Genomic copy number alterations with transcriptional deregulation at 6p identify an aggressive HCC phenotype

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Abbreviations: CGH, comparative genomic hybridization; ER, early recurred group; FFPE, formalin-fixed, paraffin-embedded; NT-CTRL, non-targeting control; HB, hepatoblast; HCC, hepatocellular carcinoma; L.R, late recurred group; ROI, region of interest; shRNA, short hairpin RNA; TM, T-statistic map; qPCR, quantitative PCR.

Materials and methods

DNA copy number profiling

We analyzed the genomic DNA profiles of 15 HCC samples and a normal reference liver used in the previous study (7), which had ultra-high-resolution genome-wide tiling array comparative genomic hybridization (CGH) profiles with >3 million probes. The regional copy number alteration was estimated by T-statistic-based sliding window approach, that is, T-statistic map (TM) as described previously (7). In brief, TM scores were calculated as the T-statistic values, which were obtained by applying one-sample T-test on the probe values for HCC samples within a moving window (100 kb). The threshold for significant TM score was determined as the highest TM score from 100 random data sets, which were generated by randomly ordering the probe positions. The significant TM regions were determined using a segmentation algorithm. The probe positions neighbors within 5 Mb by the above threshold of TM score (2Mb for two-sample TM) were regarded as same segment. Before segmentation, the probes were excluded whose TM scores were above threshold but not neighboring by the thresholded probes within 100 kb. The segments sized <100 kb were also excluded from subsequent analysis (For details, see Supplementary Methods, available at Carcinogenesis Online).

DNA preparation from frozen tissues and formalin-fixed, paraffin-embedded tissues

DNA copy numbers were examined from 21 cases of formalin-fixed, paraffin-embedded (FFPE) samples of tumor and paired non-tumoral surrounding tissues obtained from Korea University Medical Center. The study protocol was approved by the institutional review board. The genomic DNAs from FFPE were extracted using the RecoverAll™ Total Nucleic Acid Isolation Kit (Ambion, Grand Island, NY) following the manufacturer’s protocol, and the DNA concentrations were measured by Nanodrop (Thermo-Fisher, Wilmington, DE). In addition, 52 cases of genomic DNAs from frozen HCC tissues obtained from National Cancer Institute were examined.

Quantitative PCR for genomic DNA copy number estimation

DNA copy numbers of HCC samples were estimated by real-time quantitative PCR (qPCR). PCR reaction was performed on an ABI PRISM 7000 machine (Applied Biosystems, Foster City, CA) using SYBR Green Kit. PCR conditions were 95°C for 10 min and 40 cycles of 95°C for 15 s, and 60°C for 1 min.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world (1). The inherent heterogeneity of HCC in genomic level produces difficulties in characterizing molecular pathogenesis of the disease and developing efficient treatment modalities. Ablant genetic and epigenetic events occur and accumulate during HCC development, which may contribute to the development of HCC heterogeneity (2). Particularly, genomic copy number aberrations ranging from deletion of one or both copies of chromosomal regions to gains of numerous additional copies have been considered to play critical roles during tumor development, linking to disease pathogenesis and clinical behavior in many tumor types. However, many of the genes residing in the recurrently amplified or deleted regions are expressed neither in normal nor in tumor tissues, making the functional role of these copy number–altered genes unclear. Thus, the approach to identify the genes, which have concomitant transcriptional deregulations as well as DNA copy number alterations, might be more reliable analysis strategy (3–6). In this context, we have previously identified the DNA copy number–dependent gene expression alterations by performing integrative analyses combining gene expression and DNA copy number profiles (7). Although we have successfully identified the DNA copy number–dependent gene expression alteration in the previous study, the genomic heterogeneity of cancers at transcriptional level was not considered. Therefore, we decided to further investigate the DNA copy number–dependent gene expression from the more homogeneous subgroups of HCC. Previously, 139 cases of HCC have been classified based on gene expression similarity, which revealed subclasses of subtype A with poorer survival and subtype B with less aggressive phenotype (8). In addition, a subtype hepatoblast (HB), which had been identified among the A type HCC, harbored the stem cell–like gene expression trait and showed the worst prognostic outcome, implying the stem-like cell of origin of the tumors (9). In this study, we extended the previous work by integrating the DNA copy number profiles of the distinct transcriptomic subtypes A, B and HB. This analysis could identify novel chromosomal regions of subtype-specific DNA copy number alteration and concomitant transcriptional deregulation.

