High-resolution mapping of copy number aberrations and identification of target genes in hepatocellular carcinoma

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SUMMARY **Hepatocarcinogenesis involves complex combinations of molecular events, such as genetic aberrations, epigenetic changes, and alterations in gene expression. To elucidate the mechanism of hepatocarcinogenesis, it is necessary to reconstruct these molecular events at each level. This article presents a review of copy number analyses of hepatocellular carcinoma (HCC) using traditional comparative genomic hybridization (CGH), array-based CGH (aCGH), and single nucleotide polymorphism (SNP) arrays. A number of studies have applied CGH technology for copy number analysis of HCC and have indicated the significance of correlations of frequent genomic aberrations with various clinicopathological parameters, prediction of recurrence and prognosis, and treatment selection, followed by comprehensive genomic analysis using aCGH with much higher resolution. Furthermore, we present our data regarding genomic aberrations of HCC obtained using the Genome Imbalance Map (GIM) algorithm, which simultaneously detects DNA copy number alterations and loss of heterozygosity using SNP arrays, and the Expression Imbalance Map (EIM) algorithm, which detects mRNA expression imbalance correlated with chromosomal regions. Using these two algorithms, we integrated the expression profiles, locus information, and genomic aberrations in a systematic manner, which is effective for detecting structural genomic abnormalities, such as chromosomal gains and losses, and showed that gene expression profiles are subject to chromosomal bias.**

Key Words: Liver cancer, karyotyping analysis, high-resolution mapping, copy number alterations

Introduction

Cancer is a genetic disease of somatic cells arising from accumulation of genetic changes, and abnormalities of suppressor genes and oncogenes are frequently associated with carcinogenesis. To stratify patients and select the most appropriate treatment options for hepatocellular carcinoma (HCC), many staging systems from the standpoint of clinical information and pathological classification have been proposed (*1*,*2*). However, despite improvements in these trials, prognostic predictions for HCC are still not fully acceptable for selection of individualized treatments (*3*). Therefore, there has been a great deal of effort using molecular biological technologies to establish prognostic models for HCC.

Many researchers have reported genomic decoding regarding carcinogenesis, invasion, and metastasis of liver cancer (*4-14*). Furthermore, considering the complexity of carcinogenesis, many other genes may be involved in both the initiation and progression of cancer, and comprehensive expression analysis using microarray technology has great potential for the discovery of new genes involved in carcinogenesis (*15*).

In addition to identification of novel candidate genes for biomarkers and the discovery of therapeutic targets, which are helpful for improvement of clinical diagnosis and treatment (*16*,*17*), classification and selection of predictor genes for HCC using genome-wide expression analysis have been reported (*18*). Okabe *et al*. reported gene expression profiling analysis

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Received June 6, 2007
Accepted June 25, 2007

of liver cancer etiology, including hepatitis B and hepatitis C viral infection (*19*). Using comprehensive expression analysis, gene prediction sets for anti-cancer drug sensitivity (*20*) or intrahepatic recurrence (*21*) were demonstrated. Thus, comprehensive expression analysis has enabled us to perform clustering analysis based on clinicopathological features, identification of candidate genes for therapy, and diagnosis, and selection of predictor genes for tailor-made therapy.

On the other hand, by integration of expression profiles with gene loci, it has been shown that gene expression profiles are subject to chromosomal bias (*22-26*). In addition, genes in regions of chromosomal aberration with altered gene expression levels are more likely to represent oncogenes or tumor suppressor genes. Therefore, it is necessary to investigate the copy number information in addition to expression profile in the same samples. Comparative genomic hybridization (CGH) has been used extensively to detect genome-wide copy number alterations in various types of cancer and to determine the localization of expression of many oncogenes and tumor suppressor genes (*27*), and there have been a number of reports of chromosomal analysis using CGH in HCC (*28-40*). These previous studies investigated the associations between chromosomal alterations and various clinicopathological factors, such as tumor progression (*29,30,32,36,37,40*), prognosis (*35*), and viral infection (*31,33*) in liver cancer.

