Accumulation of Molecular Aberrations Distinctive to Hepatocellular Carcinoma Progression

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ABSTRACT

Cancer develops through the accumulation of genetic and epigenetic aberrations. To identify sequential molecular alterations that occur during the development of hepatocellular carcinoma (HCC), we compared 52 early and 108 overt HCC samples by genome sequencing. Gene mutations in the p53/RBI pathway, WNT pathway, MLL protein family, SWI/SNF complexes, and AKT/P13K pathway were common in HCC. In the early phase of all entities, TERT was the most frequently upregulated gene owing to diverse mechanisms. Despite frequent somatic mutations in driver genes, including CTNNB1 and TP53, early HCC was a separate molecular entity from overt HCC, as each had a distinct expression profile. Notably, WNT target genes were not activated in early HCC regardless of CTNNB1 mutation status because β-catenin did not translocate into the nucleus due to the E-cadherin/β-catenin complex at the membrane. Conversely, WNT targets were definitively upregulated in overt HCC, with CTNNB1 mutation associated with downregulation of CDH1 and hypomethylation of CpG islands in target genes. Similarly, cell-cycle genes downstream of the p53/RB pathway were upregulated only in overt HCC, with TP53 or RBI gene mutations associated with chromosomal deletion of 4q or 16q. HCC was epigenetically distinguished into four subclasses: normal-like methylation, global-hypomethylation (favorable prognosis), stem-like methylation, and CpG island methylation. These methylation statuses were globally maintained through HCC progression. Collectively, these data show that as HCC progresses, additional molecular events exclusive of driver gene mutations cooperatively contribute to transcriptional activation of downstream targets according to methylation status.

Significance: In addition to driver gene mutations in the WNT and p53 pathways, further molecular events are required for aberrant transcriptional activation of these pathways as HCC progresses.

Introduction

Early hepatocellular carcinoma (HCC) is defined as a highly differentiated liver cancer with stromal invasion into the remaining Glisson’s triad inside the tumor (1). When these small precursor lesions reach 1.5 to 2.0 cm in diameter, less differentiated cancerous lesions reach 1.5 to 2.0 cm in diameter, less differentiated cancerous lesions are still considered as early HCC (2). As has been previously asserted in the colorectal cancer (3), early HCC arises from chronic liver disease and progresses with accumulated mutations to overt HCC in a stepwise manner (1, 4). Using such samples, we and others have characterized genetic and epigenetic alterations associated with early versus late molecular events of hepatocarcinogenesis (5–7), and TERT promoter mutation is the most frequent molecular aberration in early HCC (7).

With recent advances in biological technologies, high-throughput genome sequencing has been used to elucidate the genetic basis of many diseases. To date, several researchers have used next-generation sequencing (NGS) technologies to identify molecular aberrations in HCC specimens (8–15), and a large number of mutations in genes involved in the p53 and β-catenin pathways and somatic mutations of genes involved in chromatin remodeling (16), histone methylation (9), AKT/P13K signaling (16), and JAK/STAT signaling (11) were common in HCC. Furthermore, it was attempted to classify overt HCC into subtypes mainly with respect to etiological background of the underlying liver disease and pathologic aspects of HCC. However, NGS has not been used to characterize genetic aberrations associated with early HCC for any large-scale set of samples; consequently, the genetic mechanisms of progression from early to overt HCCs is still unknown.

Besides the gene expression profile and mutational signature, landscape of aberrant methylation of epideivhers has been elucidated by genome-wide methylation profiling (17, 18). In these reports, utility of panel of methylated genes for early biomarkers of HCC using plasma DNA were determined (17), and predictive biomarkers based on promoter DNA methylation of several epideivhers were identified for patients with HCC (18).

Here, we characterized the genetic and epigenetic driver aberrations responsible for initiation and progression of live cancer using...
chronic liver disease, early HCC, and overt HCC samples. Moreover, the sequential molecular changes were confirmed using HCC with nodule-in-nodule appearance (NIN) samples and identified the pathways that were frequently responsible for liver cancer progression.