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Primers for IER3 (forward: 5'-CGCCGAAATGGAGGAA-3', reverse: 5'-ACTGCGCAGATACTGAGA-3'), HP1A1 (forward: 5'-CGACCTGTA ACAAGAGCCTC-3', reverse: 5'-AAAGTCTGGCTCGTCTGTG-3'), CD37 (forward: 5'-ACTCTCCGTGCTCTCTAAA-3', reverse: 5'-ATCTTGTTGTTGTTGAGG-3'), SL231A1 (forward: 5'-CCCTGAGACAGCCTGGAAC-3', reverse: 5'-GACACCGGGAAGGACGAAG-3') and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, forward: 5'-CGACCATTTGTCAGCTA-3', reverse: 5'-AGAGTGGTCAGGCGCTT TT-3'). Each sample was assayed in duplicate and a control normal liver DNA was included in every assay. DNA copy number was measured as 2^−ΔCt and normalized with that of the GAPDH in each sample, and the relative values compared with that of a normal liver sample was calculated. For FFPE samples, each of paired non-tumoral DNA copy number was assayed and used for data normalization.

Gene set enrichment analysis

The enriched expression of stem cell trait in HCC was evaluated by calculating the enriched expression of HB signatures (9,10). HB subtype with stemness trait was determined based on the enrichment of both up- and downregulated HB signatures, which was estimated by calculating the hypergeometric P-values in each sample as described previously (P < 0.01) (11). The Gene Ontology analysis for biological processes was performed using DAVID software (12).

Short hairpin RNA-mediated knock-down experiment

The short hairpin RNA (shRNA) expression vectors targeting IER3 were constructed as follows. To obtain shRNA-expressing plasmid, pSME lentiviral vector was used as a backbone plasmid with minimal modification as described elsewhere (13). In brief, shRNA insertion site on original vector was replaced to Pacl and Nhel site by introducing new multi cloning site. Two independent IER3 target sites were designed from the website (http://katahdin.cshl.org/shRNA/RNAi.cgi?type=shRNA) and synthesized (Supplementary Table 1, available at Carcinogenesis Online). To generate double-strand shRNA, each synthesized template was subjected into PCR using following primers: Pacl-FW, 5'-CAGAAAGTTAATTAAAAAAAAAGGTATATGCTGTTGACAGTGAGCG-3' and Nhel-RE, 5'-CTAAATGATGCCGTTGAGGACATGAGC-3'. Underlines indicate each restriction enzyme site, pSME-Nt-expressing scramble shRNA was used as a non-targeting control (NT-CTL).

qPCR for messenger RNA expression level estimation

Forty-eight hours after transfection, messenger RNA was extracted from the transfected HepG2 cells using the mirVana™ miRNA Isolation Kit (Ambion) as per manufacturer’s protocol. First-strand complementary DNA synthesis was performed using High-Capacity cDNA Reverse Transcription Kit (Invitrogen, Foster City, CA) following the manufacturer’s instruction. Messenger RNA expression level was quantified using a Rotor Gene Q (Qiagen, Foster City, CA) according to the manufacturer’s instruction. Western blot analysis

