predominant in HCC incidence (29). However, there was no significant difference in AFP levels between HBV- and HCV-related HCC in this study. We re-evaluated the predictive factors of recurrence after resection by stratifying this cohort into HBV- or HCV-related HCC. ASRI ≥ 20 was significantly associated with overall recurrence after resection in the HBV cohort, and this result was similar in HCV. Therefore, we consider ASRI as the useful index regardless of the viral aetiology, even in an HBV-endemic area.

Patients with advanced recurrence had a poor prognosis because of limitation and resistance of treatment. The overall survival rates were lower (35.1% per 5 years) in the advanced recurrence group than in the minor or the no recurrence group, in this study. On the other hand, patients with minor recurrence had a relatively good prognosis because it was possible to conduct reresection or percutaneous ablation therapy for recurrent tumour. Therefore, adjuvant therapy to prevent advanced recurrence after resection is needed. Although a number of studies of adjuvant therapy have been reported, none is effective for preventing intrahepatic metastasis after resection of HCC. Pre-/post-operative chemoembolization and chemotherapy had no benefit for tumour recurrence (30–32). Although a few authors including our hospital have reported that interferon is effective for preventing recurrence of HCC after resection, it is assumed that interferon itself suppresses de novo carcinogenesis (33–35). Recently, it was reported that sorafenib, which was a multikinase inhibitor, improved the overall survival rates in patients with advanced HCC (36). Sorafenib is expected to have the potential of effective adjuvant therapy to prevent tumour recurrence by intrahepatic metastasis, and a future report is awaited.

In conclusion, tumour number, ASRI and tumour differentiation were identified as risk factors for advanced recurrence of HCC. In particular, ASRI was easy to calculate and a useful index to predict advanced recurrence after curative resection of small HCC and to choose patients requiring adjuvant therapy after resection.

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Integrative Transcriptome Analysis Reveals Common Molecular Subclasses of Human Hepatocellular Carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is a highly heterogeneous disease, and prior attempts to develop genomic-based classification for HCC have yielded highly divergent results, indicating difficulty in identifying unified molecular anatomy. We performed a meta-analysis of gene expression profiles in data sets from eight independent patient cohorts across the world. In addition, aiming to establish the real world applicability of a classification system, we profiled 118 formalin-fixed, paraffin-embedded tissues from an additional patient cohort. A total of 603 patients were analyzed, representing the major etiologies of HCC (hepatitis B and C) collected from Western and Eastern countries. We observed three robust HCC subclasses (termed S1, S2, and S3), each correlated with clinical parameters such as tumor size, extent of cellular differentiation, and serum α-fetoprotein levels. An analysis of the components of the signatures indicated that S1 reflected aberrant activation of the WNT signaling pathway, S2 was characterized by proliferation as well as MYC and AKT activation, and S3 was associated with hepatocyte differentiation. Functional studies indicated that the WNT pathway activation signature characteristic of S1 tumors was not simply the result of *β-catenin* mutation but rather was the result of transforming growth factor-β activation, thus representing a new mechanism of WNT pathway activation in HCC. These experiments establish the first consensus classification framework for HCC based on gene expression profiles and highlight the power of integrating multiple data sets to define a robust molecular taxonomy of the disease. [Cancer Res 2009;69(18):7385–92]

Introduction

Hepatocellular carcinoma (HCC) affects approximately half a million patients worldwide and is the most rapidly increasing cause of cancer death in the United States owing to the lack of effective treatment options for advanced disease (1). Numerous lines of clinical and histopathologic evidence suggest that HCC is a heterogeneous disease, but a coherent molecular explanation for this heterogeneity has yet to be reported. Genomic approaches to the classification of HCC therefore hold promise for a molecular taxonomy of the disease.

Mutations in the WNT signaling pathway have been found to be common in HCC, but other DNA level classification approaches have proven challenging. This relates to the enormous complexity of the genomic alterations observed in HCC, likely attributable to the accumulation of chromosomal rearrangements resulting from decades of chronic viral hepatitis and cirrhosis. This complexity makes it difficult to identify the causal genetic events promoting HCC development and progression (2, 3). An alternate approach to HCC classification has been to study tumors at the level of their gene expression profiles. Although several such profiling efforts have been reported (4–11), a cohesive view of expression-based subclasses of HCC has yet to emerge. In part, this is because each of the reported studies analyzed different patient populations (most of them small) on a different microarray platform, with a different primary biological or clinical question in mind. Perhaps not surprisingly then, each study reported a somewhat different view of the heterogeneity of HCC, and it has been therefore impossible to see whether there exists a common biological thread that links these disparate studies.

We believe that any biologically or clinically meaningful classification system should be informative across multiple patient populations and should be independent of any particular microarray platform. In the present study, we therefore set out to define molecular subclasses of HCC that existed across all available HCC data sets, including eight previously reported studies and one new one reported here, totaling 603 patients. We report that indeed there exist three distinct molecular subclasses of HCC that are present in all nine data sets examined, regardless of technical differences between the microarray platforms used to generate the profiles. We show that these subclasses are correlated with histologic, molecular, and clinical features of HCC, and we highlight the important role of transforming growth factor-β (TGF-β) signaling in one of the HCC subclasses. These findings thus create a solid foundation for HCC classification on which to build informed clinical trials for patients with HCC and also suggest new opportunities for therapeutic intervention.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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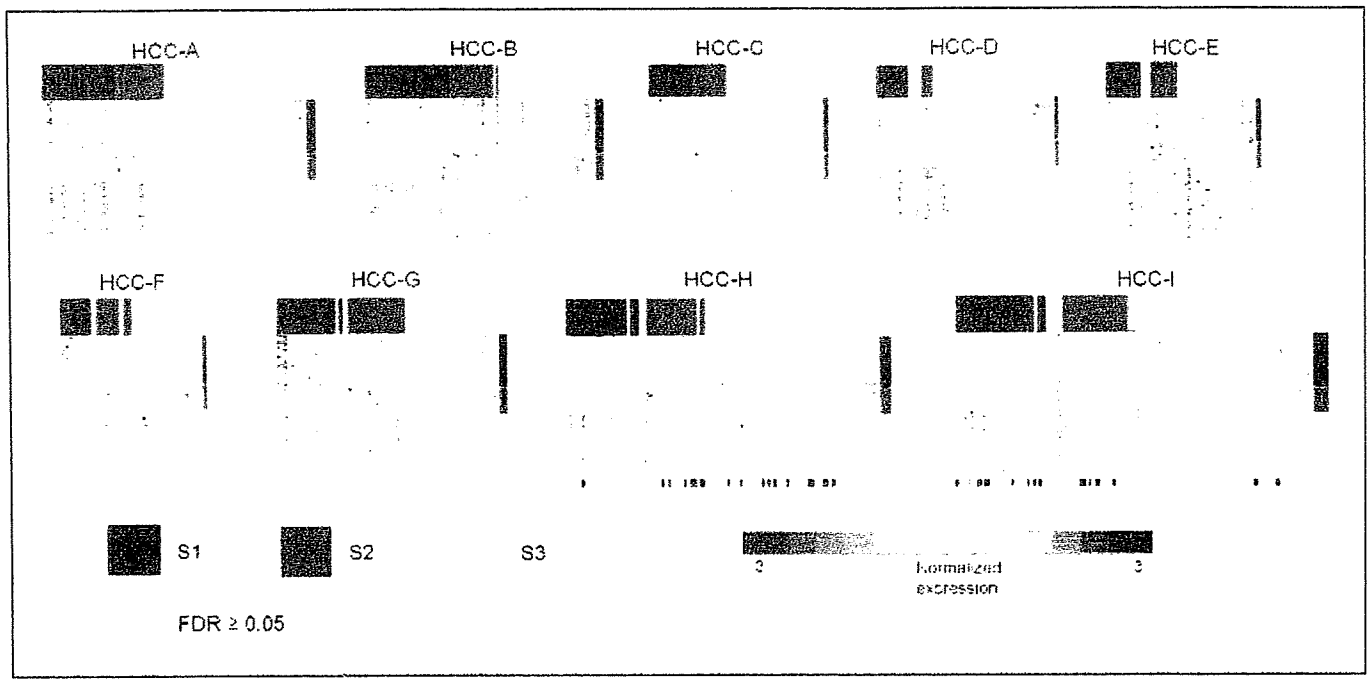

Figure 1. HCC subclasses predicted in nine independent data sets. Predicted subclasses are shown in red (S1), blue (S2), and yellow (S3) with expression pattern of the HCC subclass signature. The proportion of the cases with confident prediction (FDR < 0.05) in HCC-A, HCC-B, HCC-C, HCC-D, HCC-E, HCC-F, HCC-G, HCC-H, and HCC-I were 96%, 96%, 90%, 81%, 79%, 87%, 94%, 83%, and 83%, respectively. Red bars attached to HCC-H and HCC-I indicate positive *β-catenin* mutations and nuclear staining of p53, respectively.

Materials and Methods

Microarray Data Sets and Statistical Analysis

Identification of common HCC subclasses. To define and validate a gene expression–based model of common molecular subclasses of HCC, we collected publicly available gene expression data sets from eight independent cohorts profiled on a wide variety of microarray platforms (HCC-A, HCC-B, HCC-C, HCC-D, HCC-E, HCC-F, HCC-G, and HCC-H; see Supplementary Tables S1 and S2 for details; refs. 4–11). Between the training data sets (HCC-A, HCC-B, and HCC-C) chosen as larger data sets covering major etiologies of HCC to avoid overfitting a model to any particular cohort or microarray platform, corresponding subgroups of the samples were defined by subclass mapping (SubMap) method (12) based on subclasses that identified three unsupervised clustering methods: hierarchical clustering, k-means clustering, and nonnegative matrix factorization, which finds clusters of samples after collapsing the data set into representative "meta-genes" (13).

For each subclass defined by SubMap, meta-analysis marker genes were selected as overexpressed genes compared with the rest of the subclasses (HCC subclass signature) in all the three clustering methods to avoid defining gene expression–based subclasses that were unique to a particular clustering algorithm. Prediction of the subclass was performed for each sample using nearest template prediction method (14, 15) to accommodate the diverse microarray platforms (see Supplementary Data for details).

Molecular annotation of HCC subclasses. Functional characterization of the HCC subclasses was performed using gene set enrichment analysis (GSEA; ref. 16). Two categories of gene sets in Molecular Signature Database (MSigDB)[11] were used: target gene sets regulated by experimental perturbations (377 gene sets) and literature-based manually curated molecular pathway gene sets (150 gene sets).