Materials and Methods

Patients

The study group comprised 160 patients with HCC who were undergoing liver resection in the Department of Digestive Surgery, Nihon University; each participant provided written informed consent, and this study received approval (protocol number: 131) by the institutional review boards of Nihon University (Supplementary Table S1). All clinical investigations were conducted according to the principles of the Declaration of Helsinki.

Early HCC was histologically determined according to the criteria defined by International Consensus Group for Hepatocellular Neoplasia (1): 52 and 108 liver tumors were independently diagnosed as early or overt HCCs, respectively, by two trained pathologists. In addition to 160 HCC samples, randomly selected 31 liver samples were analyzed for gene expression analysis and methylation analysis. Therefore, 191 samples were analyzed in total. Surgical specimens were immediately cut into small pieces after liver resection, then snap frozen in liquid nitrogen and stored at −80°C.

DNA and RNA extract

QiAamp DNA Mini Kit (Qiagen) and TRizol (Invitrogen) were used according to the manufacturer’s protocols to isolate genomic DNA and total RNA, respectively. Genomic DNA and total RNA concentrations were determined by dsDNA BR Assay using the Qubit (Life Technologies) and the NanoDrop Spectrophotometer (NanoDrop Technologies), respectively; RNA integrity was assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies).

DNA and RNA sequencing

An Illumina HiSeq 2000 (Illumina) and 1 μg of genomic DNA or total RNA were used for each sequencing procedure. Exome sequencing was performed on 160 pairs of HCC tumor specimens and matched adjacent nontumorous liver specimens according to the manufacturer’s protocol. Briefly, DNA was fragmented using a Covaris SS Ultrasonicator. Exome capture was performed using Agilent SureSelect V4/V5 (Agilent Technologies) or HGSC VCRome 2.1 design1 (42 Mb; NimbleGen). Each sample was sequenced as Agilent SureSelect V4/V5 (Agilent Technologies) or HGSC VCRome 2.1 design1 (42 Mb; NimbleGen). Each sample was sequenced as 100-bp pair-ended reads. RNA sequencing was performed to investigate expression of genes and gene fusion events. An RNA sequence library was prepared using a TrueSeq Stranded mRNA Library Prep Kit (Illumina).

Mutation calling

Exome sequence data were used to identify somatic mutations and chromosomal aberrations as described previously (15); we used Burrows-Wheeler Aligner (19) and Novoalign software to align NGS reads to the human reference genome GRCh37/hg19. After removal of PCR duplicates, SRMA [1] was used to improve variant discovery through local realignments. To identify somatic mutations, we used the integrated genotyper software karkinos (http://github.com/genome-rcast/karkinos) as described previously (15). Briefly, variant allele frequencies of somatic mutation were adjusted by estimated tumor content ratios and were filtered with a heuristic algorithm and statistical filtering algorithm such as Fisher exact test; tumor read depth >4 and allele frequency >0.12 were used, and low mapping quality reads and orientation biased reads were removed.

P value for driver mutation

An initial probability for observing recurrent somatic mutation on the same gene was calculated via a binomial probability equation, gene length, and the background mutation rate; FDRs were calculated via simulation as described previously (20).

Copy number analysis

After normalizing and adjusting GC content bias, a tumor-to-normal tissue depth ratio was calculated, and copy number peaks were estimated using wavelet analysis with complex Gaussian models. Hidden Markov model with calculated Gaussian models were constructed, and copy number peaks were annotated as absolute integer copy numbers.

Gene expression

NGS reads were mapped to a cDNA database (UCSC genes) and reference genome GRCh37/hg19 independently using the Burrows-Wheeler Aligner. After cDNA coordinates were converted to genomic positions, an optimal mapping result was chosen either from cDNA or genome mapping by comparing the minimal edit distance to the reference. Local realignment was then performed with an in-house short reads aligner with a smaller k-mer size (k = 11). Finally, fragments per kilobase of exon per million fragment-mapped values were calculated for each UCSC gene while considering strand-specific information. Batch information and patient background were shown in Supplementary Table S2.