The short hairpin RNA-expressing plasmid pSME was used as a backbone plasmid with minor modification as described previously (14). To obtain shRNA-expressing plasmid, pSME lentiviral vector was used as a backbone plasmid with minimal modification as described elsewhere (13). In brief, shRNA insertion site on original vector was replaced to Pacl and Nhel site by introducing a multi-cloning site. Two independent IER3 target sites were designed from the website (http://katahdin.cshl.org/shRNA/RNAi.cgi?type=shRNA) and synthesized (Supplementary Table 1, available at Carcinogenesis Online). To generate double-strand shRNA, each synthesized template was subjected into PCR using following primers: Pacl-FW, 5'-CAGAAAGTTAATTAAAAAAAAAGGTATATGCTGTTGACAGTGAGCG-3' and Nhel-RE, 5'-CTAAATGATGCCGTTGAGGACATGAGC-3'. Underlines indicate each restriction enzyme site, pSME-Nt-expressing scramble shRNA was used as a non-targeting control (NT-CTL).

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First, to exclude the HB-like subtype (i.e., stemness-positive tumors), we evaluated the expression of HB-like signatures by calculating the hypergeometric P-values in each sample as described previously (11). After excluding the HB subtype, which express both upregulated and downregulated HB signatures (P < 0.01), we stratified the 48 cases of stemness-negative HCC samples into two groups by the median DNA copy numbers of IER3. The HCC samples with high DNA copy numbers of IER3 showed shorter overall survival compared with those with low DNA copy numbers of IER3 (P = 0.056, log-rank test) (Figure 3B). The stemness-harboring HB-type tumors had no significant changes in IER3 copy numbers (Supplementary Figure 1, available at Carcinogenesis Online).

The clinical implications of IER3 were also evaluated at transcriptional level. After leaving out the HCC samples with HB signature, the stemness-negative HCC samples (n = 135) were stratified into two groups based on the expression levels of IER3. The IER3-positive patients (expression levels > 0, n = 47) showed shorter overall survival (P = 0.002, log-rank test) as well as recurrence time (P = 0.049, log-rank test) compared with those of the IER3-negative patients (expression levels < 0, n = 88) (Figure 3C). In addition, by using the same method, we further validated our result with the LEC data set (data from the Laboratory of Experimental Carcinogenesis, National Cancer Institute, n = 139). After excluding the 21 samples expressing HB signatures, the HCC patients with increased IER3 expression showed shorter overall survival (P = 0.048, log-rank test) and earlier recurrence time (P = 0.156) with marginal statistical significance compared with those with IER3-negative patients (Figure 3D). Taken together, we could suggest that the DNA copy number alteration and concomitant transcriptional alteration of IER3 may contribute to the aggressive progression particularly in the stemness-negative HCC.

Next, we evaluated the biological relevance of the IER3 expression in HCC progression. Knock-down experiment of IER3 was performed by using two shRNAs targeting different sites. Morphological changes were observed under microscopic examination resulting in decreased number of cells and shrunken cells as compared with control (Figure 4A). The effect on cell viability and proliferation was examined by using Cellceim AM and WST-1 assay. Both of shRNA mediated knockdown of IER3 revealed significant inhibition of HepG2 liver cancer cell viability and proliferation (Figure 4B and C). The shRNA-mediated targeted down-expression of IER3 messenger RNA was validated by real-time qPCR (Supplementary Figure 2, available at Carcinogenesis Online). In addition, we examined the possible involvement of apoptotic pathways, which may lead to abnormal cell proliferation. Indeed, IER3 has been notified to have controversial effects on pro-apoptotic or anti-apoptotic signaling (15). We observed that IER3 knockdown evoked the apoptosis by detecting the cleaved form of caspase proteins (i.e. 3, 7 and 9 isoforms) although those signals were not much prominent (Figure 4D). Furthermore, we could observe that the invasive property of HepG2 cells was significantly suppressed by IER3 knockdown by performing Matrigel invasion assay (Figure 4E and F). Taken together, these results suggest that the IER3 has a functional role in HCC aggressiveness, and which may be at least in part through the regulation of signaling pathways for cancer cell proliferation and apoptotic cell death. As a proof-of-principle, our data suggest that IER3 is a candidate biomarker for aggressive HCC subtype with functional evidence.