Clinical data analysis. The hazard of tumor recurrence was calculated to estimate the pattern of HCC recurrence over time after the surgery as previously reported (17, 18). Continuous and proportional data were tested by Wilcoxon rank sum test and Fisher's exact test, respectively. All clinical data analyses were performed using the R statistical package version 2.4.0.[12]

Microarrays for Fixed Tissues

We generated a ninth data set of formalin-fixed, paraffin-embedded (FFPE) tumors (HCC-I), reasoning that any meaningful classification system should be applicable to routinely collected fixed (as opposed to frozen) tissues. We analyzed FFPE tissue blocks from 118 HCC patients who consecutively underwent surgical resection during 1990 to 2001 at Toranomon Hospital. Ethical approval for use of the FFPE tissues, obtained and archived as part of routine clinical care, was acquired from the institutional review board granted on condition that all samples be made anonymous. Total RNA was extracted from macrodissected 10-μm tissue slices (three to four slices for each sample) using High Pure RNA Paraffin kit (Roche). Expression of transcriptionally informative 6,000 genes, selected to capture global state of transcriptome (14), was profiled using DNA-mediated annealing, selection, extension, and ligation (DASL) assay (Illumina; see Supplementary Data; ref. 19).

Microarrays for Cell Lines

Total RNA was isolated using Trizol reagent (Invitrogen) according to the manufacturer's instruction. Microarray experiment was performed using HT_HG-U133A High-Throughput Arrays (Affymetrix). The raw data were normalized using BioConductor affy package.[13]

All microarray data sets are available through National Center for Biotechnology Information Gene Expression Omnibus[14] with accession numbers of GSE10186 (DASL), GSE10393 (U133A), and GPL5474 (transcriptionally informative gene panel for DASL) and our Web site.[15]

[11] http://www.broad.mit.edu/gsea/msigdb/

[12] http://www.r-project.org
[13] http://www.bioconductor.org/
[14] http://www.ncbi.nlm.nih.gov/geo/
[15] http://www.broad.mit.edu/cancer/pub/HCC

Table 1. Clinical phenotypes associated with HCC subclasses

Variable	S1	S2	S3	*P*
Tumor size (cm)*	3.0 [2.0,4.5]	4.5 [2.5,7.0]	2.5 [1.8,4.3]	0.003
Tumor differentiation*				
Well	8 (16%)	4 (10%)	37 (44%)	
Moderate	27 (53%)	23 (59%)	45 (53%)	<0.001
Poor	16 (31%)	12 (31%)	3 (4%)	
AFP (ng/mL)†	50 [14,332]	171 [27,1,251]	13 [5,43]	<0.001
Hepatitis B virus infection‡	39 (38%)	27 (36%)	39 (25%)	0.05
Hepatitis C virus infection‡	55 (53%)	44 (58%)	109 (69%)	0.03

NOTE: Median [25%,75%]. Wilcoxon rank sum test for continuous data, and Fisher's exact test for categorical data.
*HCC-F, HCC-H, and HCC-I: S1, $n = 55$; S2, $n = 46$; S3, $n = 96$.
†HCC-H and HCC-I: S1, $n = 48$; S2, $n = 39$; S3, $n = 83$.
‡HCC-B, HCC-C, HCC-F, HCC-H, and HCC-I: S1, $n = 103$; S2, $n = 76$; S3, $n = 158$.

Immunostaining

Immunohistochemical staining was performed on 10-μm FFPE sections using antibodies for β-catenin (Becton Dickinson), phospho-AKT (Cell Signaling), and p53 (Immunotech) followed by detection using the Envision+ DAB system (Dako). The stains were evaluated by a pathologist blinded to the results of the gene expression profiling, and the results were scored in a binary system. For immunofluorescence staining, cells grown on multiwell chamber slides were fixed by 4% paraformaldehyde and stained for β-catenin (see Supplementary Data for details).

Cell Culture

Human HCC cell lines, Huh-7 (Riken Bioresource Center) and SNU-387 (American Type Culture Collection), were grown in DMEM supplemented with 10% heat-inactivated fetal bovine serum at 37°C in a 5% CO_2 atmosphere. No *β-catenin* mutation has been found in these cell lines.

Western Blotting

Cell lysates were separated on NuPAGE 4% to 12% gels (Invitrogen) and transferred to polyvinylidene difluoride membranes (Bio-Rad) and blotted for α-fetoprotein (AFP; Santa Cruz Biotechnology), β-catenin, phospho-SMAD3 (Cell Signaling), and proliferating cell nuclear antigen (Santa Cruz Biotechnology) antibodies.

β-Catenin Knockdown

Cells were infected with the indicated short hairpin RNA (shRNA) vectors (construct 1, TRCN0000003843; construct 2, TRCN0000003844; The RNAi Consortium[16]) and puromycin selected. Ninety-six hours after infection, cells were counted and seeded in triplicate (20,000/six well). After 10 d, cells were fixed in paraformaldehyde and stained with crystal violet. Dye was extracted with 10% acetic acid and absorbance was determined at 600 nm.

Luciferase Assay

Cells were transfected using Lipofectamine 2000 (Invitrogen) with either TOP-flash or FOP-flash constructs and stimulated with 100 pmol/L TGF-β (R&D Systems) for 48 h. Luciferase activity was measured using Dual-Glo kit (Promega). All transfections were performed in triplicate and measurements were normalized to a SV40 promoter-driven *Renilla* luciferase construct.

Results

Three common molecular subclasses of HCC. The SubMap method identified three robust subclasses in each of the three initial data sets analyzed. We refer to these subclasses as S1, S2, and S3 (Fig. 1), and the complete list of genes that correlated with each of the subclasses is available in Supplementary Table S3. As with any unsupervised clustering–based definition of cancer subclasses, it is essential to establish the validity of the newfound classification system. In the following sections, we describe three independent validations of the three-class structure of HCC. First, we show that the three subclasses are detected with statistical significance in each of the six remaining HCC data sets (totaling 371 patients). Second, we show that the subclasses are associated with clinical parameters. Third, we show that the subclasses are associated with biological mechanism known to be operative in the pathogenesis of HCC.

Statistical validation of subclasses across nine HCC cohorts. As a statistical measure of the validity of the three subclasses, we determined the confidence with which HCC samples could be classified into one of the three subclasses using the HCC subclass signature–based classifier, including 619 genes. As expected, subclass labels were assigned with high confidence [false discovery rate (FDR) < 0.05] to 94% of the training samples (HCC-A, HCC-B, and HCC-C), which were used to define the subclasses in the first place. More importantly, high confidence subclass labels were assigned to 84% of the 371 samples in the validation set (HCC-D, HCC-E, HCC-F, HCC-G, HCC-H, and HCC-I). In contrast, a classifier based on the same number of genes chosen randomly yielded high confidence predictions in <1% of the samples. In addition, our classification system was superior to those reported previously (10, 11, 20, 21) when those classifiers were tested across all of the validation data sets (high confidence predictions using reported signatures were 27–75%; Supplementary Fig. S1). These results, taken together, indicate the statistical significance of our three subclasses and point to the limitation of defining subclasses based on only a single cohort, where overfitting often leads to failure of the classifier to validate on new samples, particularly when profiled on a different microarray platform.

Clinical relevance of HCC subclasses. Having established the statistical validity of the HCC subclasses, we next asked whether any of the subclasses were associated with clinical parameters to add the validity of the subclasses. Clinical data were available for 197 patients, as summarized in Table 1.

Our first observation was that tumors in subclass S2 were larger than the others, whereas tumors in S3 were smaller compared with the rest ($P = 0.003$). In addition, subclass S3 included the majority of well-differentiated tumors (37 of 49; $P < 0.001$), whereas there was no

[16] www.broad.mit.edu/genome_bio/trc/rnai.html

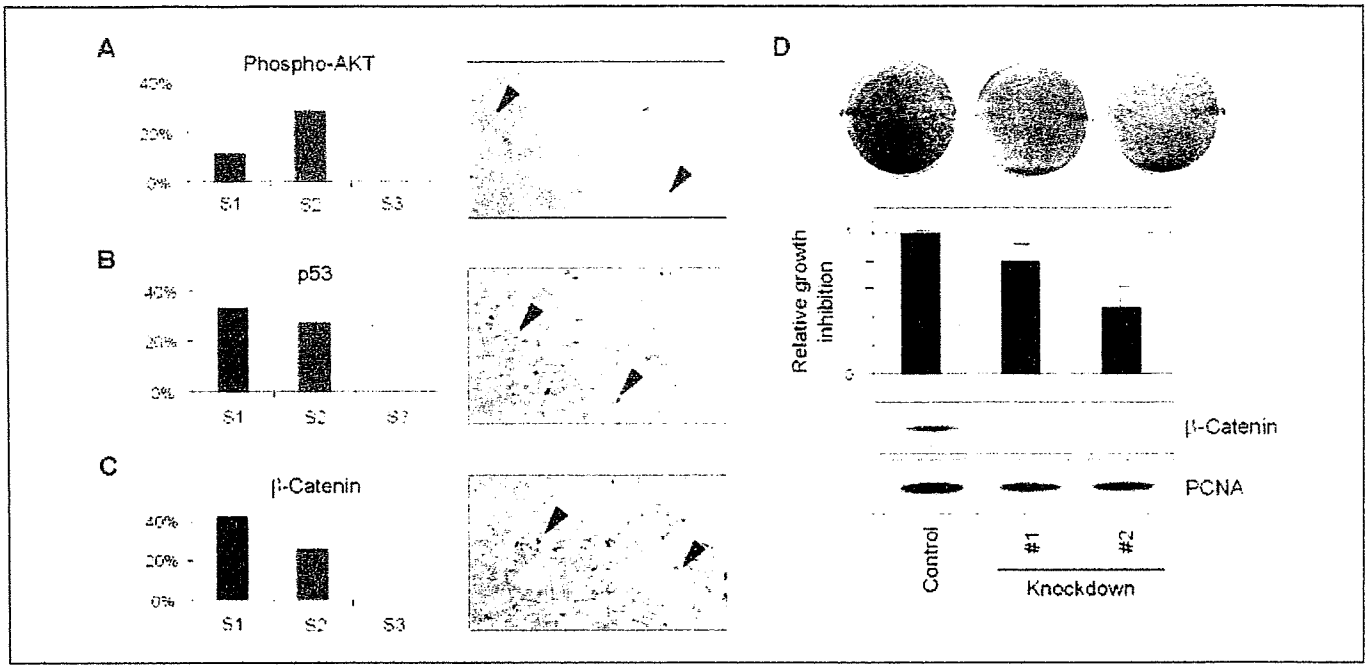

Figure 2. Molecular pathways associated with HCC subclasses. Immunohistochemical analysis of phospho-AKT (*A*), p53 (*B*), and β-catenin (*C*) proteins in HCC-I. *Left*, proportions of the cases with positive staining in each HCC subclass; *right*, representative positive staining (*arrowheads*). Magnification, ×20. *D*, growth inhibition of SNU-387 cells (predicted to be subclass S1) by knocking down β-catenin protein using two different shRNA constructs.

histologic difference between S1 and S2 ($P = 0.73$). We also examined the serum levels of the one clinically used serum biomarker of HCC—AFP. Serum AFP was the highest in S2 (0.001), further supporting the notion that our subclasses are clinically relevant.