Reverse-transcription PCR for fusion genes

Transcription of TERT fusion genes were detected by reverse-transcription PCR (RT-PCR); FastStart Taq Polymerase (Roche) and the following parameters were used for RT-PCR: denaturing at 95°C for 30 seconds, annealing at 60°C or 56°C for 30 seconds, respectively, and extension at 72°C for 1 minute. Primers used for RT-PCR are as follows: a forward primer, 5'-GATCGGTGAATGTCGGAGAC-3' and a reverse primer, 5'-TACACACTCATCAGCCAGTG-3' for SLCl2A7-TERT fusion, and a forward primer, 5'-TAGGCGCTTGG-GACCTCAC-3' and a reverse primer, 5'-AAAGACCTGAGGAC-TGCAC-3' for fusion of 10q25.2 and TERT.

IHC

To detect TERT in the HCC cells, cancerous specimens with TERT fusion genes were subjected to IHC using an automated Immunohistochemical Staining Methods (Dako) according to the manufacture’s protocol. Sections from HCC formalin-fixed paraffin-embedded specimens were incubated with anti-TERT antibody (Lifespan Bioscience).

Methylation profiling

Methylation status was analyzed using HumanMethylation450 BeadChip (Illumina) as described previously (21). Briefly, 500 ng of genomic DNA was bisulfite-converted using an EZ DNA Methylation Kit (Zymo Research). The converted DNA was amplified, fragmented, and hybridized to a BeadChip. The raw signal intensity for methylated and unmethylated DNA was measured using a BeadArray Scanner (Illumina). After color-bias correction, background subtraction of the signal intensities, and interarray normalization on Genome Studio (Illumina), the raw methylation value for each CpG was defined as $\beta = M/(M + U + 100)$, where $M$ and $U$ were the intensities of methylated and unmethylated probes, respectively.
Statistical analysis

Pearson’s chi-square test, Student t test, and Wilcoxon rank-sum test were used to assess the statistical significance of data collected from early and overt HCC groups. Survival curves were generated using the Kaplan–Meier method and compared by the log-rank test. Statistical significance was set at $P < 0.05$.

Sequence data

Sequence data are available on the Japanese Genotype-phenotype Archive (JGA, http://trace.ddbj.nig.ac.jp/jga) under accession number JGAS00000000233.

Results

Expression profile, molecular karyotyping, and mutation spectrum

Hierarchical clustering of the expression data indicated that the three different specimen types (Fig. 1A; Supplementary Fig. S1A) were mostly classified (Fig. 1B). For overt HCCs, WNT target genes and cell cycle related genes were enriched by mutation of the $\text{CTNNB1}$ and $\text{TP53}$ genes, respectively. Consistent with our previous data using SNP array (6), copy number analysis showed that chromosomal aberrations of early HCC were much smaller than those of overt HCC ($P < 0.001$; Fig. 1C). Similarly, the number of mutation events and fusion events per tumor was significantly frequent in overt HCC ($P < 0.001$; Supplementary Fig. S1B), whereas the distribution did not significantly differ between the two HCC types ($P = 0.946$; Table 1).

Gene mutations in accordance with HCC progression

Mutation analysis showed that five pathways (denoted p53/RB, WNT, MLLs, SWI/SNF, and AKT/PI3K), in addition to the $\text{TERT}$ promoter, were frequently altered in HCC samples (Fig. 1D). Although mutation of $\text{CTNNB1}$ was common in the two groups, mutations in the WNT pathway genes were significantly more common in overt HCCs than in early HCCs ($P = 0.014$). The p53/RB pathway and histone methylase genes were also frequently mutated in both groups. Conversely, mutations in the SWI/SNF complex genes ($P = 0.026$) and seven AKT/PI3K genes ($P = 0.045$) were more common in overt HCCs. Most notably, $\text{ARID2}$ mutations were significantly more frequent in overt HCC ($P = 0.002$).

Figure 1.

