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Regional over-representations on chromosomes 1q, 3q and 7q in the progression of hepatitis B virus-related hepatocellular carcinoma

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Hepatocellular carcinoma is a highly malignant tumor that is prevalent in Southeast Asia and China, where hepatitis B viral infection is the main etiologic factor. Despite a high incidence of hepatocellular carcinoma developing in patients with viral hepatitis B-induced liver cirrhosis, the molecular events underlying the malignant liver progression remain largely unclear. In an effort to characterize the genetic abnormalities involved in the hepatitis B-related liver carcinogenesis, we performed genome-wide explorations by the technique of comparative genomic hybridization (CGH) on 100 hepatocellular carcinoma tumors that arose from hepatitis B-induced liver cirrhosis. According to the American Joint Committee on Cancer staging, four cases were classified as stage I, 69 as stage II, 23 as stage III and four as stage IV. CGH analysis indicated chromosomal instability in both early (stages I/II) and advanced (stages III/IV) stage tumors, with common gains on 1q, 8q and 17q23–q25, and losses on 4q22–q35, 8p21–p22, 13q14–q21, 16q and 17p identified in both groups ($P > 0.05$). Nevertheless, preferential sites of chromosomal defects in relation to hepatocellular carcinoma progression were also identified. Statistical correlations suggested a higher incidence of regional 1q21–q22, 3q22–q28, 7q21–q22 and 7q34–q36 over-representations in association with the advanced stage tumors ($P < 0.05$). In this study, our novel identification of specific chromosomal aberrations in relation to the advanced stage tumors may represent a first step towards mapping genes linked to the progression of hepatocellular carcinoma.
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Hepatocellular carcinoma is a primary liver malignancy that is highly malignant and rapidly fatal. The dismal clinical outcome is largely due to the majority of hepatocellular carcinoma tumors being asymptomatic during the natural course of the disease, consequently rendering most patients not being diagnosed in time for curative surgery.[1] By the time of clinical presentation, intra- and extrahepatic metastases are also common, which in turn has limited the scope of curative surgery.[2] In addition, the relatively high incidences of hepatocellular carcinoma recurrence, possibly from micrometastasis prior to surgical treatment, has further lowered the 5-year survival rate for individuals diagnosed with hepatocellular carcinoma.[3] The understanding of molecular events involved in the malignant liver progression thus would hold much value in the prognosis for patients with hepatocellular carcinoma.

Studies on the molecular pathogenesis have guided the development of gene-based biomarkers in a number of human cancers including breast, colon, prostate and lung.[4] In hepatocellular carcinoma, although genetic characterizations have indicated frequent chromosomal over-representations on 1q, 6p, 8q, 17q and 20q, and deletions on 1p, 4q, 8p, 13q, 16q and 17p,[5–11] emphasis on the molecular

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changes that underlie the tumor progression has been somewhat limited. This is mainly because the genomic basis of hepatocellular carcinoma is considered heterogeneous owing to its mixed background of histological precursors, namely cirrhosis and chronic hepatitis, and the different actions of causative agents such as viral hepatitis types B or C inductions. Nevertheless, the main etiologic factor associated is chronic hepatitis B virus infection, where hepatitis B virus-induced liver cirrhosis is considered to be a strong predisposing factor in the development of hepatocellular carcinoma. While our group has attempted to evaluate the genetic changes involved in the progression of hepatocellular carcinoma, our previous study did not suggest progressive alterations in relation to disease staging.[12] The absence of correlative changes might have been due to the utilization of conventional T staging, which was probably less appropriate in hepatocellular carcinoma than other malignant tumors, since the prognosis of hepatocellular carcinoma is related to the state of underlying liver disease as much as to the tumor itself. In this study, we have revised our approach to examine a homogeneous series of cirrhotic hepatocellular carcinoma tumors derived from chronic hepatitis B virus-infected patients, and adopted the American Joint Committee on Cancer Classification (AJCC) in tumor staging. While most of our cases accrued represented early resectable (stages I/II) tumors, we have also managed to secure a considerable series of more advanced (stages III/IV) cases, of which curative surgery can still be offered. By expanding our previous series to a larger cohort of patients, we have been able to identify in this study a number of previously undescribed genetic alterations that participated in the progression of hepatocellular carcinoma.