Next, we sought to determine whether the HCC subclasses were associated with clinical outcome following surgical resection. We were careful to analyze the two major patterns of HCC recurrence: early recurrence, which is related to residual dissemination of primary tumor cells within the liver, and late recurrence, which is attributable to new primary tumors arising in a hypercarcinogenic state of a cirrhotic liver (17, 18, 22). "Early" recurrence is associated with malignant characteristics of the primary tumor itself, and we reported that it has less effect on patient survival in earlier-stage HCC, in which "late" recurrence is the major determinant of survival (14, 23). We found that subclass S1 was associated with a significantly greater risk of earlier recurrence ($P = 0.03$ within 1 year; Supplementary Fig. S2). This association remained to be significant even after correcting for tumor size in multivariate Cox regression modeling (Supplementary Table S4). Consistent with this observation, S1 tumors exhibited more vascular invasion and satellite lesions (both known risk factors for early recurrence; Supplementary Table S5). These results may suggest that the S1 subclass is associated with a more invasive/disseminative phenotype. Interestingly, we found that a recently reported signature of poor survival defined in patients with more advanced HCC, where early recurrence is the major determinant of survival (4), was associated with S1 and S2, whereas the good survival signature defined in that study was enriched in S3 tumors (Supplementary Table S6), lending further credence to our subclass model. Importantly, our HCC subclasses were not associated with late recurrence, consistent with our recent study indicating that late recurrence is determined not by the characteristics of the tumor itself but rather by the biological state of the surrounding liver at risk (14). Furthermore, consistent with our previous observation, the HCC subclasses showed no association with survival ($P = 0.12$) in our data set (HCC-H) consisting mostly of early-stage tumors.

Molecular pathways associated with HCC subclasses. We next asked whether we could ascribe biological meaning to our validated HCC subclasses. The GSEA results indicated that indeed our HCC subclasses were associated with distinct biological processes, several of which have been implicated in HCC pathogenesis (Supplementary Tables S7 and S8). For example, S2 were tumors associated with a relative suppression of IFN target genes (7), of interest because of the use of IFN as an experimental chemopreventive strategy for HCC. S2 tumors were also enriched in MYC target genes, suggesting that MYC activation is a feature of S2 tumors. Consistent with this observation, we found that a recently reported mouse model of HCC based on MYC overexpression (24) exhibited the S2 subclass signature (Supplementary Fig. S3). S2 tumors were also strongly enriched in a signature of EpCAM positivity (Supplementary Table S6; ref. 25), and in addition, we found that S2 tumors overexpressed *AFP* (consistent with S2 patients having elevated serum AFP levels; Table 1). Lastly, S2 tumors were enriched in a signature of AKT activation (10), and validation experiment indicated a trend toward elevated phosphorylation of AKT as determined by immunohistochemistry ($P = 0.07$; Fig. 2A). An AFP-AKT association has been previously observed (10, 26), and we see here that this association is being driven primarily by S2 tumors. The mechanism of AKT activation in these tumors is not known but likely reflects upstream signaling of the phosphatidylinositol 3-kinase (PI3K) pathway (27). As PI3K inhibitors are now entering clinical development, it may be of value to examine their role in S2 tumors in particular.

GSEA also identified differential activation of p53 and p21 target gene sets, with these genes being more abundantly expressed in S3 tumors compared with S1 and S2, consistent with our observation that S3 tumors tend to be lower grade (Table 1). To further validate this result, we performed

immunohistochemistry for p53, wherein nuclear accumulation of p53 protein is well known to reflect inactivating p53 mutation (28). As predicted by the GSEA analysis, S1 and S2 tumors exhibited significantly greater nuclear p53 staining compared with S3 ($P = 0.001$; Fig. 2*B*). The more well-differentiated nature of S3 tumors was also reflected in the S3 gene expression profile, with S3 tumors exhibiting relatively higher levels of expression of hepatocyte function-related genes involved in glycogen/lipid/alcohol metabolism (*APO/ALDH/ADH* family genes), detoxification (*CYP* family genes), coagulation, and oxygen radical scavenging (*CAT, SOD1*; Supplementary Tables S3, S7, and S8).

WNT pathway activation in S1. The WNT signaling pathway is perhaps the best characterized oncogenic pathway in HCC, with pathway activation occurring through *β-catenin* mutation (specifically via mutation in exon 3 in up to 44% of cases) and less frequently in *AXIN1* (<10% of cases: refs. 2, 3). We addressed WNT status with regard to HCC subclass in two ways. First, we performed GSEA analysis using an experimentally defined WNT activation signature. We found strong enrichment of the WNT signature in subclass S1 compared with S2 or S3 (FDR = 0.03; Supplementary Table S7), suggesting preferential WNT activation in S1 tumors. We validated this result via immunohistochemistry for β-catenin (the principal downstream effector of WNT in HCC) and found that S1 tumors indeed had higher levels of cytoplasmic β-catenin protein expression compared with the other subclasses (0.001), again indicating preferential activation of the canonical WNT pathway in S1 (Fig. 2*C*; ref. 29). In addition, we found that shRNAs targeting *β-catenin* resulted in growth inhibition when introduced into the SNU-387 cell line (predicted to be subclass S1), thereby further supporting the hypothesis that WNT activation is functionally important in S1 tumors (Fig. 2*D*).

Mechanisms of WNT activation in S1 tumors. Having determined that S1 tumors exhibit preferential activation of the WNT/β-catenin pathway, we next addressed potential mechanisms for this activity. We first asked whether the S1 tumors were associated with *β-catenin* mutation in HCC-H data set, for which we previously sequenced exon 3 of *β-catenin* gene (11). Surprisingly, *β-catenin* mutations were preferentially found in S3 tumors, consistent with previously reported "CTNNB1" class representing a subset of S3 (11, 30). This result is also consistent with recent evidence indicating that *β-catenin* mutations do not regulate the canonical WNT target genes (e.g., *cyclin D1* and *MYC*) that characterize our S1-associated WNT activation signature (31). These results further suggest that the WNT pathway is activated in S1 tumors by a mechanism other than *β-catenin* mutation.

To explore alternate explanations for WNT pathway activation, we again turned to GSEA, asking whether other gene sets (signatures) enriched in S1 tumors might provide insight into WNT activation in these tumors. Strikingly, we observed strong overexpression of TGF-β target gene sets (i.e., genes expressed as a result of experimental activation of TGF-β) in S1 tumors (Supplementary Table S7). We similarly observed enrichment of a gene set associated with epithelial-to-mesenchymal transition, a phenomenon implicated in tumor invasion and metastasis (32) and known to be regulated by TGF-β signaling in HCC (Supplementary Table S7; refs. 33, 34). Furthermore, a previously reported TGF-β activation signature associated with an invasive phenotype (35) showed strong enrichment in S1 (Supplementary Table S6). We observed no genomic copy number change associated with S1 subclass in *TGFB1* locus, suggesting that chromosomal aberration is not the causative mechanism of the activation (Supplementary Fig. S4). These results indicate that TGF-β and WNT signaling co-occur in the same HCC subclass (subclass S1), and suggest the hypothesis that TGF-β might in some way lead to WNT activity that defines the S1 molecular phenotype.

TGF-β-WNT interactions. We next explored the hypothesis that TGF-β regulates WNT pathway activity in HCC cells. First, we treated the HCC cell line Huh-7 with intact WNT pathway components (classified as subclass S2 and with no activation of S1 and WNT activation signature) with TGF-β and monitored the genome-wide expression consequence. As predicted, TGF-β stimulation induced expression of WNT target genes (FDR < 0.001; Fig. 3*A*) and induced the expression of genes characteristic of subclass S1 (FDR = 0.04; Fig. 3*B*; Supplementary Data) characterized by WNT/

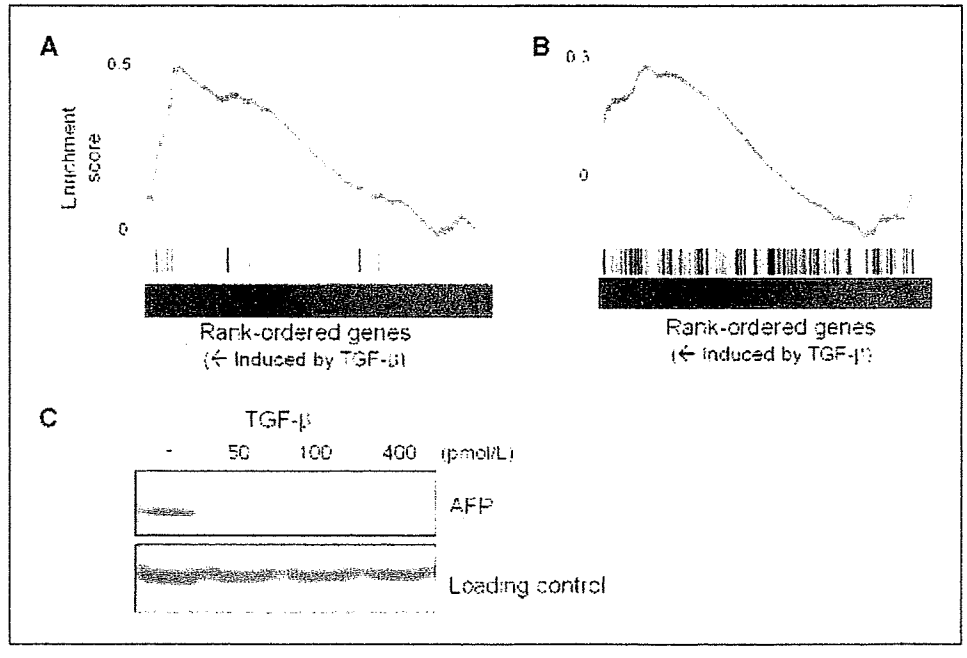

Figure 3. Interaction between WNT pathway and TGF-β. *A*, up-regulation of an experimentally defined WNT target gene set, "KENNY_WNT_UP" (FDR < 0.001), by TGF-β. Genes were rank ordered based on differential expression between TGF-β–treated and untreated Huh-7 cells (predicted to be subclass S2). A database of target gene sets for experimental perturbations (377 gene sets) was assessed by GSEA. *B*, up-regulation of the S1 signature by TGF-β treatment. Genes were rank ordered based on differential expression between treated and untreated Huh-7 cells, and induction of the subclass signature was evaluated by GSEA (FDR = 0.04). *C*, suppression of AFP protein expression by TGF-β treatment. Loading control is nonspecific for AFP antibody to show that equal amounts of protein were loaded.

