Clustering of Minimal Deleted Regions Reveals Distinct Genetic Pathways of Human Hepatocellular Carcinoma

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ABSTRACT

Systematic scan and statistical analysis of loss of heterozygosity (LOH) has been widely used to define chromosomal aberrations in various cancers for cloning of tumor suppressor genes and for development of prognostic markers. However, the establishment of novel strategies is needed, so that the nonrandom but heterogeneous chromosomal aberration data could provide significant insights into our understanding of molecular pathogenesis of cancers. After comprehensive allelotyping of recurrent allelic losses with 441 highly informative microsatellite markers and overlapping LOH regions on human hepatocellular carcinoma (HCC) chromosomes, 33 minimal deleted regions (MDRs) were revealed. Five and 15 of the 33 MDRs have physical intervals in less than 5 and 10 Mb, respectively, with the smallest MDR9p1 of 2.2 Mb located at 9p21.3-p21.2. Statistical and Kaplan-Meier survival analysis revealed a significant association between the loss of MDR15q1 (15q21.1-q22.2) and the HCC patient survival (adjusted P = 0.033). After cluster analysis of 33 MDRs that represented LOH profiles of each HCC tissue based on clinicopathological features and p53 mutations, two major genetic pathways, low-stage and advanced-stage HCC, were uncovered based on high concordance of MDR clusters. We propose that the definition of genome-wide MDRs on the cancer genome not only narrows down the location of existing tumor suppressor genes to facilitate positional candidate cloning and develop potential prognostic markers after statistical association of MDRs with clinicopathological features but also dissects genetic interactions and pathways of chromosomal aberrations in tumorigenesis.

INTRODUCTION

Human hepatocellular carcinoma (HCC) is one of the most prevalent malignancies worldwide, especially in parts of Asia and Africa, with an estimated 0.5 million new cases and around 1 million deaths annually (1). The average incidence of HCC in Asia is about 10–30 versus 1–3 per 100,000 person-year in western countries (2). In an endemic area like Taiwan, the incidence of HCC rises to 200–820 cases per 100,000 person-year with patients suffering chronic hepatitis and around 4,500 persons dying of HCC every year (3–5). Recent epidemiological studies have also projected an alarming increase of HCC in Japan and western countries for the next decade (6, 7). The etiology of HCC is a long-term process of chronic hepatitis and cirrhosis resulting from prolonged exposure of risk factors such as persistent hepatitis virus infection, dietary exposure of mycotoxins, and alcohol abuse (2, 8). Although early HCC is curable by surgical resection, the asymptomatic feature of HCC progression results in poor prognosis and a low 5-year survival rate (12–15%). Therefore, early diagnosis developed from molecular genetic studies of tumorigenesis should improve the clinical management and treatment of HCC.

Recent studies have identified numerous genetic and/or epigenetic changes, even though these likely represent only a fraction of multifactorial and multistage features of HCC progression (4, 8). Among them, mutations of p53 and β-catenin genes (9–11) and inactivation of Rb1 and INK4a-ARF genes by aberrant methylation or deletion (12–14) occur most frequently and are likely to contribute to major genetic mechanisms of hepatocarcinogenesis. Other genetic and epigenetic changes include mutations of M6P/IGF2R (15), BRCA2 (16), Smad2/4 (17), LPTS (18), HCCSI (19), DLC-1 (20), PTEN (21), Axin1 (22), and TCF1 (23) genes; gene amplification of c-myc (24), gankyrin (25), and cyclin D1 (26); and gene silencing by hypermethylation of SOCS-1 (27), GSTP1 (28), and 14-3-3 sigma (29) genes. Over the last few years, techniques that allow systematic analysis of chromosomal aberrations at a genome-wide level were applied to HCC (30, 31). Notably, results obtained from loss-of-heterozygosity (LOH) analysis using microsatellite markers are well correlated with those from comparative genomic hybridization analysis and point out common chromosome losses on 1p, 4q, 6q, 8p, 13q, 16q, and 17p.