Table 1 Clinicopathological information on 100 hepatocellular carcinoma

	Early stage HCC	*Late stage HCC*	P-*value*
Gender			0.730
Male	65 (89.0%)	23 (85.2%)	
Female	8 (11.0%)	4 (14.8%)	
Age			0.972
Median (range)	56 (30–76)	57 (37–70)	
AFP (ng/ml)			0.345
Median (range)	46.5 (<10–59 200)	197.0 (<10–14 600)	
HbsAg			
Yes	73 (100.0%)	27 (100.0%)	
No	0 (0%)	0 (0%)	
Cirrhosis			
Yes	73 (100.0%)	27 (100.0%)	
No	0 (0%)	0 (0%)	
Tumor encapsulation[a]			0.533
Yes	44 (80.0%)	21 (87.5%)	
No	11 (20.0%)	3 (12.5%)	
AJCC staging			
Stage I	4 (5.5%)	—	
Stage II	69 (94.5%)	—	
Stage III	—	23 (85.2%)	
Stage IV	—	4 (14.8%)	
Multinodular presentation[a]			<0.0005
Yes	9 (15.5%)	23 (92.0%)	
No	49 (84.5%)	2 (8.0%)	
Vascular invasion[a]			0.035
Yes	5 (9.1%)	7 (26.9%)	
No	50 (90.9%)	19 (73.1%)	

[a]Applicable to cases with available information.

Materials and methods

Patients

Tumorous liver tissues were collected from 100 patients who underwent curative surgery for primary liver malignancy at the Prince of Wales Hospital, Hong Kong. Serological analysis indicated the presence of hepatitis B virus surface antigen in all cases. Diagnosis of hepatocellular carcinoma was confirmed by an experience pathologist (KF To) who also confirmed the presence of underlying liver cirrhosis. Among these 100 cases, patients' age ranged from 30–76 years (median age 56.5 years) with a male to female ratio of 5.67 (88 males and 12 females). Clinicopathological information of patients was summarized in Table 1.

The AJCC tumor–node–metastasis staging system was utilized in tumor grading of cases recruited.[13] Staging criteria included the pathologic characteristics of tumor size at greater or less than 2 cm, number and location of tumor nodules, presence of vascular invasion, perforation of visceral peritoneum and invasion of adjacent organs. According to these criteria, four out of the 100 hepatitis B virus-infected tumors collected were classified as stage I, 69 as stage II, 23 as stage III and four as stage IV. Stage I and II tumors were grouped as early stage tumors, and stage III and IV tumors were grouped as advanced stage tumors.

Comparative Genomic Hybridization

The CGH protocol was carried out according to the method of Kallioniemi *et al*[14] with modifications previously described from our laboratory.[12] Briefly, differentially labeled tumor and normal DNA with biotin-16-dUTP (Boehringer Mannheim, Mannheim, Germany) and digoxigenin (dig)-11-dUTP (Boehringer Mannheim) were cohybridized onto normal metaphase chromosomes. Following hybridization, biotin signals were detected through avidin-conjugated-FITC antibodies (Sigma, St Louis, USA), and

dig-labeled DNA visualized by TRITC conjugated antibodies (Sigma). Chromosomes counterstained with DAPI were captured through a cooled CCD camera mounted on a Leitz DM RB (Leica, Wetzlar, Germany) fluorescence microscope. Three band pass filter sets (4′,6-diamidino-2-phenylindole (DAPI), fluorescein isothiocyanate (FITC), tetramethylrhodamine isothiocyanate (TRITC)) arranged in an automated filter-wheel were employed for image acquisition. The CGH software ver3.1 on Cytovision (Applied Imaging Ltd., Sunderland, UK) was used in the digital image analysis of fluorescence intensities. The average ratio profiles of 10–12 metaphases were calculated based on chromosome identification of the computer-generated inverted DAPI images. Thresholds for gains and losses were defined as the theoretical value of 1.25 and 0.75, respectively. High-level gains of chromosome arm or regional amplifications were considered when ratios exceeded 1.5.