Overview of genetic aberrations evident in 160 HCC and 31 liver samples. A, Macroscopic findings of early and overt HCCs. White arrowheads, the boundary of early HCC. B, Hierarchical clustering of gene expression. Expression levels for each gene were normalized across the samples and values are shown as relative scales. The concentration of gene category is shown on the right side, and mutations of $\text{CTNNB1}$ and $\text{TP53}$ are shown below the heat map. C, Frequency of copy number alterations. Copy number analysis with exome sequence data from early HCC specimens revealed frequent copy number gain on 1q (29.8%) and LOH on 17p (27.7%). However, gains on 1q (41.5%) and 8q (39.0%) and LOH on 1p (27.6%), 4p (22.5%), 16q (33.3%), and 17p (52.4%) were each common in classical HCC specimens. D, Landscape of significantly altered pathways. The color of a box indicates the proportion of mutated cancer cells within the tumor sample according to the scale below. Number of mutation per 10 Mb base is shown on the top.
Activation of WNT target genes

To examine the relationship between gene expression and mutation in HCC, an integrated analysis of the different types of sequence data was performed. Nonnegative matrix factorization from the expression data showed that 160 HCC samples were divided into three clusters; PROLIF_UP, CTNNB1_UP, and OTHERS, which are registered in MSigDB by Gene Set Enrichment Analysis (22). The PROLIF_UP and CTNNB1_UP clusters were enriched by TP53 (P < 0.001) and CTNNB1 (P < 0.001) gene mutations, respectively (Fig. 2A; Supplementary Figs. S2A and S2B). Conversely, the OTHERS cluster included most of early HCC samples (94.2%; P < 0.001).

Notably, despite the frequent mutations of CTNNB1 (13.7%) and TP53 (18.7%) genes in early HCC, CTNNB1_UP and PROLIF_UP gene sets were not upregulated (Supplementary Fig. S2C).

For each HCC type, we compared gene expression patterns between HCC samples with and without mutations in the WNT pathway genes; specifically, we assessed expression differences in the WNT target gene cluster as "CHIANG_LIVER_CANCER_SUBCLASS_CTNNB1_UP (M16496)" in MSigDB (22, 23). WNT targets (154 genes) were not activated in early HCC, regardless of WNT status; notably, this gene cluster was definitively upregulated only in overt HCC with CTNNB1 mutations associated with hypomethylation of CpG sites (4.9%) of the target genes (Fig. 2B), whereas 35.8% of CpG sites of WNT targets

Table 1. Mutation pattern of HCC (nonsynonymous).

| Mutation type | Early HCC (n = 52) | Overt HCC (n = 108) | P value |
|---------------|--------------------|---------------------|---------|
| C>G>T>A (%)   | 15.5 ± 5.8 (27.3)  | 20.0 ± 6.7 (25.8)   |         |
| T>A>C>G (%)   | 15.2 ± 8.2 (23.3)  | 22.6 ± 9.7 (29.1)   | 0.046   |
| C>G>A>T (%)   | 13.5 ± 9.5 (23.9)  | 15.1 ± 7.3 (19.4)   |         |
| T>A>G>C (%)   | 4.1 ± 3.2 (7.2)    | 5.6 ± 4.9 (7.2)     |         |
| T>A>A>T (%)   | 5.7 ± 3.8 (10.2)   | 7.7 ± 5.7 (9.9)     |         |
| SNV           | 56.6 ± 26.5        | 77.6 ± 27.8         | <0.001  |
| Indel         | 5.4 ± 3.5          | 6.4 ± 5.0           | 0.043   |
| Total         | 62.0 ± 28.0        | 84.0 ± 29.6         | <0.001  |

Note: Data were shown by average with SD.

Figure 2.