TGF-β activity while suppressing expression of AFP protein, one of the top markers for S2 (Fig. 3C).

Second, we asked whether TGF-β could regulate the activity of a T-cell factor-lymphoid enhancer factor (TCF-LEF) reporter, further reflecting WNT/β-catenin activity. TGF-β stimulation of Huh-7 cells resulted in activation of a wild-type (TOP-flash) but not mutant (FOP-flash) TCF-LEF luciferase reporter (Fig. 4A). Interestingly, the superactivation of TCF-LEF activity was also observed in the presence of cotransfected mutant β-catenin. These results validate the hypothesis that TGF-β enhances WNT activity in HCC, consistent with the subclass S1 molecular profile.

We next explored the mechanism by which TGF-β augments WNT/β-catenin activity. A simple explanation would be that TGF-β induced expression of β-catenin RNA or protein levels, but we found no evidence for this (Fig. 4B). Strikingly, however, TGF-β treatment resulted in a marked change in β-catenin subcellular localization. Specifically, TGF-β treatment induced a shift from membranous β-catenin staining to a cytoplasmic distribution with focal perinuclear aggregation (Fig. 4C). This suggests that TGF-β enhances WNT signaling by modulating the intracellular pool of free β-catenin.

Taken together, these results validate the observation that TGF-β and WNT activity together typify the S1 subclass of HCC, and further suggest that TGF-β augments WNT activity via alteration of the subcellular localization of β-catenin, consistent with the cross-talk between these pathways observed in other biological contexts (34, 36). This implies that therapeutic cotargeting TGF-β and β-catenin in S1 tumors might be explored as a strategy for the treatment of S1 subclass HCC.

Discussion

Advances in genome technologies are now supporting a breadth of cancer genome characterization studies, including those focusing on HCC. Along with this proliferation of studies has come, however, a certain confusion in the field—different studies often report different results relating to the same set of underlying questions. For example, ~10 articles on the gene expression–based classification of HCC have been published in recent years, but a consensus molecular taxonomy of the disease has yet to emerge. This might lead some to believe that either expression technologies are insufficiently stable or HCC is so hopelessly heterogeneous and complex that regular, reproducible patterns in the data are nonexistent. We report here that in fact a highly reproducible molecular architecture of HCC is identifiable and is detected across all available HCC data sets.

Our analysis of nine HCC data sets totaling 603 patients indicated that there exist three major subclasses of HCC, which we refer to as subclasses S1, S2, and S3 (Fig. 5). Importantly, although the proportion of each subclass varied slightly from study to study, the subclasses were identifiable regardless of the geographic location of the study patients (Asia versus Europe versus United States) or the technology platform used (cDNA versus oligonucleotide arrays, and frozen versus FFPE tissues). Notably, the new data set generated in the present study used FFPE tissues, thereby showing that the three-class structure is readily detectable in specimens collected and stored in the routine clinical setting. This is relevant because the future deployment of diagnostic tests aimed at cancer classification should ideally be applicable to the standard FFPE specimens that are obtained in clinical practice.

Several biological insights can be made from the observed three-class structure of HCC. Class S1 is particularly notable for the prominence of a WNT activation gene expression signature. This is notable because such WNT activation is not simply explained by the presence of activated *β-catenin* mutations, suggesting that additional mechanisms of WNT activation seem to be at play,

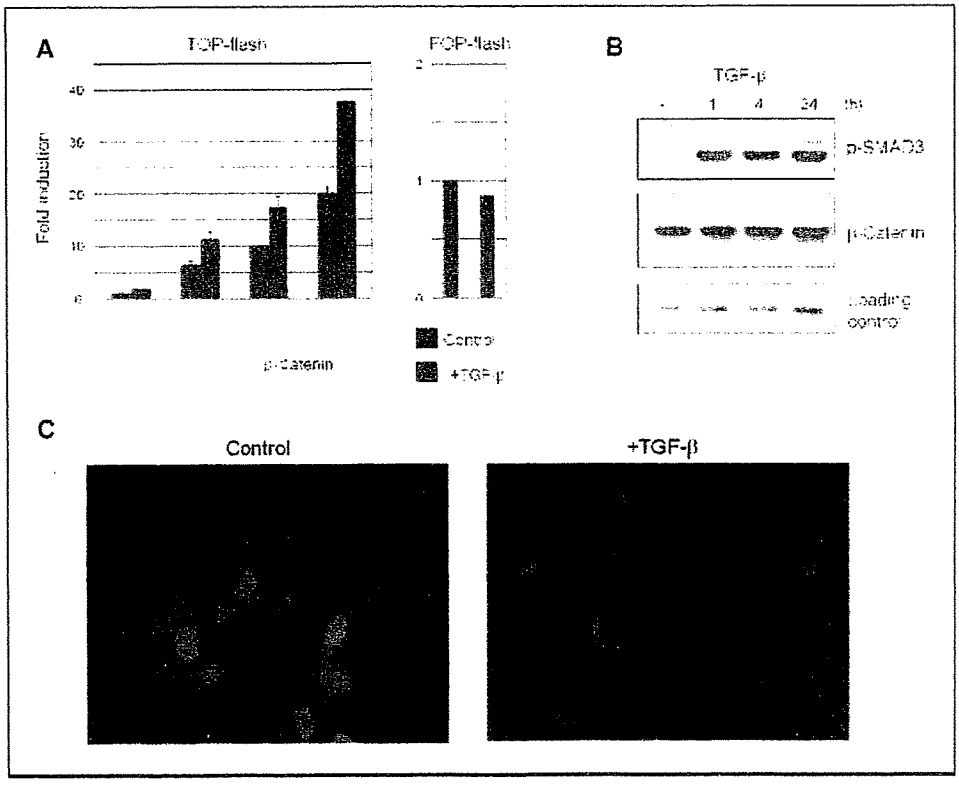

Figure 4. Activation of WNT pathway by TGF-β. *A*, Huh-7 cells were transfected with the indicated reporter constructs and increasing amounts of mutant β-catenin (2, 5, and 10 ng of plasmid). *B*, TGF-β pathway activation was confirmed by phosphorylation of SMAD3. Abundance of β-catenin protein was not changed by TGF-β treatment (100 pmol/L, 48 h). Loading control is nonspecific for phospho-SMAD3 antibody to show that equal amounts of protein were loaded. *C*, Huh-7 cells were stimulated as above and stained for β-catenin. Cellular distribution of β-catenin changed from predominantly membranous to cytoplasmic and perinuclear, and clustered cells spread out with more elongated and flattened morphology.

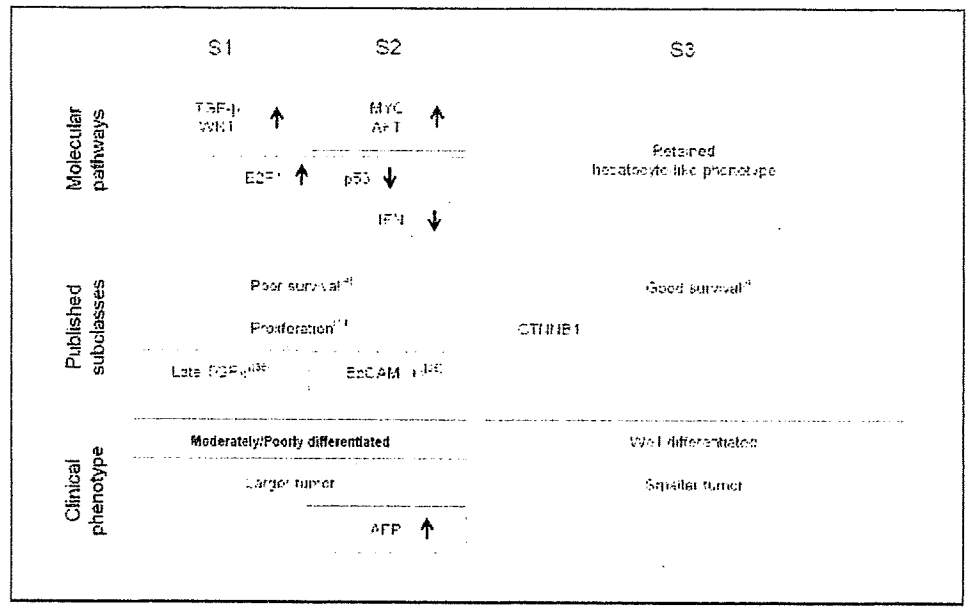

Figure 5. Schematic summary of the characteristics of HCC subclasses.

including TGF-β activation. This may be particularly important in the setting of clinical trials testing β-catenin inhibitors in HCC. Our data suggest that such inhibitors may be worth exploring in HCC beyond those patients harboring *β-catenin* mutation. Although additional mechanistic studies are clearly required, our data support the existence of an interaction between WNT activation and TGF-β activation in S1 tumors, an interaction that has been recently proposed in HCC (34).

Class S2 tumors were notable for their high level of expression of *AFP*, associated with elevated plasma levels of AFP protein compared with non-S2 patients. S2 tumors also tended to be enriched in MYC tumors harboring a MYC activation signature. This is of relevance because it suggests that genetically engineered mouse models of HCC based on MYC activation may be used to interrogate biological basis of the S2 subclass of human HCC. In addition, the finding of an AKT activation signature in S2 tumors suggests that AKT or PI3K inhibitors might be particularly worthy of exploration in this subclass. Further studies are required to establish the mechanism by which AKT is activated in these tumors.

S3 tumors were notable for their relative histologic evidence of differentiation, and the S3 gene expression program was accordingly suggestive of a molecular program of differentiated hepatocyte function. It is tempting to speculate that these tumors might be particularly well suited to differentiation therapy with agents such as retinoids, as has been previously suggested (37). Whether S3 tumors have distinct mechanisms of transformation or rather simply allow for more complete cellular differentiation remains to be determined. The preserved p53 function in S3 suggests that the abrogation of p53 is associated with stepwise malignant transformation of well-differentiated tumors rather than initiation of carcinogenesis. The less frequent *β-catenin* mutations in S1 and S2 may suggest that these tumors arose through different carcinogenic mechanisms compared with S3.

