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Microarray-based analysis for hepatocellular carcinoma: From gene expression profiling to new challenges

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Abstract

Accumulation of mutations and alterations in the expression of various genes result in carcinogenesis, and the development of microarray technology has enabled us to identify the comprehensive gene expression alterations in oncogenesis. Many studies have applied this technology for hepatocellular carcinoma (HCC), and identified a number of candidate genes useful as biomarkers in cancer staging, prediction of recurrence and prognosis, and treatment selection. Some of these target molecules have been used to develop new serum diagnostic markers and therapeutic targets against HCC to benefit patients. Previously, we compared gene expression profiling data with classification based on clinicopathological features, such as hepatitis viral infection or liver cancer progression. The next era of gene expression analysis will require systematic integration of expression profiles with other types of biological information, such as genomic locus, gene function, and sequence information. We have reported integration between expression profiles and locus information, which is effective in detecting structural genomic abnormalities, such as chromosomal gains and losses, in which we showed that gene expression profiles are subject to chromosomal bias. Furthermore, array-based comparative genomic hybridization analysis and allelic dosage analysis using genotyping arrays for HCC were also reviewed, with comparison of conventional methods.

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INTRODUCTION

Cancer is a genetic disease of somatic cells arising from accumulation of genetic changes, and abnormalities of suppressor genes, *TP53*^[1], *RB*^[2], and *IGF2R*^[3], and oncogenes, *c-myc*^[4], *CCND1*^[5], *CTNNB1*^[6], and *c-Met*^[7], have been reported in hepatocarcinogenesis. On the other hand, activation of the matrix metalloproteinase (MMP) family^[8], angiopoietin^[9], and vascular endothelial growth factor (*VEGF*)^[10], and inactivation of *E-cadherin*^[11] have been demonstrated to play pivotal roles in invasion and metastasis of liver cancer. Considering the complexity of carcinogenesis, many other genes may be involved in 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.

cDNA microarray analysis for hepatocellular carcinoma (HCC) was first reported by Lau *et al*^[12], using approximately 4000 known human genes in 10 pairs of HCC and non-tumorous tissues, which was followed by many subsequent studies^[13-16]. Thereafter, using HCC samples, clustering analysis based on clinicopathological features, including viral infection, tumor differentiation grade, and vessel invasion, were reported^[17,18]. Identification of novel candidate genes for biomarkers and discovery of therapeutic targets are helpful for improvement of clinical diagnosis and treatment^[19,20]. Tailor-made therapy becomes possible if predictor genes can anticipate therapeutic responses and prognosis precisely^[21,22]. Furthermore, bioinformatics technology has enabled the integration of expression profiles with various types of gene information, such as gene ontology, function, and locus^[23,24].

Comparative genomic hybridization (CGH) is a molecular cytogenetic analysis of screening neoplasms for genetic changes, and has been used extensively to localize regions of oncogenes and tumor suppressor genes in various types of cancer^[25]. Recently, array-based CGH (aCGH) using genomic DNA or cDNA clones has been developed with much higher resolution than conventional

CGH, and accurate identification of genes with DNA copy number changes in carcinogenesis is now possible^[26,27].

In addition to aCGH, we and others have developed novel algorithms for global and high-resolution analysis of copy number changes using single nucleotide polymorphism (SNP) arrays^[28-31], which were originally designed for high-throughput SNP analysis^[32,33]. In comparison to aCGH, the newly developed Genome Imbalance Map (GIM) algorithm has advantages for detecting not only copy number changes but also allelic imbalance, including loss of heterozygosity (LOH) and uniparental disomy (UPD)^[28].

In this article, we review the outcomes of microarray analysis for HCC through a literature search of published reports, *i.e.*, clustering analysis based on clinicopathological features, identification of candidate genes for therapy and diagnosis, selection of predictor genes for tailor-made therapy, and integration of microarray data with other types of gene information. Furthermore, we discuss the chromosomal bias of gene expression and pitfalls of gene clustering.