Statistical Analysis

Clinicopathological factors were assessed by Mann–Whitney test and χ^2 test between early and advanced stage hepatocellular carcinoma. Total number of aberrations per tumor, including numbers of copy gains, losses and amplifications, were compared using Student's *t*-test to categorize potential differences between specific groups. To facilitate statistical analysis, conventional CGH interpretation was recoded as discrete numerics. The nomenclature adopted for each chromosome was according to the ISCN nomenclature.[15] Regions rich in heterochromatin were excluded from our analysis. The genomic alterations, that is, copy number gains, copy number losses and balanced regions were recoded as 1, −1 and 0 respectively. In this regard, CGH interpretation was assessed in a continuous manner across the remaining 322 cytobands that spanned the 24 chromosomes (22 autosomes, and sex chromosomes X and Y). Comparison on genomic alterations between early and advanced stage tumors was performed with 2×2 contingency tables constructed between tumor stage and the presence or absence of genomic alteration on each genomic locus. χ^2 statistic was used to determine *P*-values. A difference was considered significant when *P*-value was less than 0.05. All statistical analyses were performed using SPSS for Windows 10.0 software (SPSS Inc., Chicago, USA).

Results

The overall pattern of genomic changes in the 100 cirrhotic hepatocellular carcinoma examined suggested common gains on 1q (66%), 6p (26%), 7q (32%), 8q (44%), 17q (29%) and 20q (25%), and losses on 4q (33%), 8p (32%), 13q (32%), 16q (34%) and 17p (37%). Examples of common genetic alteration observed in hepatocellular carcinoma are shown in Figure 1.

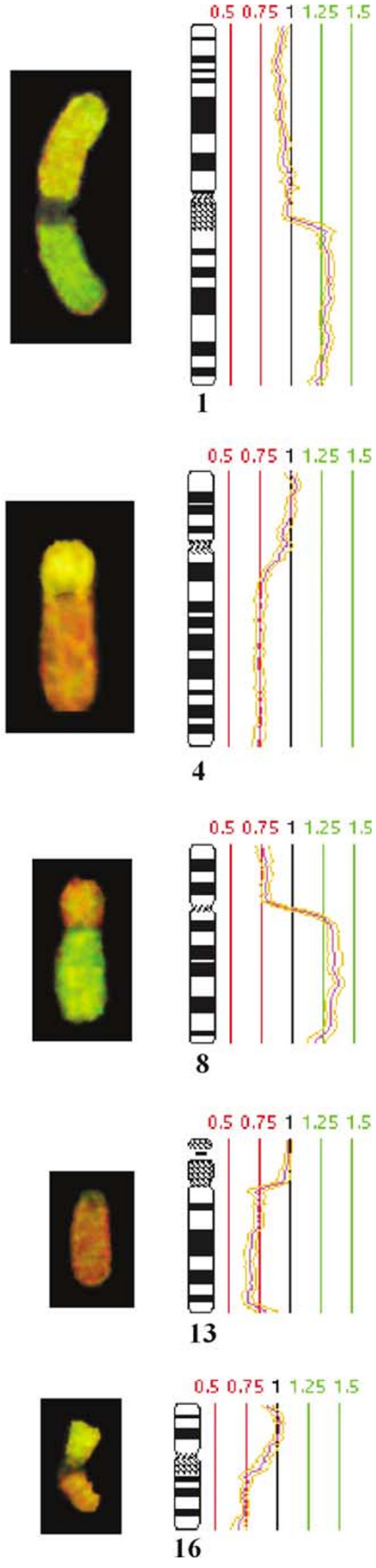
Since similar 5-year survivals had been suggested in hepatocellular carcinoma patients with stage I and II tumors, and that the survival of patients with multiple tumors from stage III matched that of stage IV patients,[16] we have hence examined the genomic alterations in the 100 cirrhotic hepatocellular carcinoma by grouping cases of stage I and II as early stage tumors (totaling 73 cases) and compared findings to those derived from stage III and IV as advanced stage tumors (totaling 27 cases). No difference in clinical factors such as age, gender and pathologic factors, including serum alpha-fetoprotein (AFP) level, and tumor encapsulation was observed with the above stratification (Table 1). Available information on vascular invasion and multinodular presentation suggested significant difference between the early and advanced stage hepatocellular carcinoma, which was in concordant with the AJCC staging criteria for stage III and IV tumors.