Clearly, much remains to be learned about the biological basis of our observed HCC subclasses. But the fact that they are observed in all studies of HCC examined to date suggests that they represent a reproducible classification framework for the disease. We therefore propose that it will be important to know the subclass of HCC patients entering clinical trials for the treatment of HCC because the response to targeted agents (e.g., β-catenin and PI3K) is likely to be different across the subsets (38). Early observations of differential sensitivity of these distinct tumor types may help guide the design of future clinical trials aimed at targeting agents to distinct patient populations.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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＜速　報＞

核酸アナログ療法中のB型関連肝癌に対する肝癌再発予測マーカーとしてのHBコア関連抗原の有用性

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荒瀬　康司[1)]　池田　健次[1)]　小林万利子[2)]　熊田　博光[1)]

緒言：B型肝疾患に対する核酸アナログ療法の有効性は広く知られており，ラミブジンにおいては投与により発癌率を抑制することが既に報告されている[1)2)]．しかしながら経過観察期間が長くなるにつれ肝発癌例も増加しつつある．また血中HBV-DNA量が抑制されているにもかかわらず，肝癌根治後の再発例も散見される．そこで今回我々は核酸アナログ投与中の肝癌について，肝癌根治療法後の再発予測マーカーとしてのHBコア関連抗原（HBcrAg）の有用性を検討した．

対象と方法：2001年～2008年までに当院で初発の肝細胞癌と診断されたB型肝癌症例で核酸アナログ投与中に肝発癌した54例を対象とした．肝癌発症時の核酸アナログ投与内容の内訳はラミブジン29例，ラミブジン＋アデフォビル併用17例，エンテカビル8例であった．肝癌治療法の内訳は外科切除36例，経皮的局所治療18例であった．HBcrAg測定は既報のごとくCLEIA法を[3)]，HBV-DNA量はアンプリコア法を用いた．肝癌根治後の再発に寄与する因子についてCox比例ハザードモデルを用いて，単変量及び多変量解析を行い検討した．

結果：発癌時のAST/ALT値は31/29 IU/l(中央値)，genotype Cが92.6%（50/54）で，HBe抗原陽性例は42.6%（23/54），血清HBV-DNA量は＜2.6 log copies/ml（中央値）であった．血清HBcrAg量は5.0 logU/ml（中央値）であった．血清HBV-DNA量＜2.6 log copies/mlであった症例35例中，HBcrAg量≧3.0 logU/mlであった症例が29例（82.9%），≧4.8 logU/mlであった症例は13例（37.1%）であった．核酸アナログ投与開始から発癌までの投与期間は2.2年（中央値）であった．

肝癌再発は38.9%（21/54）で認め，根治後から再発までの期間は14カ月（中央値）であった．再発に寄与する因子について単変量解析を行ったところ，HBV-DNA量≧3.0 log copies/ml，HBcrAg≧4.8 logU/ml，腫瘍数多発，門脈浸潤ありの4因子が抽出され，さらに多変量解析を行ったところ，独立因子としてHBcrAg≧4.8 logU/ml，門脈浸潤の2因子が抽出された(Table)．

考察：今回の検討では核酸アナログ投与中の発癌例は血清HBV-DNA量が低値に抑制されているにもかかわらず，HBcrAg量は十分抑制されていない例が認められた[4)]．核酸アナログが投与されていないB型肝癌において，血清HBV-DNA量が肝癌再発に関係するという報告はされている[5)]．しかしながら今回の対象症例のように核酸アナログ投与中の場合はHBV-DNA量よりHBcrAg量の方が肝癌根治後の再発予測マーカーとして有用であると考えられる．

索引用語：HBコア関連抗原，肝癌再発予測，核酸アナログ

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Table Factors associated with recurrence of HCC by univariate and multivariate analysis.

	Univariate		Multivariate	
factors	Hazard Ratio (95%CI)	P	Hazard Ratio (95%CI)	P
HBeAg (Positive)	1.53 (0.63-3.70)	0.343		
HBV DNA ($\geq$ 3.0 logcopies/mL)	2.49 (1.03-6.00)	0.042		
HBcrAg ($\geq$ 4.8 logU/mL)	10.4 (2.39-45.0)	0.002	8.50 (1.95-37.1)	0.004
AST ($\geq$ 50 IU/L)	2.47 (0.98-6.20)	0.055		
ALT ($\geq$ 40 IU/L)	2.37 (0.99-5.71)	0.054		
Platelets count ($< 10^5$ /mm^3)	2.20 (0.81-6.02)	0.123		
Serum Albumin ($<$ 3.5 g/dl)	1.39 (0.53-3.63)	0.505		
Serum bilirubin ($\geq$ 1.5 mg/dl)	1.11 (0.62-2.00)	0.713		
Prothorombin time ($<$ 80%)	2.23 (0.51-9.82)	0.286		
ICG-R 15 ($\geq$ 30%)	0.54 (0.16-1.87)	0.332		
AFP levels ($\geq$ 100 ng/mL)	1.81 (0.74-4.44)	0.194		
DCP levels ($\geq$ 100 mAU/mL)	2.09 (0.81-5.39)	0.129		
Tumor size ($\geq$ 21 mm)	2.02 (0.81-5.07)	0.133		
Tumor number (multiple)	4.03 (1.31-12.4)	0.015		
Presence of portal vein invasion	5.39 (1.69-17.2)	0.004	3.63 (1.15-11.5)	0.028

Abbreviation: AST, aspartate aminotransferase; ALT, alaine aminotransferase; ICG-R15: indocyanine green retention test at 15 min; AFP, alpha-fetoprotein; DCP, des-γ-carboxylprothorombin,

英文要旨

Low hepatitis B virus core-related antigen is a predictor of absence in post-treatment recurrence of hepatocellular carcinoma during antiviral therapy

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The tumor recurrence rate of hepatocellular carcinoma (HCC) is still high even in patients who receive a curative therapy. We analyzed predictive value of HBV-related viral markers, including HBcrAg, HBV DNA, and HBeAg, for HCC recurrence in the patients who developed HCC during antiviral nucleot(s)ide analogues therapy. By univariate analysis, HBV DNA, HBcrAg, tumor number and presence of portal vein invasion were significant predictive factors. By multivariate analysis, HBcrAg and presence of portal vein invasion were independent and significant predictive factors of recurrence after curative therapy for HCC. We conclude that HBcrAg is useful as a predictor of post-treatment recurrence of HCC after curative therapy in patients who received antiviral therapy.

Key words: HB core-related antigen, prediction of recurrence of HCC, nucleot(s)ide analogues

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ORIGINAL ARTICLE

Development of hepatocellular carcinoma in elderly patients with chronic hepatitis C with or without elevated aspartate and alanine aminotransferase levels

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Abstract

Objective. Hepatocellular carcinoma (HCC) in the elderly infected with hepatitis C virus (HCV) is expected to increase globally within the next two decades. The purpose of the study was to define the natural history of elderly patients with chronic hepatitis C needs in order to prevent HCC from arising in these patients. ***Material and methods.*** Treatment-naive patients aged ≥65 years with platelet counts $>120\times10^3/mm^3$ were classified as 120 with aspartate and alanine aminotransferase (ASAT and ALAT) levels ≦40 IU/l (group A) and 212 with either or both levels ≥41 (group B) and followed-up for 3 years or longer without antiviral treatment. ***Results.*** Cirrhosis and HCC developed more frequently in group B than in group A ($p<0.001$ for both). In particular, of the patients aged 65–69 years at entry, cirrhosis and HCC developed more frequently in group B than in group A ($p<0.001$ and $p=0.001$, respectively). Liver-related causes of death were more common in group B than in group A (20/34 (59%) versus 1/9 (11%), $p=0.021$). HCC developed more frequently in men than in women ($p=0.033$). ***Conclusions.*** In elderly patients with chronic hepatitis C, cirrhosis and HCC develop more frequently in those with elevated transaminase levels than in those without elevated transaminase levels. Therefore, transaminase levels need to be suppressed below ≦40 IU/l, using antiviral treatments or other agents, in order to prevent cirrhosis and HCC arising in these patients. In view of rare liver-related deaths, aggressive antiviral treatment would not be necessary in the elderly with chronic hepatitis C who have normal transaminase levels.

Key Words: *Age, chronic hepatitis, cirrhosis, hepatitis C virus, hepatocellular carcinoma*

Introduction

There are an estimated 170 million people persistently infected with hepatitis C virus (HCV) worldwide, and approximately 30% of them develop serious complications during their lifetime, such as decompensated cirrhosis and hepatocellular carcinoma (HCC) [1]. The incidence of HCC in HCV carriers increases with age and is particularly high in those aged 65 years or older. Based on the shift in age-specific distribution of HCV carriers with time [2–4], HCC is expected to increase in the next 20 years, globally.

The natural history of infection with HCV is influenced by host and virological factors including age and gender [5–7], as well as viral loads and genotypes [8–10]. Thus, hepatitis proceeds slowly in HCV infections contracted by children and young women. During follow-ups carried out over 20 years, liver damage developed in a mere 3% of children who were infected with HCV during heart surgery [7], and cirrhosis emerged in only 2% of pregnant women infected with anti-D immune globulin contaminated with HCV [5].

As the average life span of human beings continues to extend, owing to improvements in sanitary

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conditions and efficient management of ailments, difficulties in the treatment of chronic hepatitis C in elderly individuals are increasingly coming to the fore. This is attributable, at least in part, to liver fibrosis accelerating in parallel with age [11], as well as less tolerability and more side effects of combined interferon (IFN) and ribavirin in these patients [6,11,12].

These constraints notwithstanding, there is a pressing need for treatment of aged individuals with antiviral agents in order to prevent the development of cirrhosis and HCC and to promote better survival with an increased quality of life. When planning antiviral treatment of the elderly, weighing its merits against untoward effects, it is essential to understand the natural history of HCV infection in these patients. However, there have been virtually no reports on the natural history of HCV infection in older adults, nor are there any solid guidelines for antiviral treatment in these patients [13].

In the 42 years from 1964 to 2005, we have followed-up 332 patients who were persistently infected with HCV and had not received any antiviral treatment. They included the 120 patients with aspartate and alanine aminotransferase (ASAT and ALAT) levels $\leqq$40 IU/l (group A) and the 212 with ASAT and/or ALAT $\geq$41 (group B), and were followed-up for 3 years or longer without receiving any antiviral treatment. It is hoped that the evolution of chronic hepatitis in these patients, with special reference to the baseline transaminase levels, will shed light on how they should be treated for the prevention of cirrhosis and HCC in the coming era of global longevity.