Recently, array-based CGH (aCGH) using genomic DNA or cDNA clones has been developed and provided much higher resolution detection of copy number alterations than conventional CGH. Therefore, accurate identification of genes with DNA copy number changes in carcinogenesis is now possible (*41-43*). Using aCGH, high-resolution mapping of copy number aberrations in HCC has been reported, especially in measuring high-level amplification and homozygous deletion (*44-48*).

Single nucleotide polymorphism (SNP) arrays, which were originally designed for high-throughput SNP analysis (*49,50*), can provide high-resolution analyses of loss of heterozygosity (LOH) in a genome-wide fashion (*51-55*). We and other groups have developed novel algorithms for global and high-resolution analysis of copy number changes using SNP arrays (*56-59*). In comparison to aCGH, the newly developed Genome Imbalance Map (GIM) algorithm (*56*) has advantages for detecting not only copy number aberrations but also allelic imbalance, including LOH and uniparental disomy (UPD) (*24*).

This article presents a review of the outcomes of copy number analysis for HCC through a literature search of published reports, especially with regard to identification of candidate genes for oncogenes and tumor suppressor genes using aCGH and SNP arrays. Furthermore, we propose an algorithm for integration of expression data with gene loci, and discuss the chromosomal bias of gene expression and pitfalls of gene clustering.

Molecular karyotyping analysis for hepatocellular carcinoma using conventional comparative genomic hybridization

A number of studies of chromosomal alterations in HCC using conventional CGH have been reported (*28-44*), which were summarized according to etiology, histological grade, and tumor stage (*60,61*). A meta-analysis based on 31 CGH analyses of 785 HCC nodules showed that gains on chromosome arms were observed on 1q (57.1%), 8q (46.6%), 6p (23.3%), and 17q (22.2%), while losses were detected on 8p (38%), 16q (35.9%), 4q (34.3%), 17p (32.1%), and 13q (26.2%) (*60*). Through this meta-analysis, Moinzadeh *et al*. further classified chromosomal alterations according to clinicopathological parameters, including hepatitis virus infection (*31,33*), tumor differentiation grade (*32*), and tumor progression (*30,36,37,62*). Comparison between HBV-positive and -negative cases indicated that losses at 4q, 8p, 13q, and 16q were positively correlated with HBV-positive HCC, whereas only 8p loss was more frequent in HCV-positive cases. With regard to tumor histological grade, chromosomal losses at 4q and 13q were significantly associated with tumor dedifferentiation. Although the number of dysplastic nodules analyzed by CGH was low, 1q gains were characteristic of the initiation of hepatocarcinogenesis (*60*). In addition to the clinical features described above, Pang *et al*. reported that gains at 1q and 6p were independent factors for liver cancer invasion (*29*).

If copy number analysis can predict the recurrence of HCC after resection, individualized therapy may be possible. Kusano *et al*. reported that recurrence was linked to loss at 13q, which was a variable independent of other factors on multivariate analysis (*35*). Furthermore, Poon *et al*. reported a tumor progression model for HCC using bioinformatics analyses using the self-organizing tree algorithm (SOTA) in a large-scale study. Based on the patterns of significant chromosomal aberrations derived, they identified 4 HCC classes at 3 different evolution levels, one group of which had poorer recurrence-free survival than the other 3 groups. They also showed that patients with 3q22-24 gain have both poorer recurrence-free and overall survival rates (*40*).

Thus, CGH analysis can make it possible not only to classify the clinicopathological parameters of the tumor but also to predict the prognosis of HCC patients, which will facilitate individualized therapy.