IDENTIFICATION OF ALTERED GENE EXPRESSION IN HEPATOCELLULAR CARCINOMA

Gene expression profile analysis has made it possible to identify novel genes with altered expression that have not been reported in liver cancer. For example, aberrations of *MARKL1* and *MARK3*^[34], *VANGL1*^[35], *PEG10*^[36], *BMAL2*^[37], *DDEFL1*^[38], *RhoC*^[39], *GEP*^[40], *HLA-DR*^[41], *Claudin10*^[42], and *Ephrin A1*^[43] were demonstrated by microarray analysis.

Through comprehensive expression analysis in HCC, we also identified up-regulated genes, *GPC3*^[20], *ROBO1*^[19], and *SP-5*^[44]. *GPC3* is a member of the heparan sulfate proteoglycans and binds to the cell membrane *via* glycosyl phosphatidylinositol anchors. We demonstrated that *GPC3* works as a co-receptor, modulating signaling pathways of growth factors, such as FGF2 and BMP-7, and contributes to hepatocarcinogenesis^[20]. Up-regulated genes in HCC, compared to the background hepatocytes, are candidate diagnostic markers, and we confirmed that monoclonal antibodies against *GPC3* generated in our laboratory recognize the *GPC3* molecule in HCC, and demonstrated the feasibility of *GPC3* as a marker for HCC in routine histological examination^[45]. Furthermore, this molecule is secreted due to the signal peptide in its N-terminus, and we succeeded in measuring serum *GPC3* levels by enzyme-linked immunosorbent assay in HCC patients^[46].

In addition to the availability of the neural cell adhesion molecule *ROBO1* as a diagnostic serum marker for HCC, this molecule is also a clinical gene therapy target as a newly generated anti-*ROBO1* monoclonal antibody induced complement-dependent cytotoxicity in the *ROBO1*-expressing liver cancer cell line, PLC/PRF/5^[19].

Abnormalities in β -catenin are observed in about 40% of liver cancers, and several genes, including *c-myc*^[47], *CCND1*^[48], *MDR1*^[49], *WISP1*^[50], *L1*^[51], *GPR49*^[52], and *DKK1*^[53], have been identified as downstream targets in

the Wnt signaling pathway, which may be important for understanding the role of Wnt signaling in carcinogenesis because these genes are involved in cell proliferation, differentiation, and migration. As new downstream target genes of the Wnt signaling pathway, we observed up-regulation of *SP-5*^[44] in liver cancer with Wnt signaling abnormality and showed that this molecule is a direct target for β -catenin/TCF4 complex. Especially, transfer of *SP-5* into MCF-7 cells, in which *SP-5* protein is not detectable, resulted in significant growth promotion, and we, therefore, concluded that *SP-5* plays a pivotal role in the progression of liver cancer.

FROM GENERAL CLUSTERING TO PREDICTION OF PROGNOSIS AND TREATMENT EFFECT BASED ON GENE EXPRESSION PROFILING

Most cases of HCC originate from chronic liver disease caused by hepatitis viral infection, including hepatitis B virus (HBV) and hepatitis C virus (HCV), exposure to aflatoxin B1 in mold, and alcohol abuse. After a long period of inflammation of the liver, early-stage HCC, which is small with indistinct margins and consists of well-differentiated cancerous tissues, may occur and develop to less well-differentiated HCC. Through the progression of liver cancer with dedifferentiation, cancer cells may metastasize to the lungs, adrenal glands, bone, and other segments of the liver parenchyma. Many researchers have attempted to determine the associations between gene expression profiles and such clinicopathological features.

Viral hepatitis is one of the most important epidemiological factors, and Okabe *et al*^[17] classified 20 HCC specimens into HB- and HC-based liver cancers by two-way clustering after data mining and selected genes that were differentially expressed between the two viral-based HCC, and this was followed by studies by other groups^[18]. Takemoto *et al*^[54] reported that sex affects not only the incidence of HCC, but also the outcome after treatment, and performed clustering analysis based on gender. After chronic infection forces hepatocytes to regenerate with consequently excessive replication, regenerative nodules appear as precancerous lesions. Genes responsible for hepatocarcinogenesis may be identified through comparison of expression profiles between regenerative nodules and early liver cancer^[55-57]. On the other hand, we and other groups have highlighted HCC with nodule-in-nodule appearance to investigate gene alterations in the course of liver cancer progression^[58,59].