Because nonrandom but heterogeneous chromosomal aberrations are attributed to mutation accumulation and clonal selection in tumor progression, statistical analysis of chromosomal aberrations associated with various clinical features such as viral infection, cirrhosis, tumor stage and size, tumor cell differentiation, and patient survival were applied to develop prognostic markers for early diagnosis and therapeutic treatment (32). For example, McGlynn et al. (30) applied a phylogenetic approach on genome-wide LOH data to conclude the heterogeneity of HCC with nonrandom allelic loss and cluster four definable branches characterized by distinctive rates of loss in hepatocarcinogenesis. Laurent-Puig et al. (33) used mutations in specific genes (p53, Axin 1, and β-catenin) to index the stability of chromosome indexed by fraction allelic loss, and allelic losses on a specific chromosome arm to classify HCC into two major genetic pathways in relation with HCC clinical features. To further improve our understanding of molecular pathogenesis of HCC, cloning of unidentified tumor suppressor genes and their interactions based on recurrent chromosomal aberrations in HCC are critical to dissect heterogeneous HCC pathways (31, 32). However, cloning of candidate cancer genes was hampered by the difficulty of further refining these regions due to the low resolution of chromosome aberrations detected by comparative genomic hybridization and the large LOH deletions detected by microsatellite markers. We, therefore, carried out genome-wide minimal deleted region (MDR) analysis on the HCC genome and discovered 33 MDRs that have pinpointed many small intervals to aid in future cloning of putative tumor suppressor genes. Furthermore, cluster analysis of these MDRs not only revealed interesting interactions but also elucidated potential genetic pathways that they participate in the hepatocarcinogenesis. Our systematic strategy of revealing genome-wide MDRs and their potential interactions involved in distinct tumorigenic pathways of HCC should be applicable to genomic studies of other cancers.
We used the permutation adjustments to adjust the same data set, it would increase the probability of declaring false significances. copathological features. Because we performed many hypothesis tests on the patients, we excluded features of gender and hepatitis B virus and hepatitis C virus negative, 15% (7/48) hepatitis C virus positive, and 15% (7/48) female.

Finally, the alignment of continuous LOH regions from multiple HCC tissues served by plotting number of markers as a function of LOH percentage in examining the distribution of LOH frequency, a biphasic distribution was observed (Fig. 1A). When both sets of ratios were greater than 2-fold, the marker region was considered to be a stringent LOH locus. For determination of LOH by determining the difference of allelic ratio in 1.5-fold was applied. To group into pathways of HCC progression. All of the 1584 segments of data (33 MDRs in 48 HCC cases) were each assigned a number and a color code as follows: (a) the existence of MDR in a chromosomal region of HCC was assigned +1 and red color; (b) the retention of a chromosomal region in contrast to MDR in HCC was assigned +1 and green color; and (c) the noninformative data of contiguous microsatellite markers in MDR was assigned no data and gray color (Fig. 3). The number-coding system allows us to describe the aberrant profile of particular HCC tissues by 33 digits of −1 or +1 of MDRs for cluster analysis. The color-coding system provides interactive graphical analysis of clustering results in the TreeView program (40). Based on a previously described clustering algorithm (35, 40), the similarity of MDR profiles of a group of tumor samples (such as a subgroup of HCC features) was calculated and clustered according to the average concordance value of 33 MDRs that ranges from 1 (full concordance) to −1 (total discordance). After cluster analysis of all HCC tissues with or without subgrouping of clinicopathological parameters, a concordance value of at least 0.75, which denotes excellent reproducibility for potential genetic interaction, was selected for grouping into pathways of HCC progression.