Material and methods

Patients

During 42 years, from 1964 through 2005, 7358 patients with HCV-RNA in the serum visited the Department of Hepatology at the Toranomon Hospital in Metropolitan Tokyo. Of these patients, 843 (11.5%) were $\geq$65 years of age at presentation, and 512 (60.7% of the elderly) had not received antiviral agents or other drugs that might suppress the replication of HCV. In order to rule out cirrhosis, 180 patients with platelet counts $<120\times10^3/mm^3$ were excluded. The remaining 332 patients were classified into the 120 with ASAT and ALAT levels $\leqq$40 IU/l (Group A) and the 212 with ASAT and/or ALAT levels $\geqq$41 IU/l (group B); they included 22 patients (10.4%) with ASAT levels $\leqq$40 IU/l and 18 (8.5%) with ALAT levels $\leqq$40 IU/l. Baseline transaminase levels were determined at least twice, 2–3 months apart, in the course of 6 months. The patients were followed-up for 3 years or longer without receiving any antiviral treatment, and tested monthly for liver function, HCV-RNA and α-fetprotein (AFP) or protein induced by the absence of vitamin K or antagonist-II (PIVKA-II). Screening for cirrhosis and HCC was carried out yearly using ultrasonography and/or computed tomography. Angiography was implemented when HCC was strongly suspected by imaging modalities. During follow-ups, herbal medicine (intravenous Stronger Neo-Minophagen C (SNMC) or oral Shousaikotou) and/or ursodeoxycholic acid was given to 51 (42.5%) patients in group A and 139 (65.6%) patients in group B. Three (2.5%) patients in group A and 24 (11.2%) patients in group B, in whom IFN was started after they had been followed-up for 3 years or longer, left the study cohorts at the initiation of treatment. Informed consent was obtained from each patient who participated in this study, and the protocol conformed to the ethics guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the Human Research Committee of the institution.

Markers of HCV infection

Qualitative assay for HCV-RNA was performed using polymerase chain reaction (PCR) with nested primers and the results were recorded as positive or negative, with the detection limit at 100 copies/ml. Quantification of HCV-RNA was carried out with the branched-DNA assay version 2.0 (Chiron Corp., Calif., USA), and the results were expressed in megaequivalents (MEq) per milliliter over a range from <0.5 to 120 MEq/ml.

Statistical analysis

Since certain data in the analysis were regarded to comply with non-Gaussian distribution, categorical variables at baseline were compared with the Fisher exact test and numerical values were analyzed with the Mann-Whitney U-test and the Kruskal-Wallis test. Cumulative rates of cirrhosis, HCC, and death were calculated using the Kaplan-Meier technique, and differences between curves were evaluated by the log-rank test. A p-value <0.05 with the two-tailed test was considered significant. All the analyses were carried out using the computer program SPSS ver.11.0 (SPSS Inc., Ill., USA).

Results

Treatment-naive patients older than 65 years infected with HCV

During the 42 years from 1964 through 2005, the Department of Hepatology at the Toranomon Hospital in Metropolitan Tokyo admitted 332 patients aged 65 years or older with HCV who had not received any antiviral treatment, and in whom cirrhosis had not developed. In Table I we compare demographic, clinical, and virological characteristics between the 120 patients with baseline transaminase levels ≦40 IU/l and the 212 patients with levels ≧41 IU/l. ASAT and ALAT levels were higher, while platelet counts were lower in the patients with elevated transaminase levels compared with in patients without elevated transaminase levels.

When patients with baseline transaminase levels ≦40 IU/l were stratified by age, the median follow-up period was shorter in those aged 75–80 years than in those aged 65–69 or 70–74 years (4.5 versus 8.6 or 7.0 years, $p=0.011$) (Table II). Although the baseline transaminase levels were within normal limits in all of them, the median ASAT level was higher in patients aged 70–74 years than in those aged 65–70 or 75–80 years (35 versus 27 or 28 IU/l, $p=0.040$). In patients with baseline levels of both or either transaminase ≧41 IU/l, the median albumin level was lower in those aged 75–80 years than in those aged 65–69 or 70–74 years (3.9 versus 4.1 or 4.1 g /dl, $p=0.005$) (Table III).

Development of cirrhosis and HCC

Cirrhosis developed more frequently in elderly patients aged 65 years or older, with elevated transaminase levels at baseline, during follow-ups for longer than 3 years (Figure 1A). At 5 and 10 years of follow-up, cirrhosis developed in, respectively, 26% and 27% of the patients with the baseline transaminase levels ≧41 IU/l in contrast to only 4% and 13% of the patients with levels ≦40 IU/l ($p<0.001$). Likewise, HCC developed more frequently in elderly patients with elevated transaminase levels at baseline (Figure 1B). At 5 and 10 years of follow-up, HCC developed in, respectively, 22% and 26% of the patients with the baseline transaminase levels ≧41 IU/l, contrasting with only 3% and 5% of the patients with levels ≦40 IU/l ($p<0.001$).

Development of cirrhosis is compared between patients with and without elevated transaminase levels at baseline who were stratified by age (Figure 2). Cirrhosis developed more frequently in the patients with elevated transaminase levels than in those without elevated transaminase levels who were aged 65–69 years ($p<0.001$). In patients aged 70–74 years, cirrhosis tended to occur more often in those with elevated transaminase levels than in those without elevated transaminase levels during 5 years (27% versus 0%), but the difference fell short of being significant owing to the small number of patients in both groups.

Likewise, development of HCC is compared between patients with and those without elevated transaminase levels at baseline who were stratified by age (Figure 3). HCC developed more frequently in the patients with elevated transaminase levels than in those without elevated transaminase levels who were aged 65–69 years ($p=0.001$). In patients aged 70–74 and 75–80 years, HCC tended to occur more often in those with elevated transaminase levels than in those without elevated transaminase levels during 5 years (20% versus 5% and 19% versus 0%, respectively), but the difference was not significant, owing to the small number of patients in both groups.

Influence of gender on the development of cirrhosis and HCC

Figure 4 shows a comparison of the development of cirrhosis and HCC between 155 male and 177

Table I. Characteristics of patients with HCV-RNA aged 65 years or older with or without elevated transaminase (ASAT and ALAT) levels.

Features	≤40 IU/ml ($n=120$)	≥41 IU/l ($n=212$)	Differences p-value
Men	51 (42.5%)	104 (49.1%)	0.513
Follow-up (years)	7.8 (3–31.5)	8.7 (3–18.9)	0.181
ASAT (IU/l)	23 (6–40)	76 (27–496)	<0.001
ALAT (IU/l)	28 (11–40)	63 (22–411)	<0.001
Albumin (g/dl)	4.1 (2.4–4.9)	4.1 (3.2–5.3)	0.189
Platelets ($\times 10^3/mm^3$)	184 (120–343)	173 (120–313)	0.001
HCV RNA (MEq/ml)	4.5 (<0.5–120)	5.6 (<0.5–49)	0.168
HCV genotypes (1b:2a:2b:ND)	85:20:3:7	176:28:12:9	0.970

Abbreviations: HCV = hepatitis C virus; ASAT = aspartate aminotransferase; ALAT = alanine aminotransferase; MEq = megaequivalents; ND = not determined. Data are expressed as the number (%) or the median with the range in parentheses.

Table II. Characteristics of patients aged 65 years or older with HCV-RNA and without elevated baseline transaminase levels (ASAT and ALAT ≤40 IU/l) stratified by the age.

Features	65–69 years (n=79 (65.8%))	70–74 years (n=25 (20.8%))	75–80 years (n=16 (13.3%))	Differences p-value
Men	29 (36.7%)	11 (44.0%)	11 (68.8%)	0.062
Follow-up (years)	8.6 (3–31.5)	7.0 (3–12.6)	4.5 (3–17.6)	0.011
ASAT (IU/l)	27 (11–39)	35 (16–40)	28 (15–40)	0.004
ALAT (IU/l)	22 (6–40)	25 (9–40)	22 (9–37)	0.604
Albumin (g/dl)	4.1 (3.2–4.9)	4.1 (3.0–4.4)	4.0 (2.4–4.5)	0.247
Platelets ($\times 10^3/mm^3$)	193 (120–298)	177 (120–343)	182 (120–263)	0.408
HCV RNA (MEq/ml)	4.2 (<0.5–34.6)	6.5 (<0.5–120)	4.0 (<0.5–17.1)	0.181
HCV genotypes (1b:2a:2b:ND)	51:19:2:4	21:1:1:1	13:0:0:2	0.074

Abbreviations: HCV =hepatitis C virus; ASAT =aspartate aminotransferase; ALAT =alanine aminotransferase; MEq =megaequivalents; ND =not determined. Data are expressed as the number (%) or the median with the range in parentheses.

female patients aged 65 years or older. Cirrhosis tended to occur more frequently in male than in female patients. There were marked gender differences in the development of HCC. At 5 and 10 years of follow-up, HCC occurred more frequently in men than in women (18% and 25% versus 9% and 9%, respectively, p=0.033).

Complications and death in patients with the baseline transaminase levels ≦40 IU/l and ≧41IU/l

Of the 120 patients with baseline transaminase levels ≦40 IU/l, 33 (27.5%) developed complications during follow-up (hypertension in 9 (27%), diabetes in 7 (21%), both complications in 1 (3%), pulmonary disease in 4 (12%), heart disease in 4 (12%), and other illnesses in the remaining 8 (24%)). At 5, 10, and 15 years of follow-up, respectively, death occurred more frequently in the patients with complications than in those without complications (10%, 18%, and 45% versus 0%, 5%, and 5%, p=0.015) (Figure 5).

Among 9 of the 120 (7.5%) patients who died, liver disease was the cause of death in only one. Of the remaining 8 (89%) patients, 4 died of heart failure or infarction, and one each of pneumonia, cerebral hemorrhage, renal insufficiency, and decrepitude. Death was more frequent in the patients aged ≥70 years than in those aged <70 years at presentation (p=0.006) (Figure 6).

Complications and death in patients with the baseline transaminase levels ≧41 IU/l

Of the 212 patients with baseline tranasaminase levels ≧41 IU/l, 83 (39.2%) developed complications during follow-up (hypertension in 18 (22%), diabetes in 23 (28%), both complications in 10 (12%), extrahepatic malignancies in 12 (15%), and other diseases in the remaining 20 (24%)). There were no differences in the frequency of death between the patients with and those without complications, however (Figure 7).

Among 34 of the 212 (14.0%) patients who died, liver disease was the most frequent cause of death and occurred in 20 (59%); the frequency was higher than that (11% (1/9)) in the patients with transaminase levels ≦40 IU/l at baseline (p=0.021). There were no differences in the frequency of death among

Table III. Characteristics of patients with HCV-RNA aged 65 years or older and with elevated baseline transaminase levels (ASAT and/or ALAT ≥41 IU/l) stratified by the age.