Intrahepatic recurrence is one of the main causes of poor prognosis in HCC, and Iizuka *et al*^[21] reported a gene set consisting of 12 genes that can predict intrahepatic recurrence within 1 year after curative surgery using a supervised learning method to construct a predictive system. On the other hand, Cheung *et al*^[60] identified 90 genes that are correlated with intrahepatic metastasis and may provide clues to identify patients with increased risk of developing metastasis.

Patients with advanced HCC with tumor thrombi in the major portal branches are no longer candidates for surgical

resection, and combination chemotherapy with intra-arterial 5-fluorouracil (5-FU) and subcutaneous interferon- α is one of a few effective chemotherapeutic regimens for such advanced HCC. Kurokawa *et al.*^[22] selected 63 genes capable of predicting chemotherapeutic responses to 5-FU and IFN- α combination therapy using PCR-based array, which will lead to tailor-made medicine for advanced HCC.

KARYOTYPING ANALYSIS OF HEPATOCELLULAR CARCINOMA USING ARRAY-BASED COMPARATIVE GENOMIC HYBRIDIZATION AND SINGLE NUCLEOTIDE POLYMORPHISM ARRAY

In addition to expression profiling analysis, genome dosage analysis using aCGH for HCC has been reported^[26,27]. Takeo *et al.*^[61] analyzed 20 HCC samples by genomic DNA microarray analysis using an array containing 57 oncogene spots and emphasized the utility of microarray technology compared to conventional CGH. Katoh *et al.*^[62] investigated the significance of correlations of frequent chromosomal aberrations with various clinicopathological features, and demonstrated that chromosomal loss on 17p13.3 and gain on 8q11 were independent prognostic indicators by multivariate analysis. Furthermore, Patil *et al.*^[63] correlated gene expression with aCGH data and identified high-level expression of *JAB1* on 8q, which was shown to have a potential role in the development of HCC by functional analysis.

GIM is a novel algorithm for detecting copy number changes at both gene and allele levels using SNP arrays^[28]. We applied GIM to 36 HCC samples, and analyzed copy number alterations and allelic imbalance accurately in the liver cancer genome in a single experiment^[64]. That is, 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 contains 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 a tumor suppressor gene in HCC was elucidated.

SYSTEMATIC INTEGRATION OF EXPRESSION PROFILES WITH OTHER TYPES OF GENE INFORMATION

As described above, strong correlations between expression profiles and various HCC classifications, including hepatitis viral infection, tumor differentiation grade, and prognosis, have been reported. The next

era of gene expression analysis will involve systematic integration of expression profiles and other types of gene information. Patil *et al.*^[24] analyzed gene ontology categories for the 703 over-expressed genes selected by microarray analysis and concluded that metabolism, cell cycle, growth, and proliferation may be involved in HCC development.

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 chromosomal region^[65]. 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, which is consistent with previous reports using CGH in liver cancer. Furthermore, more poorly differentiated liver cancer contains more chromosomal alterations, which are accumulated in a stepwise manner in the course of HCC progression. Taken together, we demonstrated that gene expression profiles are subject to chromosomal bias in EIM analysis^[23].

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. Furge *et al.*^[66] obtained regional expression biases (REBs) from a multiple span moving binomial test and demonstrated that REBs overlapped genetic abnormalities identified using aCGH in HCC. Comparing the expression intensity with the genome dosage obtained from GIM directly, we also confirmed that alterations in mRNA expression level reflect gain or loss of genomic copy number, and substantiated our assertion in the previous report using EIM^[23,64].

CONCLUSIONS

Candidate genes for gene therapy and diagnostic markers are selected through microarray analysis and shown to be available for clinical application. In addition, clustering analysis based on clinicopathological features is performed. However, bioinformatics technology indicates that gene expression profile is subject to chromosomal bias, *i.e.*, clustering analysis involves the risk of being affected by gene structural abnormalities, such as genomic gains and losses.

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