RESULTS

LOH Allelotyping and MDRs Identification. Allelotyping analysis was conducted using 441 (91% successful rate) highly informative microsatellite markers with an average inter-marker distance of 7.53 cM and only one marker displaying an interval greater than 20 cM (21.36 cM). Based on the stringent LOH definition (Fig. 1A), the average genome-wide LOH frequency is 34.01%. LOH analysis by chromosome arms demonstrated that 13 arms, 17p (67.12%), 4q (57.84%), 16q (54.67%), 13q (54.51%), 8p (49.93%), 10q (45.10%), 1q (43.06%), 9p (42.95%), 14q (42.17%), 18q (41.63%), 22q (40.22%), 16p (35.16%), and 15q (35.15%), displayed a LOH frequency above average. When the numbers of markers were plotted with the frequencies of LOH, we observed a biphasic distribution, in which two peaks were separated at a frequency of ~50% (Fig. 1B). According to previous LOH studies on other tumors (35), markers appearing in the peak of low LOH frequencies detect mostly random changes reflecting the genetic instability of tumors, whereas markers in another peak are likely to show LOHs associated with cancer-specific phenotypes. Therefore, only markers with a LOH frequency >50% were used to be annotated onto chromosomes of individual tumor tissues based on the comprehensive human genetic map published by the Center for Medical Genetics at Marshfield (41). After aligning LOH data of all cancer tissues on each chromosome, 33 MDRs were defined, and Fig. 1C shows the annotation of these MDR data along each chromosome (Supplement 2). The average genetic and physical intervals of 33 MDRs are 18.08 cM and 12.82 Mb, respectively (Table 1). Among 33 MDRs, 23 were reported by conventional LOH analysis on HCC previously and are located on 1p (MDR1p1), 1q (MDRs 1q1 and 1q2), 4q (MDRs 4q1 and 4q2), 6q (MDR6q1), 7p (MDR7p1), 8p (MDRs 8p1 to 8p3), 9p (MDR9p1), 10q (MDR10q1), 13q (MDRs 13q1 to 13q5), 14q (MDRs 14q2 and 14q3), 16q (MDRs 16q1 and 16q2), and 17p (MDRs 17p1 and 17p2). In addition, we identified 10 novel MDRs including 2q1, 5q1, 10q1, 12p1, 14q1, 15q1, 17q1, 18q1, 18q2, and 21q1. Using the annotation
of Ensembl database to estimate the physical distances of MDRs, we found that five MDRs are within 5 Mb and 15 are within 10 Mb. Many newly discovered candidate tumor suppressor genes (TSGs) identified in various human tumors were located in these MDRs.

**Loss of MDR15q1 Associated with HCC Patient Survival.** We next examined correlation of MDRs with clinicopathological parameters for developing potential HCC prognostic markers. To avoid false association from multiple hypothesis tests, permutation adjustments were conducted to adjust the \( P \) values (38, 39). Except for a marginal association of MDR1p1 (1p36.23–1p36.21) loss with serum AFP concentration \( < 20 \) ng/ml (adjusted \( P = 0.0560 \)) in HCC patients, we observed that a loss of MDR15q1 (15q21.1–15q22.2) correlated significantly with poor survival (i.e., \(< 2 \) years, adjusted \( P = 0.0330 \)) of HCC patients. Kaplan-Meier survival analysis indicated that the loss of MDR15q1 is significantly associated with 2-year poor survival of HCC patients (adjusted \( P = 0.0024 \)), and all three markers within MDR15q1 (6.71 cM) are associated with the tendency of poor survival, especially for allelic loss of \( D15S126 \) (adjusted \( P = 0.0054 \); Fig. 2).