**Discussion**

In this study, we employed a high-resolution whole-genome tiling array CGH to map the regions with chromosomal gain or loss in HCC genome. The array CGH includes >3 million probes across whole genome and the multiple probes corresponding to genomic regions of encoding genes increase not only resolution but also sensitivity in detecting the chromosomal alterations. The chromosomal regional patterns of DNA copy number alteration and transcriptional deregulation were determined by using the sliding window approaches, that is, TM and TCM, respectively. The samples were selected from the molecular subtypes with homogeneous gene expression pattern; therefore,
we could define the molecular subtype-specific DNA copy number profiles. We found that the HCC subtype with stemness trait (HB-like subtype) displayed minimal alteration in DNA copy numbers. This suggests that the pathogenesis of the subtype HB tumors might be different from the conventional HCC progression model of the multi-step accumulation of genetic aberration such as DNA copy numbers.

Fig. 1. DNA copy number profiling in HCC subtypes. Genome-wide TM for HCC subtypes HB (A), A (B) and B (C) is constructed by applying one-sample TM with sliding window 10 Mb. TM for HB versus A (D) and A versus B (E) are constructed by applying two-sample TM with sliding window 2 Mb. The threshold for each subtype is determined based on 100 random simulation tests using randomly ordered data sets. (Detailed methods are described in Supplementary Methods, available at Carcinogenesis Online.)
Fig. 2. HCC subtype–dependent copy number alteration and gene expression levels. (A) TM profiles of chromosome 6 for each subtype HB, A and B (left panel), and the TM profiles for A versus B at chromosomes 19 and 20 are shown (right panel). (B) Box plots indicate the distribution of the log2-transformed DNA copy numbers of HSPA1A, IER3, CD37 and SLC31A1 in the 52 HCC samples of subtypes HB (n = 10), A (n = 20) and B (n = 22). The significances of the subtype difference are estimated by Wilcoxon rank-sum test (A versus HB and A versus B, respectively). (C) Expression profiles of the 128 genes located at 6p21–p24 (overlapped region of CR and TM A versus B region 4–9) are shown according to the subtypes HB, A and B. Pseudo-colored heat map represents the gene-centered relative values across samples. (D) Bar plot indicates average expression values of the 128 genes in each subtype HB (n = 22), A (n = 40) and B (n = 77). P-values are estimated by two-sample T-test.
Fig. 3. Clinical correlation of IER3 copy numbers and expression levels. (A) Validation of the prognostic predictability of IER3 DNA copy number by qPCR analysis using the FFPE tissues from an independent cohort \((n = 21)\). The differential expression levels of IER3 between HCC and paired surrounding non-tumoral liver tissues are determined. The patients are stratified into the ER group whose tumors were recurred <1 year after surgical resection \((n = 14)\) and the LR \((P = 0.015, \text{ Wilcoxon rank-sum test})\). (B) Clinical correlation of DNA copy numbers of IER3 is evaluated by Kaplan–Meier plot analyses of survival and tumor recurrence time with an independent validation cohort. A total of 48 cases of non-HB HCC (stemness-negative) samples were divided into IER3-positive and IER3-negative groups by the median DNA copy numbers of IER3. (C and D) The clinical correlation of the transcriptional levels of IER3 is evaluated by Kaplan–Meier plot analyses for survival and tumor recurrence time in the two independent data sets from LEC (C) and GSE14322 (D), respectively. The patients were stratified into IER3-positive or IER3-negative group based on IER3 expression level of greater than or less than 0, respectively. Statistical significance of log-rank test is indicated. The follow-up time for survival and recurrence time is truncated to 5 years after surgical resection.
and/or mutations. The innate expression of stemness trait rather than the aberrant events at DNA level may contribute to the aggressive phenotype of HB tumors. The comparison of the aggressive and less aggressive type of HCC based on gene expression patterns showed the differential aberration of copy numbers. The subtype A (aggressive) showed more prominent DNA copy number changes with concomitant transcriptional deregulation at the ROI on chromosome 6p compared with the subtype B (less aggressive). In the previous studies including ours, the amplifications at different chromosomal regions including 1q, 8q and 17q, or the deletions at 4q, 8p and 16q, were thought to play key roles in the development of the advanced HCC (7,16). In addition to these, our recapitulation of the data considering the gene expression patterns could identify a novel ROI at 6p21–p24 and a novel marker IER3. This reveals the biological distinction between the subtypes A and HB at DNA copy number level.