Comparative genomic hybridization for determination of liver cancer clonality

Multifocal cancer growth of HCC is due to either intrahepatic metastasis or multicentric origin, which

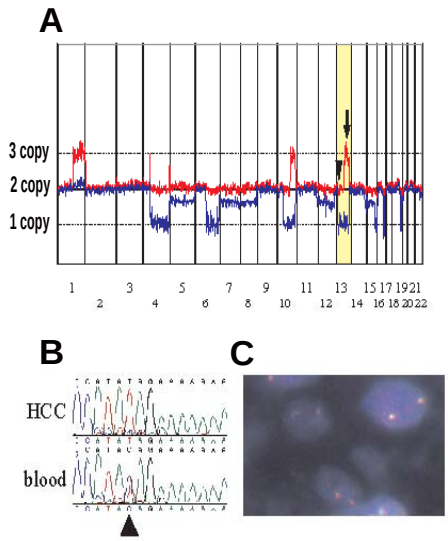

Figure 1. Genome Imbalance Map (GIM) of a representative hepatocellular carcinoma (HCC) sample. GIM can detect not only genome dosage but also allelic imbalance status more precisely than aCGH analysis. (A) Allelic dosage analysis across the whole genome showed uniparental disomy in 13q31.2-34; (B, C) Fluorescence in situ hybridization and loss of heterozygosity analysis for validation of allelic imbalance in 13q. (Modified from Reference 24 with permission)

is clinically significant. However, methods for clinicopathological and morphological discrimination have not been sufficiently reliable for physicians to determine the appropriate treatment for patients with multiple HCC. To differentiate intrahepatic metastasis from multicentric origin in HCC, it has been shown to be useful to compare the clonalities of multifocal HCC using molecular methods, such as CGH analysis

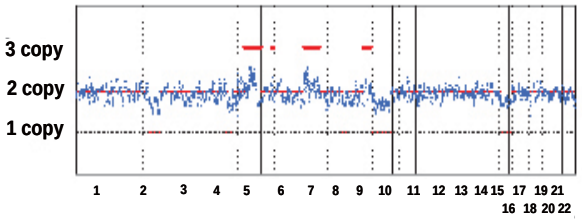

Figure 3. Comparison of genomic alteration and gene expression status. Total gene dosage and expression analysis across the whole genome of a patient. Dots represent HCC/liver expression intensity ratio and the continuous lines indicate copy numbers. Gene expression levels changed in accordance with genomic alterations. (Modified from reference 24 with permission)

(*38*,*39*,*63*), DNA fingerprinting by LOH assay (*64-66*), and hepatitis B virus integration pattern (*67-69*), as the recurrent neoplasm inherits the same altered genome from the initial HCC.

Chen *et al*. applied CGH for 31 primary and the corresponding recurrent liver tumors and calculated the clonal relationships, by which they could distinguish truly relapsed from second primary HCC in 22 of 31 cases (*39*). On the other hand, using all of the 3 molecular methods described above, Ng *et al*. succeeded in complete determination of the clonal relationships of 25 nodules from 11 patients (*38*).

Thus, evaluation of clonality of multifocal HCC using molecular methods is useful for physicians to allow precise determination of the treatment for multiple HCC. CGH is the most powerful and most readily available tool for this purpose.