**Cluster Analysis Identifies Interactions of MDRs and Their Contributions to HCC Pathways.** In addition to identifying the association of particular MDRs with clinicopathological parameters, our MDR data provide an opportunity to determine whether MDR in one chromosomal region is linked to MDRs in other chromosomal regions. We approached this by cluster analysis of MDR profiles of the 48 HCC tissues. Cluster analysis has been commonly used in microarray studies to identify functionally related genes that display similar expression patterns (40). Except for the neighboring MDR17p1 and MDR17p2 with a concordance score greater than 0.75, there is no genetic interaction from different chromosomal regions among 31 of 33 MDRs by clustering MDR profiles of 48 HCC tissues. To investigate whether interactions of MDRs could exist in more defined subgroups of HCC, we performed cluster analysis on subclassified HCC tissues according to their clinicopathological features. Fig. 3 shows a representative TreeView presentation of the clustering results of stage II and stage III/IV tissues, based on the AJCC American Joint Committee on Cancer (AJCC) cancer stage grouping of Tumor-Node-Metastasis (TNM) classification. In each case, we observed a number of MDR clusters in which MDRs in distinct chromosomal regions are lost or retained in a highly coordinated fashion. For instance, clusters MDR16q2-MDR13q5 (\( C = 0.79 \)) and MDR13q4-MDR13q2 (\( C = 0.79 \)) were found in stage II tissues, and clusters MDR4q2-MDR1p1 (\( C = 0.79 \)), MDR16q2-MDR8p1 (\( C = 0.89 \)), MDR17p2-MDR17p1-MDR18q2 (\( C = 0.76 \)), MDR16q1-MDR13q2 (\( C = 0.79 \)), and MDR8p3-MDR8p2 (\( C = 0.79 \)) were found in stage III/IV tissues. Additional cluster analysis on various HCC subgroups classified by other clinicopathological features, e.g., invasion, metastasis, \( p53 \) mutation status, recurrence, serum AFP level, and \( T_j/T_o \) classification, revealed the existence of highly concordant MDR clusters in almost every subgroup of HCC, suggesting that genetic interactions between these regions occur during the development of specific feature of HCC (Supplement 3).

By listing the MDR cluster profiles of each HCC subgroup, we...
DISCUSSION

Toward identifying genetic interactions of chromosomal aberrations and dissecting genetic pathways in tumorigenesis, we applied several novel strategies to analyze the HCC genome-wide allelotyping data. First, we distinguished LOH markers that detect mostly tumorigenic allelic losses from those detecting mostly random deletions based on the frequencies of LOH revealed by the marker, thus decreasing the noise generated by these random changes caused by genetic instability of tumors. Second, instead of analyzing LOH data per se, we aligned continuous LOH regions from multiple HCC tissues and defined MDRs. Compared with conventional LOH analysis, this MDR approach allows a more accurate determination of chromosomal losses and evaluation of the contributions of these losses in HCC progression. To our knowledge, our study provides the first genome-wide MDR data in cancer. Furthermore, a large fraction of MDRs identified (20/33; >60%) carries a physical distance of <10 Mb, which should facilitate positional candidate cloning of TSGs of HCC. Third, we applied cluster analysis based on MDR profile of each HCC tissue to examine potential interactions of MDRs involved in various clinicopathological features of HCC. Notably, this analysis not only revealed many interactions of MDRs, but also dissected distinct genetic pathways in the progression of HCC.

HCC is rapidly fatal after diagnosis; the survival rate over a 5-year interval is only an average of 15% and ranges from 22% to 73% without and with surgery, respectively (42). Development of a DNA marker for HCC prognosis will be important for HCC patient management. Our statistical analysis revealed a significant association between the presence of LOH in MDR15q1 and the outcomes of HCC patient management and with surgery, respectively (42). Development of a DNA marker for HCC prognosis will be important for HCC patient management. Our statistical analysis revealed a significant association between the presence of LOH in MDR15q1 and the outcomes of HCC patient management.

Table 1  Summary of 33 MDRs in HCC

<table>
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<th>MDRs</th>
<th>Cyto genetic loci</th>
<th>MDR regions</th>
<th>Frequencies (%)</th>
<th>cM</th>
<th>Mb</th>
<th>Known TSGs</th>
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* MDR, minimal deleted regions; HCC, human hepatocellular carcinoma; TSG, tumor suppressor gene.
The current theory of multistep tumorigenesis indicates that progression into cancer cells requires six essential alterations in cell physiology that collectively perturb regulatory circuits of normal cell proliferation and homeostasis, leading to malignant growth (47). Because linkage analysis reveals no obvious familiar predisposition of HCC, identification of critical genes located in recurrent genomic aberrant regions has been the rate-limiting step for understanding the molecular mechanism of multistage HCC. However, this task is hampered by the highly heterogeneous tumorigenesis process and the large number of genetic alterations accumulating in tumor cells as a result of genome instability. To better understand the mechanism of hepatocarcinogenesis, various models of pathways of HCC have been proposed recently for dissecting tumorigenesis of HCC based on their differences in the clinical characteristics, their aberrations of chromosome arms, and their mutations of known cancer genes (p53, Axin 1, and β-catenin; Refs. 8, 30–33, 48, and 49).