Gene expression of WNT target genes. A, Gene expression profile by nonnegative matrix factorization (top) with mutation of CTNNB1 and TP53 genes (bottom). The 160 HCC samples were divided into three clusters. B, The WNT target-gene set “CHIANG_LIVER_CANCER_SUBCLASS_CTNNB1_UP (M16496)” in MSigDB (22, 23). WNT targets (154 genes) were not activated in early HCC, regardless of WNT status; notably, this gene cluster was definitively upregulated only in overt HCC with CTNNB1 mutations associated with hypomethylation of CpG sites (4.9%) of the target genes (Fig. 2B), whereas 35.8% of CpG sites of WNT targets

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were constitutively unmethylated regardless of CTNNB1 gene mutation status (Supplementary Table S3). The positions of CTNNB1 mutations were not different between the early and overt HCC samples (Supplementary Fig. S2D). As a representative example, methylation status of CpG sites and WNT status of GLUL gene were shown (Supplementary Fig. S2E). Moreover, this cluster of genes was downregulated in specimens with AXIN1 mutations, indicating that the CTNNB1 target genes differ from the AXIN1 target genes (24).

To confirm the nuclear localization of β-catenin in early HCC with CTNNB1 mutations, an IHC study was performed on the formalin-fixed paraffin embedded samples. Immunostaining showed that overt HCC with CTNNB1 mutations exhibited strong immunoactivity in the nuclei of 15 samples (44.1%). In contrast, early HCC with CTNNB1 mutations did not exhibit any β-catenin immunoactivity in the nucleus of cancer cells (Fig. 2C). In addition, CDH1, which binds β-catenin on the cell membrane, was significantly downregulated in overt HCC samples with CTNNB1 mutations compared with early HCC samples (P = 0.004; Fig. 2D; Supplementary Fig. S2F). Decreased expression of the CDH1 gene was associated with heavy methylation of the enhancer region in both early (P = 0.027) and overt (P < 0.001) HCC samples (Fig. 2E), suggesting that the CTNNB1 target genes were not activated because of lack of aberrant β-catenin accumulation in the nucleus due to normal expression of the CDH1 gene in early HCC (25).

**Activation of cell-cycle genes downstream of the p53/RB pathway**

Integrated analysis also showed that despite the TP53 gene mutation with 17p deletion, upregulation of the cell cycle-related genes downstream of the p53/RB pathway (p53 repressed genes; ref. 8) was dominant only in overt HCC in association with 4q and 16q deletion (P < 0.001), but exiguous in early HCCs (Fig. 3A and B). Notably, the candidate suppressor genes, IRF2 (16) and WWOX (26), were included in homozygous deletion within chromosomes 4q35.1 and 16q23.1, respectively, in overt HCCs without TP53 mutations (Supplementary Fig. S3; Supplementary Table S4). Conversely, p53 induced genes were downregulated both in early (P < 0.001) and overt (P < 0.001) HCC samples with TP53 gene mutations (Fig. 3C).

Thus, the WNT pathway and cell cycle-related genes downstream of the p53 pathway were activated in overt HCC, but not in early HCC, even though the CTNNB1 and TP53 genes were often genetically altered in early HCC. Most notably, downregulation of the CDH1 gene...
and the chromosomal deletion of the 4q and 16q arms, were associated with transcriptional activation of downstream targets in the WNT and p53/RB pathways, respectively, during HCC progression.

**Aberrant overexpression of the TERT gene**

*TERT* was the most upregulated and frequently mutated gene in both early (*P* < 0.001) and overt (*P* < 0.001) HCC samples. Transcriptome sequence data revealed three types of gene-fusion events in five HCC samples involving either: a chromosomal insertion in 5p that gave rise to a *SLC12A7-TERT* fusion (Fig. 4A and B; Supplementary Fig. S4A; ref. 27), a translocation between *TERT* and a 10q25.2 loci t(5;10)(p15;q25) (Fig. 4C and D; Supplementary Fig. S4B), or a translocation between *TERT* and *PIK3R1* (Supplementary Fig. S4C). In addition, *TERT* promoter mutations (−124 or −146 bp from ATG start site; Supplementary Fig. S5A), broad gain on 5p and focal gain at 5p15.33 near the *TERT* gene (Supplementary Fig. S5B), and hepatitis B virus DNA integration near the *TERT* promoter (Supplementary Fig. S5C) contributed to *TERT* upregulation in both groups. Consequently, at least one or more aberrations occurred in 44 (84.6%) early and 80 (74.0%) overt HCCs (*P* = 0.159; Fig. 4E).