With the aid of dissecting chromosomal region in individual locus, copy number alteration along the chromosome arms was better defined. Recurrent smallest overlapping region (SOR) defined to +1q21–q22, −4q22–q32, +6p22–p11.2, +7q21–q22, −8p21–p22, +8q21.2–q22, −13q14–q21, −16q23–q24, −17p13, +17q23–q25, +20q12–q13 and +Xq23–q26 were suggested in both early and advanced stage tumors. The frequency of copy number aberrations detected in early stage and advanced stage tumors are summarized in Figure 2. In general, copy number alterations per tumor were more apparent in advanced stage tumors, although statistical correlation did not suggest significant differences (early stage, 9.52 ± 6.43; advanced stage, 11.26 ± 7.3; $P = 0.250$). Such finding was also extended to the derived gains (4.14 ± 2.95; 5.67 ± 4.02; $P = 0.080$) and losses (4.08 ± 3.48; 4.22 ± 3.52; $P = 0.859$).

While the average copy number aberrations per tumor did not reveal progressive changes with disease advancements, comparison on alteration by individual chromosomal regions between early and advanced stage hepatocellular carcinoma highlighted four regional alterations for their association with advanced stage tumors (Table 2). χ^2 statistics showed that recurring regional gains of 1q21–q22 ($P = 0.030$), 3q22–q28 ($P = 0.003$), 7q21–q22 ($P = 0.035$) and 7q34–q36 ($P = 0.010$) were considerably more frequent in advanced stage tumors.

Discussion

Examination of genomic aberrations in early stage tumors is considered important in defining the early events underlying tumor pathogenesis. CGH characterizations of 73 early stage hepatocellular carcinoma indicated common changes of gains on 1q, 8q



and 17q, and deletions on 4q, 8p, 13q, 16q and 17p (Figure 2). It is also well recognized that genetic alterations of functional relevance to tumor development will be inherited in tumor cells. In line with this principle, it appears from our CGH study that the common changes identified in the early tumors were also detected, at equally high incidences, in the advanced stage hepatocellular carcinoma, thus implying a role for these anomalies in the early pathogenic events. Allelic losses on 4q, 8p, 13q and 16q have been previously described in the cirrhotic nodules and early preneoplastic liver dysplasia. This further underscores the early nature of these changes in the development of hepatocellular carcinoma.[17–19]

In the 100 cirrhotic hepatocellular carcinoma studied, although the overall number of copy aberrations was found similar between early and advanced stage tumors, distinct genetic changes, in particular the extension of small interstitial gains, were indicated in tumor progression. Furthermore, based on the pathologic features found, our series of advanced stage tumors displayed frequent multiple lesions (~90% of cases), a feature that represents an important criterion in the classification. Early stage tumors, on the other hands, were mostly solitary tumors (~80% of cases). Molecular characterizations of multiple hepatocellular carcinoma nodules from individual patients by hepatitis B virus integration pattern, X chromosome inactivation, CGH and expression profiling have all demonstrated a strong clonal relationship among the intrahepatic metastatic nodules in the majority of patients.[20–22] Furthermore, recent expression array analysis has indicated that the molecular signatures of metastasis in human cancers could in fact be detectable in the bulk of a primary tumor.[23] Thus, genomic aberrations detected in the major lesion of advanced cirrhotic hepatocellular carcinoma tumors may be indicative not only for changes conferring more malignant characteristics but also may provide a means of identifying patients at risk for tumor dissemination.