Features	65–69 years (n=140 (66.0%))	70–74 years (n=48 (22.6%))	75–80 years (n=24 (11.3%))	Differences p-value
Men	63 (45.0%)	25 (52.1%)	16 (66.7%)	0.707
Follow-up (years)	9.0 (3–18.9)	8.4 (3–17.2)	7.7 (3–14.7)	0.061
ALAT (IU/l)	82 (28–496)	74 (27–440)	64 (30–269)	0.959
ASAT (IU/l)	67 (22–411)	67 (34–309)	71 (35–172)	0.201
Albumin (g/dl)	4.1 (3.2–5.3)	4.1 (3.4–4.6)	3.9 (3.4–4.7)	0.005
Platelets ($\times 10^3/cm^3$)	171 (120–313)	180 (120–289)	157 (120–263)	0.398
HCV RNA (MEq/ml)	5.9 (<0.5–44.8)	5.6 (<0.5–30.0)	3.0 (<0.5–49.0)	0.251
HCV genotypes (1b:2a:2b:ND)	121:19:8:6	37:7:4:1	18:2:0:2	0.294

Abbreviations: HCV =hepatitis C virus; ASAT =aspartate aminotransferase; ALAT =alanine aminotransferase; MEq =megaequivalents; ND =not determined.

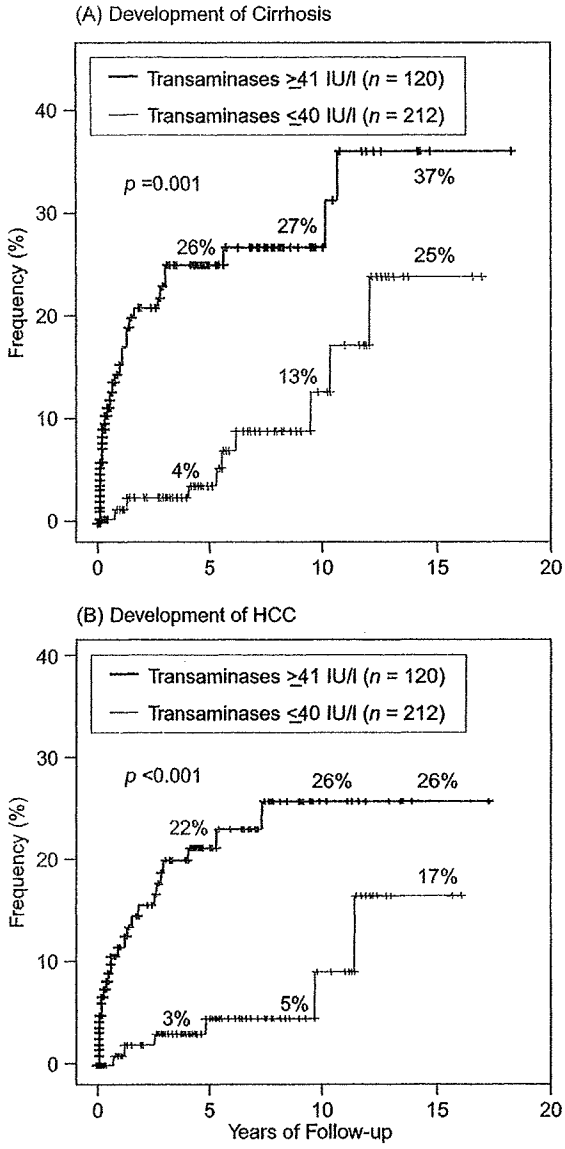


Figure 1. Development of cirrhosis (A) and HCC (hepatocelllular carcinoma) (B) in patients over 65 years of age with chronic hepatitis C who were followed-up without receiving antiviral treatment. Patients with and without elevated baseline transaminase levels are compared.

the patients in distinct age groups who had elevated baseline transaminase levels at baseline (Figure 8).

Discussion

The World Health Organization defines elderly individuals as those aged ≥65 years. In general, IFN is indicated for patients under 65 years of age, in view of frequent side effects and safety precautions. HCC develops increasingly with age and in the majority after 65 years, and in Japan approximately

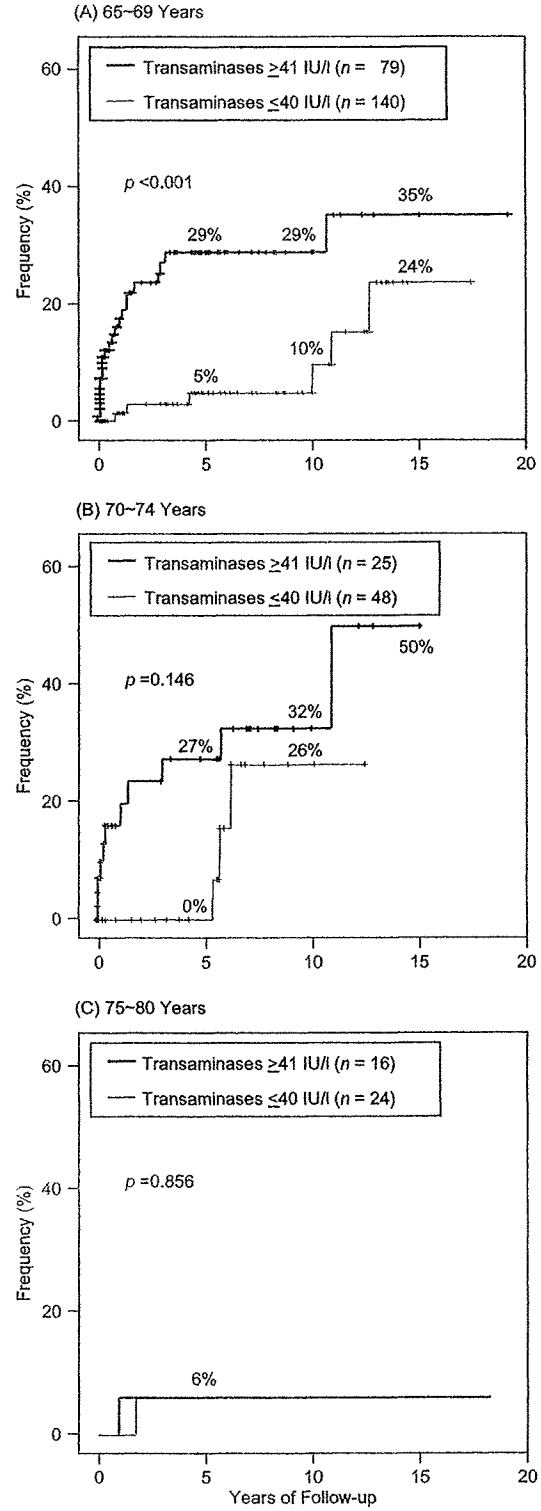
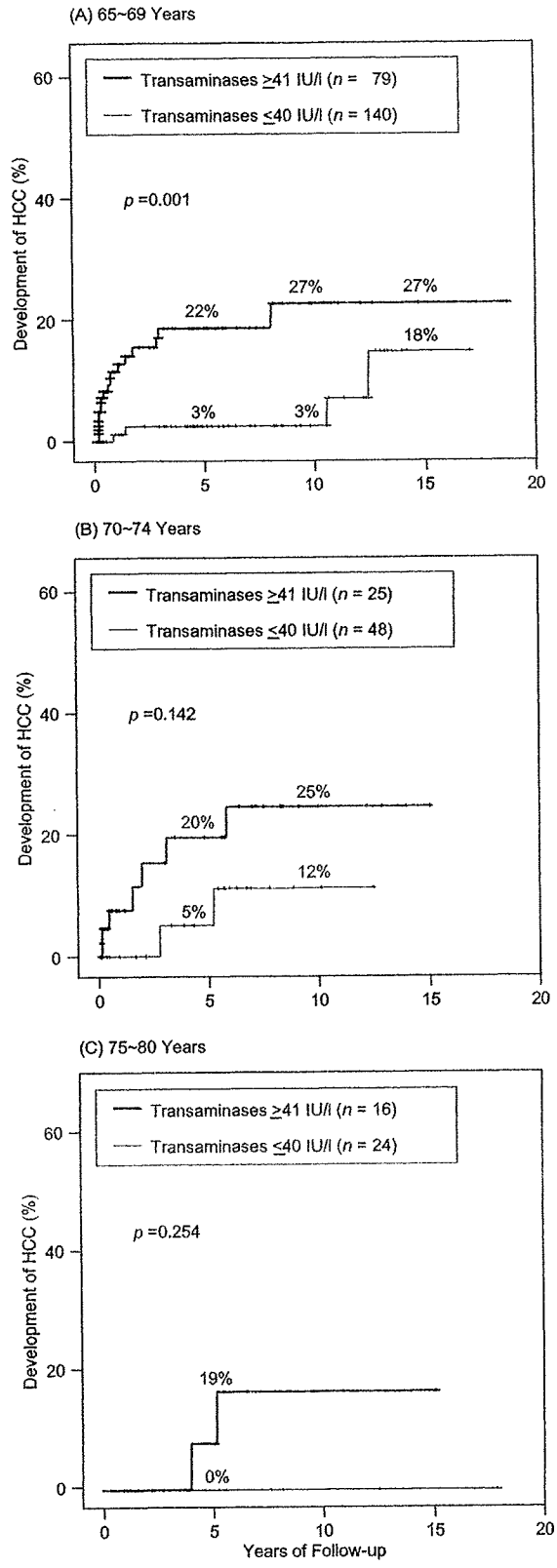


Figure 2. Development of cirrhosis in patients of more than 65 years of age with chronic hepatitis C who were followed-up without receiving antiviral treatment. Patients in different age groups are compared between those with and those without elevated transaminase levels.



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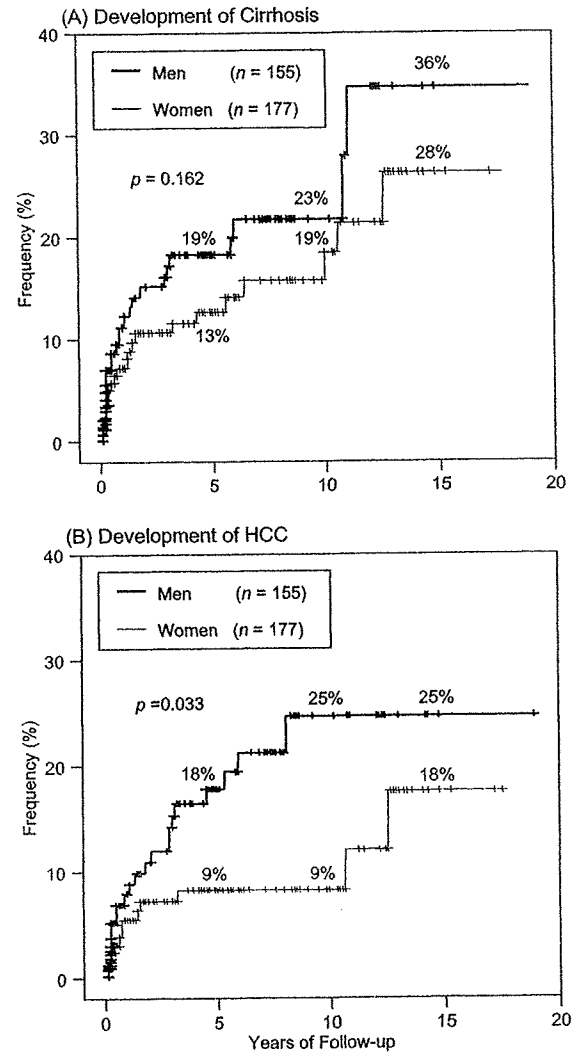

Figure 4. Development of cirrhosis (A) and HCC (hepatocelllular carcinoma) (B) in patients over 65 years of age with chronic hepatitis C who were followed-up without receiving antiviral treatment. Male and female patients are compared.

