cirrhotic livers. Normal hepatocytes did not show Jab1 expression (Figure 5F).

Jab1 function in HCC cell proliferation

Previous studies suggest that Jab1 is able to bind to p27Kip1 and promote its degradation, thereby regulating cell growth (36,37). We therefore investigated whether expression of Jab1 regulates HCC cell growth. Human HCC cell line Hep3B was chosen for the study as it does not appear to have gain or loss at the Jab1 locus.

First, Jab1 expression was inhibited using Jab1 specific siRNA and it was found that Jab1 protein expression levels could be effectively repressed using Jab1 siRNA. Cell proliferation assay demonstrated that inhibition of Jab1 expression inhibited Hep3B cell growth (Figure 6A). Next, stable Hep3B cells were transfected with a control plasmid (pcDNA3.1) or with a Jab1 expression plasmid (HA-Jab1/pcDNA3.1).

Discussion

During the past decade, CGH has been widely used to study chromosomal aberrations in human tumors. To date, there have been at least 20 reports on the application of traditional CGH to HCC (38). It is difficult to pinpoint specific candidate genes within large genome regions that may be important for tumorigenesis by traditional CGH due to its low resolution.
Functional studies of number variations and etiological background. For the future, we will need to use a larger sample set with positive. Therefore, it is difficult to correlate any chromosomal aberrations that might be associated with underlying etiology. Of the HCC samples used in this study, ~44% are HBV positive. Therefore, it is difficult to correlate any chromosomal gain or loss that is related to a specific etiological background. For the future, we will need to use a larger sample set with a variety of etiological factors, especially HCV positive samples, to make an accurate correlation between DNA copy number gains or losses. In addition, it will be important to determine any chromosomal alterations that might be associated with underlying etiology. Of the HCC samples used in this study, >80% are HBV positive. Therefore, it is difficult to correlate any chromosomal gain or loss that is related to a specific etiological background. For the future, we will need to use a larger sample set with a variety of etiological factors, especially HCV positive samples, to make an accurate correlation between DNA copy number variations and etiological background.

While in this report we focus our analysis on 8q gain and functional studies of Jab1 in HCC development, there are many other genes along commonly altered chromosomal regions whose expression levels are correlated with corresponding DNA copy number gains or losses. In addition, there are other genes on 8q that are highly expressed in HCC and show high correlation between DNA copy number and expression levels in HCC samples, especially those genes located at 8q24, which show high frequency of amplification in HCC (Figure 2 and Supplemental Table 5). One such gene is 14-3-3 zeta, a gene that has been implicated in promoting cell survival. These genes may therefore also represent candidate oncogenes at 8q in HCC. Clearly, each of these genes will require thorough analysis for their potential functions in HCC oncogenesis. Overall, the combination of array CGH and gene expression data analysis followed by validation and a functionally-guided approach is a powerful method for gene discovery from genome-wide array based studies.

Jab1/CSN5 is the fifth component of the COP9 signalosome, a highly conserved protein complex that has been shown to interact and act as a positive regulator of SCF-type E3 ubiquitin ligases (39,40). Jab1 functions by interacting with a number of diverse proteins, including c-Jun, MIF1, integrin LFA-1, progesterone receptor, SRC-1, lutropin receptor, psoriasin, Smad4 and Bcl-3 (29,37,41–46). One of the most exciting links between Jab1 and cancer development is the fact that Jab1 interacts and promotes the degradation of p27Kip1, a negative regulator of cell cycle progression (36,37). Overexpression of Jab1 has been reported in breast cancer, ovarian cancer, pancreatic cancer, embryonal rhabdomyosarcoma and oral squamous cell carcinomas (47–51). In addition, high expression of Jab1 has been associated with low levels of p27Kip1 and poor prognosis, indicating that Jab1 may play an important role in the progression of these tumor types. However, the functional significance of Jab1 in HCC pathogenesis has not yet been characterized, and there is no report on how Jab1 expression is regulated in cancer cells.

In this study, we demonstrated that the high expression of Jab1 in HCC significantly correlates with DNA copy number gain at the Jab1 locus on chromosome 8q. This is the first study to show the possible regulatory mechanism of Jab1 overexpression in tumor cells. We noticed that there were HCC samples with high expression of Jab1 but no DNA copy number gains. This suggests that Jab1 gene expression can be regulated by multiple mechanisms, of which DNA copy
number gain is one. The functional assays demonstrated that knockdown of Jab1 expression inhibits HCC cell growth, whereas overexpression of Jab1 promotes cell growth in low serum condition. The results are consistent with other reports that ectopic expression of Jab1 in mouse fibroblasts markedly reduces cell dependence on serum (37). The data support the hypothesis that Jab1 is a novel candidate oncogene for HCC. Further analysis will be required to illustrate Jab1 function in HCC. For example, a negative correlation was observed between Jab1 and p27kip1 expression in HCC tumors and cell lines. The correlation between Jab1 and p27kip1, as well as correlation between Jab1 expression and clinical pathological parameters of HCC will be reported separately (I.Gittegeman, unpublished data).

In conclusion, our study has shown that array-based CGH provides high resolution mapping of chromosomal aberrations in HCC, and demonstrates the feasibility of correlating array CGH data with gene expression data to identify novel oncogenes and tumor suppressor genes.

Supplementary material
Supplementary material is available at: http://www.carcin.oxfordjournals.org/
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