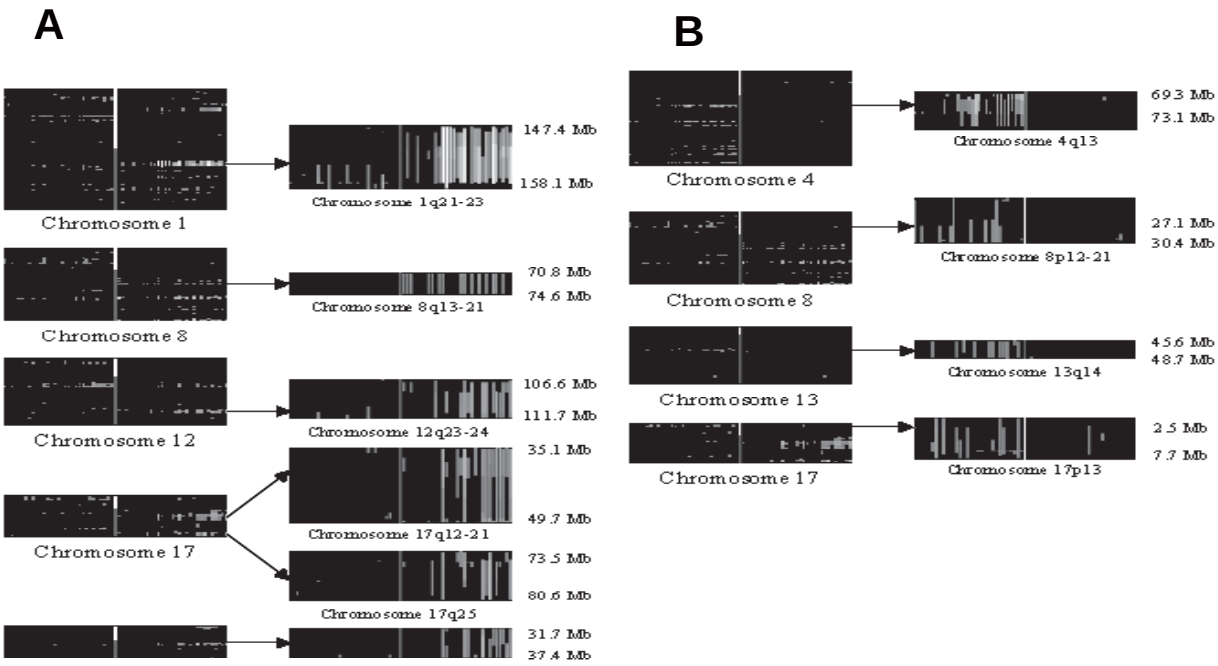

Figure 2. Expression Imbalance Map (EIM) for detecting expression imbalance region in hepatocellular carcinoma (HCC). EIM enables identification of many more genes by referring to the expanded area with lower luminance. (A) Expression imbalance region at an E value > 2 and a range of expression gain > 3 Mb. (B) Expression imbalance region at an E value > 2 and a range of expression loss > 3 Mb. (Modified from reference 23 with permission)

High-resolution mapping of copy number aberrations and identification of target genes in hepatocellular carcinoma

In comparison to traditional CGH, aCGH can detect chromosomal aberrations with high resolution. By comparison between conventional CGH and aCGH in 19 HCC samples, Hashimoto *et al*. demonstrated that 80% of the target clones identified by aCGH were included in CGH analysis, while copy number alterations for FGR/SRC2, HRAS, THRA, and GSCL, of which clones were detected by aCGH, were not found by conventional CGH (*46*).

Using aCGH, the significance of correlations of frequent chromosomal aberrations with various clinicopathological features were investigated, and Katoh *et al*. demonstrated that chromosomal loss on 17p13.3 and gain of 8q11 were independent prognostic indicators by multivariate analysis (*44*). Among the various clinicopathological features, differentiation grade is one of the best indicators of malignancy of liver cancer (*70*), and 4q and 13q were shown to be correlated with dedifferentiation of HCC (*48*).

In addition to high-resolution mapping of chromosomal aberrations, aCGH is available for identification of candidate genes correlated with DNA copy number alterations for narrowing the list of oncogenes and tumor suppressor genes. Integrating the correlation between copy number alterations and gene expression profile, Patil *et al*. identified Jab1 as a target for 8q gain, which was suggested to have a potential role in the development of HCC by functional analysis (*47*).

Molecular karyotyping analysis of hepatocellular carcinoma using single nucleotide polymorphism arrays

The detection of genome-wide LOH is possible by comparing the calls for normal control and tumor samples using SNP arrays (*51,53-55*). The accuracy of this method was validated by comparison to PCR-based microsatellite analysis by Hoque *et al*. (*52*). In addition to LOH, we and other groups have developed algorithms for detecting copy number alterations and allelic imbalance simultaneously using SNP arrays (*56-59*).