**Classification of HCC by methylation profiling**

Taking account of a comprehensive set of factorizing rank, non-negative matrix factorization from the methylation data showed that 160 HCC samples were clustered into four groups: G1, normal-like methylation; G2, global hypomethylation; G3, stem-like methylation; G4, CpG island methylation (Fig. 5A; Supplementary Fig. S6A). Most of the CpG islands were unmethylated in liver samples, whereas demethylation of non-CpG islands was common in G2 and G3. Except for G1, more sites were hypermethylated within CpG islands. G1 harbored frequent gene mutations in *TP53* (30.7%) and the WNT pathway genes (35.8%) only in overt HCC. Conversely, mutations in the *CTNNB1* (35.0% in early HCC and 57.1% in overt HCC) and *TP53* (30.0% in early HCC and 23.8% in overt HCC) genes were frequent even in early HCC in G2. Development of HCC in G3 was assumed to be *de novo* because early HCC was not included in this group. Notably,
HCC samples of G3 were enriched by upregulation of the TET1 and EZH2 genes and the high frequency of TP53 mutations (48.0%); however, EZH2 target genes (28, 29) were not significantly methylated by EZH2 upregulation (Supplementary Figs. S6B and S6C). CpG islands of HCC samples of G4 were highly methylated compared with other groups and frequently harbored mutations of TP53 (43.4%) and CTNNB1 (52.1%). Epidemiologically, infection by hepatitis B virus was significantly frequent in G1 (33.3%) compared with other groups (P = 0.011), whereas other clinical characteristics were similar through all the entities.

After a median follow-up period of 2.5 years (range: 0.3–10.1), the 3-year overall survival rates of patients with overt HCC in G1, G2, G3, and G4 were 73.4%, 93.7%, 52.1%, and 71.7%, respectively, where the overall survival period of G2 was significantly longer (favorable prognosis) and that of G3 was significantly shorter (poor prognosis; P = 0.028; Fig. 5B).

To validate the genomic aberrations as sequential changes, we investigated the epigenetic and transcriptome alterations using eight NIN samples in which the inner nodule (overt HCC) originated from the outer nodule (early HCC; Fig. 5C). Hierarchical clustering showed that samples were divided into two groups containing G1 and G2; epigenetic alterations were heritable during the HCC progression (Fig. 5D), whereas expression profiling was nearly divided by the histologic category (Fig. 5E).

Taken together, these data suggest that despite the frequent driver gene mutations in the WNT and p53 pathways in early HCC, upregulation of genes downstream of these pathways occurred in overt HCC and not in early HCC. Therefore, additional molecular events were required for aberrant transcriptional activation of the WNT pathway and inactivation of p53 pathway as HCC progressed according to the methylation status. Furthermore, upregulation of TERT was the most frequent initial event in HCC (Fig. 6).
Discussion

Findings from the gene expression data reflect the clinical peculiarities of early HCC, such as slow growth and low-grade malignancy, where several molecular alterations resulted in the morphologic change from early to overt HCC and the global methylation status of the CpG islands was maintained through the development of HCC (30). Similar to APC mutations in the multistep genetic model for the formation of colorectal cancer (31), the formation of overt HCC requires the sequential genetic aberrations in addition to the driver gene mutations.

Unlike the gene expression or copy number aberrations, simple gene mutations were relatively frequent in the early stage of HCC. Previous reports based on NGS data have demonstrated that mutations in the WNT and p53/RB pathways are common in HCC (10, 14, 15) and these driver mutations are also common in early HCC.