Fig. 2. The Kaplan-Meier survival plots of MDR15q1. The survival curves represent the survival fraction against survived months of HCC patients. The red and green lines represent loss (LOSS; MDR or LOH) and retention (RET; without MDR or RET) of the region, respectively. A, the survival plot of MDR15q1 and its cytogenetic location. B, the survival plots of microsatellite markers defined MDR15q1.

Fig. 3. A representative example of cluster analyses of MDR profiles in HCC tissues based on American Joint Committee on Cancer (AJCC) cancer stage grouping of Tumor-Node-Metastasis (TNM) classification and displayed by TreeView program. The cluster analysis is based on the profiles of 33 MDRs either with (red) or without (green) MDR in the subgroup of HCC patients (top row). The MDR subclusters with concordance score over $C = 0.75$ of stage II (left cluster) and stage III & IV (right cluster) suggested potential interactions of MDRs in HCC progression.
In our studies, we took cancer genomic approaches to pinpoint 33 recurrent deletion loci in small intervals for positional candidate cloning of TSGs in HCC. Cluster analysis of the 33 MDRs was used to reveal the interactions of these MDRs involved in the highly heterogeneous pathways of hepatocarcinogenesis. A preliminary cluster analysis on all HCC samples did not reveal any concordance except for two neighboring MDRs on chromosome 17p. Nevertheless, subsequent cluster analysis on subgroups of HCC samples classified based on their clinicopathological features has successfully identified many associated MDRs. The existence of these associated MDRs might represent different TSGs, the synergistic loss of which would provide a growth advantage during HCC development. In addition, our cluster analysis also reveals the existence of common MDR clusters in most subgroups of low-stage HCC and a distinct MDR cluster in advanced-stage subgroups. The presence of these “signature” MDR clusters not only suggests that the synergistic interactions between putative TSGs in these loci would be important in the development of low- and advanced-stage HCC but also facilitates the assignment of a given subgroup of HCC into low or advanced stage. Indeed, HCC subgroups with the p53 mutation and an aberrant serum level of AFP were both classified in the advanced stage. On the other hand, the low-stage HCCs show similar concordance of clinicopathological features with normal level of serum AFP and without mutation. Interestingly, low-stage HCC has been reported to associate to having favorable prognosis (49). Because the β-catenin mutation consists of 13% of HCC samples, our limited data of β-catenin mutation (15%, 7/48)—six cases in T2 and one case in T3 classification—confirms similar observations reported previously (data not shown).

In summary, high-density allelotyping on the HCC genome followed by a genome-wide MDR approach and cluster analysis allowed us to reveal two genetic pathways of HCC tumorigenesis. Our strategy of MDR approach and cluster analyses not only dissected genetic pathways of the molecular pathogenesis of HCC but also provided new genetic markers for tumor classification, prognosis, and positional candidate cloning of TSGs. We noticed that the definition of 33 MDRs might still underestimate cancer-related genes in HCC progression, because other mutational mechanisms such as gene amplification, haploinsufficiency, and epigenetic changes of TSG gene expression would also play important roles in the genetic pathways of HCC tumorigenesis (50). With the anticipation of having the fully sequenced and annotated human genome and newly invented tools for high-throughput genomic analyses including genome-wide aberrant studies by microarrays of single-nucleotide polymorphism markers and arrayed-comparative genomic hybridization (51, 52), integration of our current results with other genomic and proteomic approaches in conjunction with bioinformatic tools should speed up our understanding of molecular pathogenesis of cancer, including HCC.

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Clustering of Minimal Deleted Regions Reveals Distinct Genetic Pathways of Human Hepatocellular Carcinoma

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