The whole arm gain of 1q in advanced stage hepatocellular carcinoma was frequently associated with a regional amplification of 1q21–q22 ($P = 0.03$). Apart from its participation in hepatocellular carcinoma, gains of 1q21–q22 were also shown related to the progression of other human cancers. The presence of 1q21–q22 amplicon in metastatic lesions

Figure 1 Common chromosomal imbalances in hepatocellular carcinoma. Genomic imbalances of chromosomes 1q and 8q gains, and 4q, 8p, 13q and 16q losses were common in hepatocellular carcinoma. CGH images of hybridized chromosomes are shown with the corresponding fluorescence ratio profile plotted alongside the individual ideogram. Green region represents gain, whereas red region highlights loss. The mean ratio profile of analyzed chromosomes (*pink line*) is depicted with 95% confidence intervals (*gold lines*). Red and green lines represent thresholds for chromosomal loss (0.75) and gain (1.25).

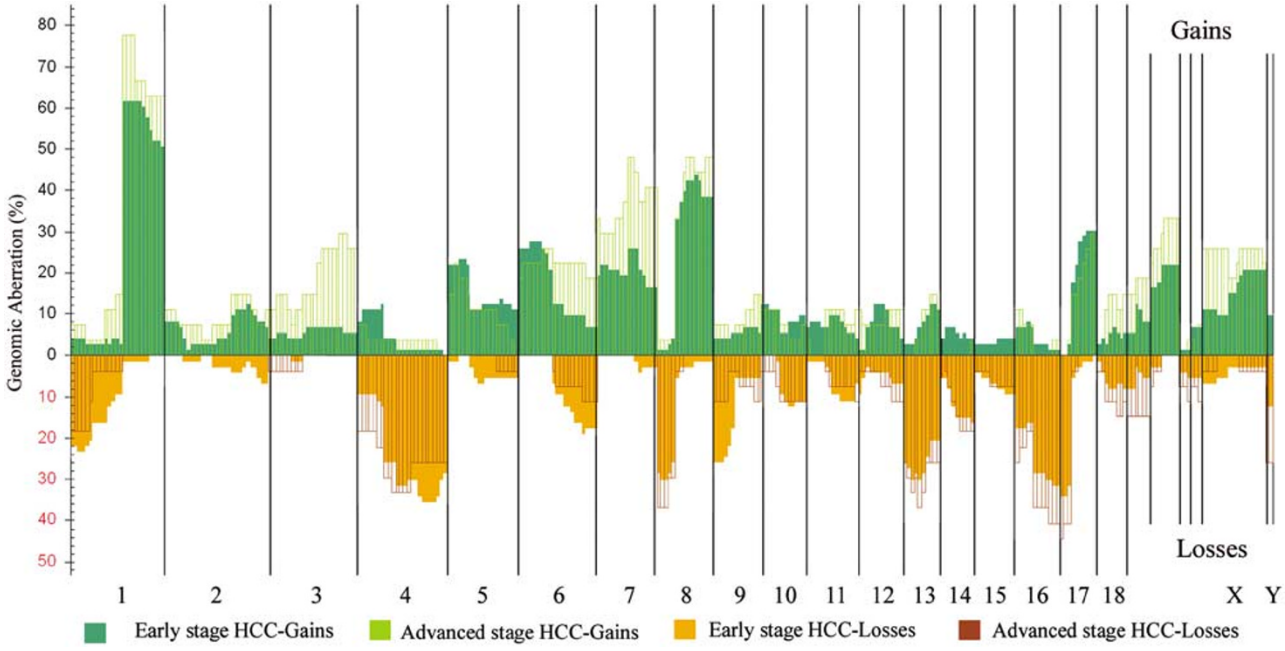


Figure 2 Frequency of genomic alterations detected in early and advanced stage hepatocellular carcinoma from CGH analysis. Percentage of hepatocellular carcinoma cases (*Y*-axis) showing particular aberration was plotted along the entire human genome (*X*-axis), from 1pter to Yqter. Copy number gains were plotted in green and copy number losses in red.