In support of our findings, frequent amplification at ROI 6p and its clinical correlation have been reported for various cancer types including HCC (17–20). Of the ROI genes (n = 128), we focused on the IER3 gene. Both the DNA copy numbers and gene expression levels of IER3 showed clinical correlation, and that was validated in an independent cohort. Functionally, IER3 (formerly IEX-1) encodes a 27 kDa glycoprotein known to regulate death receptor-induced apoptosis (21–23). However, there have been reports about the contradictory role of IER3 in apoptosis such as pro-apoptotic and antiapoptotic functions (15,24). In addition, the oncogenic potential of IER3 has been noticed. For example, IER3 is involved in the development of T-cell lymphoma, skin carcinogenesis (25) and drug responsiveness of osteosarcoma (19). IER3 rearrangements, amplification and deregulated expression were also reported in patients diagnosed with myelodysplastic syndromes (26). Higher expression of IER3 has been reported in invasive lesions than pre-invasive lesions from mouse and human tumor tissues (27). Recent study also revealed that IER3 expression facilitates the progression of pancreatic ductal adenocarcinoma (28). However, conflicting evidences are also reported. For example, the association of IER3 expression with better prognosis was shown in pancreatic cancer implying its tumor suppressive activity (29). Prominent downregulation of IER3 was also found in myelodysplastic syndromes compared with normal tissues (30). Apparently, such controversies in the role of IER3 in carcinogenesis require further elaboration to validate the clinical and
functional relevance of IER3 in cancer development and progression. Moreover, to our knowledge, the role of IER3 in liver cancers has not been studied yet. With this concern, we demonstrated the biological relevance of the IER3 by performing IER3 targeting knock-down experiments in HepG2 liver cancer cells. We also tested two different liver cancer cells of Hep3B and HuH-7 cells; however, the inhibition of IER3 has no effect on the viability of these cells (Supplementary Figure 3, available at Carcinogenesis Online). This might be due to their expression of stem cell–like features. Supporting to this, a previous study has reported the substantial expression of stemness marker, CD133+, in the Hep3B and HuH-7 by using the fluorescence-activated cell sorting analysis, whereas HepG2 showed only <10% of CD133+ expression comparing those two cell lines (31). However, further elaboration with the cells expressing differentially the stemness and/or 6p copy numbers might be required to address the mechanistic relations of IER3 expression with the expression of stemness trait or gain at 6p.

In summary, our integrative analysis has established an analytical strategy for the integration of multilayered genomic profiles of DNA and RNA levels, which provide a striking advantage for delineating the heterogeneous HCC progression and its underlying pathobiology. We successfully demonstrated the aggressive phenotype HCC could be discriminated by combining the features of the expression of stemness trait and DNA copy number aberration at the ROI 6p21–p24. By demonstrating functional and clinical significance, IER3 was identified as a putative functional biomarker for an aggressive HCC subtype without stemness trait. Our analysis suggests that DNA copy number aberration might be important in the progress of aggressive tumor particularly among the stemness-negative tumors, providing novel insight on the multilayered (DNA and RNA) tumor heterogeneity.

Supplementary material
Supplementary Methods, Tables 1–6 and Figures 1–3 can be found at http://carcin.oxfordjournals.org/

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References

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