Our method, named GIM analysis (Figure 1), was applied to 36 HCC samples and recurrent chromosomal aberrations in liver cancer were analyzed (*24*). That is, even fractional copy number, suggesting heterogeneity of cancer cells, was detected, and validated by fluorescence in situ hybridization. In this study, in addition to the gains of 1q, 5p, 5q, 6p, 7q, 8q, 17q, and 20q, and LOH of 1p, 4q, 6q, 8p, 10q, 13q, 16p, 16q, and 17p, which were significantly associated with HCC, we identified UPD and UPT on 13 regions, suggesting that genome dosage analysis misses many LOH regions with normal copy number. For example, on 6q24-25, which contained imprinting gene clusters and UPD regions in our data, we observed reduced levels of *PLAGL1* expression due to loss of the unmethylated allele. Thus, high-resolution GIM analysis can accurately determine the localizations of genomic regions with allelic imbalance, and when integrated with epigenetic information, a mechanistic basis for inactivation of tumor suppressor genes in HCC was elucidated.

Furthermore, using much higher-density arrays, it will soon be possible to elucidate micro-homozygous deletion and chromosome amplification, and boundary regions suggesting breakpoints in liver cancer.

Systematic integration of expression profiles with gene loci

We have integrated gene expression data and gene locus information, and the regions in which the numbers of up-regulated and down-regulated genes were significantly concentrated were mapped on the chromosome (*22*). This method for detection of regions of mRNA expression imbalance is called Expression Imbalance Map (EIM), and we applied EIM analysis to gene expression data from 31 HCC tissues (*23*). Our data revealed that expression gains of 1q21-23, 8q13-21, 12q23-24, 17q12-21, 17q25, and 20q11, and losses of 4q13, 8p12-21, 13q14, and 17p13 were significantly associated with HCC (Figure 2), consistent with previous reports using CGH in liver cancer (*28,32,36,37,67,71-75*). Furthermore, more poorly differentiated liver cancer contains larger numbers of chromosomal alterations, which are accumulated in a stepwise manner in the course of HCC progression.

If not only gene expression but also cytogenetic data can be obtained from the same sample, integration of expression profile with chromosomal loci will enable comparison of gene expression with gene dosage. Pollack *et al*. measured parallel mRNA levels by microarray analysis and DNA copy number alterations by aCGH in breast cancer cells, and they reported that 62% of highly amplified genes show elevated expression and that DNA copy number influences gene expression across a wide range of DNA copy number alterations (*26*).

In liver cancer tissues, we and other groups reached the same conclusions as Pollack *et al*. Furge *et al*. obtained regional expression biases (REBs) from a multiple span moving binomial test and demonstrated that REBs overlapped genetic abnormalities identified using aCGH in HCC (*25*). We have also demonstrated the effects of genome imbalance on the transcriptome by direct comparison with expression data from the same samples (*24*) (Figure 3).

On the other hand, Huang *et al*. investigated the relationship between genomic DNA copy number

changes and transcriptional levels, and found that DNA copy number alterations appeared not to parallel the corresponding gene expression profiles in either HCC specimens or cell lines (*45*).

Thus, gene expression profiles are subject to chromosomal bias and EIM can correlate gene expression to gene loci with high resolution and sensitivity.

Conclusions

Microarray analysis has contributed to identification of candidate genes and has been shown to be available for clinical application. In addition, clustering analysis of expression data and selection of predictor genes based on clinicopathological features could have been performed. However, bioinformatics technology indicated that gene expression profile is subject to chromosomal bias, *i.e.*, clustering analysis involves the risk of being affected by gene structural abnormalities. To resolve this problem, combined and well-organized reconstruction of different molecular levels, including genetic aberrations, epigenetic changes, and expression alterations, is required to narrow the candidates responsible for cancer.

Acknowledgements

This work was supported in part by a Grant-in-Aid for Scientific Research (C) 19591601 (Y.M.), and the Program of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO), by the NFAT project of the New Energy and Industrial Technology Development Organization (NEDO) and by a Special Coordination Fund for Science and Technology from the Ministry of Education, Culture, Sports, Science and Technology.

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