Table 2 Differential chromosome aberrations observed in early and late stage hepatocellular carcinoma

Genomic overrepresentation	*Early stage HCC (%)*	*Late stage HCC (%)*	P-*value*
+1q21–q22	61.6	77.8	0.030
+3q22–q28	6.8	29.6	0.003
+7q21–q22	26.0	48.1	0.035
+7q34–q36	16.4	40.7	0.010

of lung cancer,[24,25] head and neck squamous cell carcinoma[26] and prostate cancer[27,28] has further put forward a role for this region in tumor dissemination. In an attempt to elucidate for the underlying affected genes, positional mapping from our group has previously refined the 1q21–q22 amplicon in hepatocellular carcinoma to three affected loci, in which the overexpression of *JTB*, *SHC1*, *CCT3* and *COPA* was indicated.[29] While *JTB, SHC1* and *COPA* may offer the tumorous tissue with proliferative advantages, *CCT3* is a molecular chaperone that can enhance cell migration by controlling its downstream targets, namely actin and tubulin. Aside from cell motility, *CCT3* overexpression was also considered as an effective predictor between moderately and well-differentiated hepatocellular carcinoma. Its role in the dedifferentiation of hepatocellular carcinoma was further suggested.[30]

Frequent gains on chromosome 3q detected in stage III and IV tumors have also been indicated in the recurrence of hepatocellular carcinoma derived from true relapse.[22] Partial or total gain of 3q has been suggested as early genetic marker for invasion and metastasis of head and neck squamous cell carcinomas,[26] and a negative prognostic factor for patients with invasive bladder cancer.[31] A significant 3q gains has also been reported in Barrett's adenocarcinoma that subsequently developed lymph node metastasis.[32] Positional mapping has refined the 3q region in head and neck carcinomas to a functional candidate gene *PIK3CA* (3q26),[33] which holds a broad range of cancer-related functions including proliferation, cell adhesion, apoptosis, *RAS* signaling and oncogenic transformation. The role of *PIK3CA* oncogene in the hepatocellular carcinoma progression however remains to be clarified.

While gains of 7q21–q22 and 7q34–q36 could be detected in nearly half of the advanced stage hepatocellular carcinoma, the corresponding incidences in early stage tumors were significantly lower. Gains on 7q have also been signified in the advanced tumor stages of prostate cancer and appeared to be potential genetic discriminators for the prognosis of patients after radical prostatectomy.[27] It has been further suggested that the distal 7q3 regions are likely to harbor genes affecting the progression of prostate cancer from latent to invasive disease.[34] In chronic infections of the liver, elevated levels of hepatocyte growth factor (7q21) are often detected and have been shown to induce upregulation of *MET* expression in hepatocellular carcinoma.[35] Increased expression of *MET* (7q31), which encodes the receptor for hepatocyte growth factor, correlates with a poorer hepatocellular carcinoma prognosis and has been suggested to

promote tumor dissemination in liver metastases.[36] Nevertheless, *MET* expression in hepatocellular carcinoma tissues could be independent of hepatocyte growth factor stimulation, which in turn might imply alternative mechanistic actions, such as genomic gains, in its upregulation.

Our present CGH study points towards chromosomal gains being more specifically associated with the tumor progression in hepatocellular carcinoma. Genomic segments with increase and reduced gene dosage may contain critical oncogene and tumor suppressor loci relevant to carcinogenesis. Further positional mapping for putative genes in these chromosome regions would provide more information related to malignant characteristics of tumor, and allow estimation of prognosis for patients with hepatocellular carcinoma.

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