Despite frequent mutations in the CTNNB1 and TP53 genes in early HCC, WNT target genes and target genes downstream of the p53/RB pathway (cell cycle-related genes) were rarely upregulated in early HCC. Most notably, the transcriptional aberrations of the WNT pathway were associated with decreased expression of CDH1 and demethylation of the CpG islands of the target genes (32). Although it has been generally accepted that the E-cadherin/β-catenin complex at the cell membrane plays an important role in maintaining the epithelial integrity and proper cytoskeletal structure, disrupting this complex not only impairs cellular adhesion within a tissue, but also activates the Wnt pathway due to the accumulation of β-catenin in the nuclei (25, 33). Given that the losses in methylation can be a downstream consequence of transcription activation in cancer cells (34, 35), we supposed that hypomethylation of the promoter region of WNT targets could be attributed to overexpression of these genes due to CDH1 downregulation in overt HCC. By contrast, immunostaining showed strong immunoreactivity in the nuclei of overt HCC but not early HCC with CTNNB1 mutations, and therefore, we speculated that dysfunction in a component of the adherens junction at the cell membrane resulting from CTNNB1 mutations contributed to hepatocarcinogenesis at an early stage, whereas translocation of β-catenin to the nucleus through downregulation of CDH1 contributed at a later stage. In addition, consistent with the experimental data from Feng and colleagues (24), our data showed that CTNNB1 and AXIN1 target genes differ with regard to the regulation of WNT pathway targets; for example, abnormalities of the WNT pathway due to inactivation of β-catenin were varied.

At the same time, deletion of 4q and/or 16q was necessary for the transcriptional aberrations of the TP53 target genes. IRF2 on
characterized by a stem-like gene set including patients in G3 was significantly poorer. This might be because G3 was characterized by a stem-like gene set including TET1, which discriminates between an invasive and metastatic liver tumor with distinct stem-cell marker expression patterns (44). Thus, induction of reprogramming due to TET1 upregulation or hyperactivation of PRC2 caused by overexpression of EZH2 could contribute to HCC progression and poor prognosis of patients in G3. Conversely, mutations in the CTNNB1 gene were more common in G2, consistent with the previous report that prognosis of patients with CTNNB1 mutations are relatively favorable (45).

In addition, methylation statuses were globally maintained through the progression of HCC, as confirmed by comparing the methylation profile of inner nodule (overt HCC) and outer nodule (early HCC) of NIN samples. These findings suggest that several types of origin cancer cells exist, which are inherited by future generations in cancer progression, whereas the different gene expression profiles between the inner and outer nodules reflects the phenotypes of early and advanced liver cancer.

In conclusion, the most common driver gene mutations and mechanisms of TERT upregulation in early and overt HCCs were identified through transcriptome and exome sequence analyses. Notably, an integrated analysis showed that despite frequent mutations in CTNNB1 or TP53, early HCC was a separate molecular entity from overt HCC due to distinct expression profiles, while the global methylation status was maintained throughout HCC progression. These findings indicate that additional molecular events apparently cooperate for transcriptional activation of corresponding downstream targets during HCC progression according to the methylation status.

Disclosure of Potential Conflicts of Interest

S. Yamamoto reports grants from Program for an Integrated Database of Clinical and Genomic Information from AMED and collaborative research with Chugai Pharmaceutical Co., Ltd. outside the submitted work. C.J. Covington reports funding from Castle Biosciences as an employee, but the company does not work with HCC at this time. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Y. Midorikawa: Conceptualization, resources, data curation and writing—original draft. S. Yamamoto: Conceptualization, data curation and formal analysis. K. Tatsuno: Data curation. C. Renard-Guillet: Formal analysis. S. Tsuji: Formal analysis. A. Hayashi: Investigation. H. Ueda: Software and formal analysis. S. Fukuda: Resources, data curation and formal analysis. T. Fujita: Formal analysis. H. Katoh: Investigation. S. Ishikawa: Investigation. K.R. Covington: Formal analysis. C.J. Creighton: Formal analysis. M. Sugitani: Investigation. D.A. Wheeler: Supervision. T. Shibata: Writing—review and editing. G. Nagae: Conceptualization. T. Takayama: Supervision. H. Aburatani: Supervision.

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