30,000 patients infected with HCV die yearly [14]. Furthermore, HCC is steadily increasing in the United States, and the incidence is expected to double or triple in the next two decades [15]. Hence, HCV carriers aged 65 years or older should be given IFN treatment, which is proven to be efficacious in preventing the development of HCC [16,17]. Previously, we have evaluated the efficacy and safety of IFN monotherapy in patients aged 65 years or older [18]. Of the 84 patients studied, the sustained virological response was reached in 30 (36%), while

Figure 3. Development of hepaptocelluar carcinoma (HCC) in patients over 65 years of age with chronic hepatitis C who were followed-up without receiving antiviral treatment. Patients in different age groups are compared between those with and those without elevated transaminase levels.

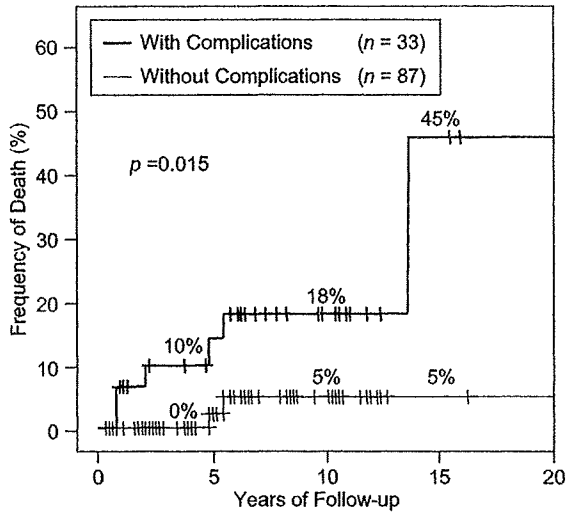

Figure 5. Deceased patients without elevated baseline transaminase levels (ASAT and ALAT <40 IU/l). Patients with and without complications other than liver disease are compared.

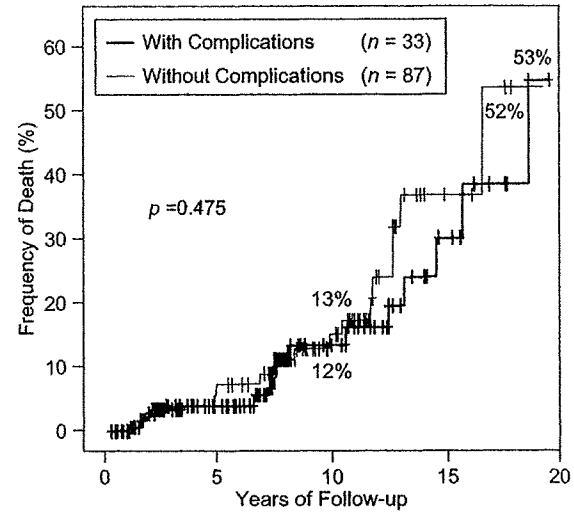

Figure 7. Deceased patients without elevated baseline transaminase levels (ASAT and ALAT <40 IU/l). Patients with and without complications other than liver disease are compared.

IFN was discontinued owing to adverse events in 11 (13%). Remarkably, the sustained virological response to combined IFN and ribavirin was comparable between the 66 patients aged ≥60 years and the 154 aged <60 years (31.8% versus 38.3%), although ribavirin had to be discontinued more frequently in the older patients (33.3% versus 20.8%, $p<0.05$) [19].

HCV spread widely in Japan around the end of World War II, at least 20 years earlier than in the other countries [4,14]. As a consequence, patients given combined IFN and ribavirin are 10–15 years older than those in Western countries [20–22]. Throughout the world, there are increasing numbers of individuals who are infected with HCV and entering the elder years. By the year 2010, the number of the elderly infected with HCV is estimated to account for 0.48 (54%) of the entire 0.89 million infected in Japan, and that in the United States for 0.78 (22%) of the 3.61 million [2–4]. These numbers will continue to increase for some time thereafter. As sequellae to this, cirrhosis and HCC will continue to increase, demanding higher medical costs. In the USA already, HCV-related

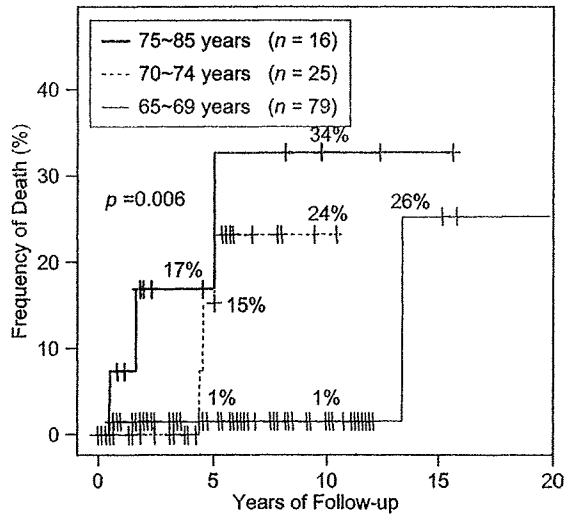

Figure 6. Deceased patients with elevated baseline transaminase levels (ASAT and/or ALAT >41 IU/l). Patients in the different age groups are compared.

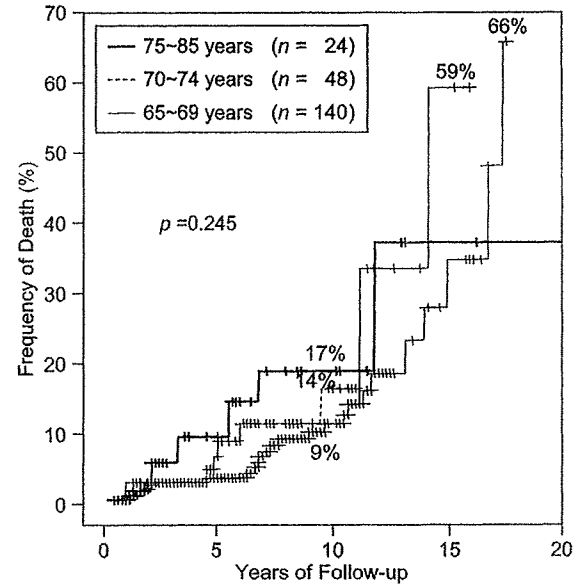

Figure 8. Deceased patients with elevated baseline transaminase levels (ASAT and/or ALAT >41 IU/l). Patients in the different age groups are compared.

end-stage liver disease is the leading cause of orthotopic liver transplantation [23]. This background demands that immediate measures should be taken to prevent fibrosis developing in the elderly with chronic hepatitis C by initiating the appropriate treatment; pegylated IFN combined with ribavirin can eliminate HCV efficiently [24,25].

Management of antiviral treatment in the elderly, however, is not without difficulties. Discontinuation of therapy or dose reduction was required frequently in the Japanese patients older than 60 years with chronic hepatitis C [21]. It is obvious that antiviral treatment needs to be administered with caution in aged patients with chronic hepatitis C, with the indication restricted to those who are likely to derive benefit from it. Early virological response at 12 weeks of treatment is predictive of sustained virological response [26]. The influence of HCV genotypes on the response to combined therapy, which increases with age [27], would have to be taken into consideration, also. In the Japanese patients infected with HCV genotype 1b, substitutions of amino acids at positions 70 and 91 are associated with a better response to combined treatment [28]. In view of the more frequent and serious side effects in elderly patients, these predictors would need to be taken into account when deciding whether to continue or discontinue combined treatment with IFN and ribavirin in elderly patients with chronic hepatitis C.

In order to plan the treatment of elderly patients, the natural history of HCV infection in these patients needs to be elucidated, which has not been done as yet. In the present study, we have followed-up treatment-naive patients aged ≥ 65 years without antiviral treatment for more than 3 years. None of them had cirrhosis at baseline. They were stratified by baseline transaminase levels $\leqq 40$ IU/l (group A ($n=120$)) and $\geqq 41$ IU/l (group B ($n=212$)) and classified further into the three age groups, 65–69, 70–74, and 75–85 years. Cirrhosis and HCC developed more frequently in the patients in group B than those in group A ($p<0.001$ for both). Of the patients aged 65–69 years at entry, in particular, cirrhosis and HCC developed more frequently in group B than in group A ($p<0.001$ and $p=0.001$, respectively). Liver-related causes of death were more common in group B than in group A (20/34 (59%) versus 1/9 (11%), $p<0.05$), and HCC developed more frequently in men than in women ($p=0.021$).

Despite the progression of fibrosis that is accelerated with age [6], liver-related deaths were infrequent in patients with normal baseline transaminase levels and much less often than in those with elevated baseline transaminase levels (1/120 (0.8%) versus 20/212 (9.4%), $p=0.002$). Development of cirrhosis or HCC was no different between patients in groups A and B who were aged 70 years or older at entry. Taken altogether, elderly patients with elevated transaminase levels who are younger than 70 years would be the best candidates for antiviral treatment. They would need to be treated, even when side effects appear, by modifying the doses of IFN and ribavirin. In contrast, antiviral treatment may not be necessary for elderly patients with normal ALAT levels, or can be discontinued in these patients when side effects emerge.

There has been some controversy over antiviral treatment for elderly patients with chronic hepatitis C, and no specific guidelines have been drawn up so far [29]. The sustained virological response to antiviral treatment in aged patients is reported to be either poorer than [30–32] or comparable with that in younger patients [19,33]. The difference is most likely ascribed to careful selection of the aged patients who would benefit from treatment [13]. Based on the natural history of elderly patients with chronic hepatitis C described herein, those with elevated transaminase levels would need treatment to prevent progression to cirrhosis and HCC, while others with normal levels may not require treatment. It is to be hoped that the results in this study might be of help in planning a reasonable treatment strategy towards the longevity, without development of cirrhosis or HCC, in elderly patients with chronic hepatitis C, whose numbers are expected to increase progressively in the foreseeable future.

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