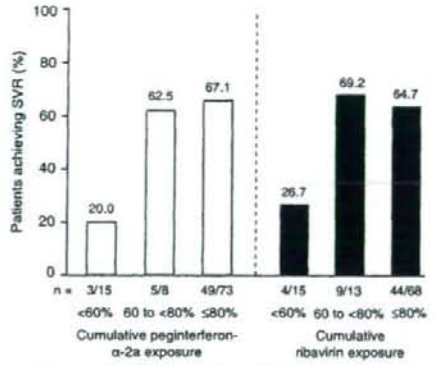

Fig. 3. Effects of peginterferon-α-2a and ribavirin exposure on sustained virological response (SVR). The cumulative exposure of patients to the study drug(s) was expressed as a percentage of the planned total dose.

severe fibrosis stage was reported in only 15.6% of patients. As a result, the small proportion of patients with severe fibrosis staging may have influenced the outcome of the current analysis.

Anaemia is a common adverse effect that can occur soon after the initiation of treatment with PEG IFN plus ribavirin for HCV infections. This complication can negatively impact patient quality of life, and is the most common reason for dose reductions and the temporary or permanent discontinuation of ribavirin. Such dose modifications have been shown to reduce the efficacy of treatment.[12] In general, females were predicted to have a higher likelihood of becoming anaemic than male patients.[13] In addition, the dose reduction rate of PEG IFN-α-2a and ribavirin is higher in elderly patients, which negatively impacts the achievement of an SVR.[5]

In a recent pooled analysis[14] of two phase III trials of 48 weeks of treatment with PEG IFN-α-2a plus ribavirin, the SVR rate was significantly reduced ($p = 0.0006$) in patients with a cumulative ribavirin dose of <60%. Prolonged periods of dose reduction, temporary interruptions or premature cessation of ribavirin were also associated with decreased SVR rates.

Previous studies have not assessed the impact of reducing the dose of PEG IFN independent of ribavirin, or differentiated between dose reduction, or interrupting or prematurely discontinuing treatment. An analysis of the HALT-C (Hepatitis C Antiviral Long-term Treatment against Cirrhosis) trial[15] investigated the impact of PEG IFN-α-2a and ribavirin dose reductions during the retreatment of patients infected with chronic HCV genotype 1 who did not respond to standard IFN with or without ribavirin treatment. A decrease in the cumulative dose of PEG IFN-α-2a received during the first 20 weeks of treatment (lead-in phase), from full dose (≥98%) to ≤60%, reduced the SVR rate from 17% to 5%. In contrast, reducing the dose of ribavirin from full dose to ≤60% did not affect the SVR rate as long as ribavirin administration was not interrupted for more than seven consecutive days. However, the premature discontinuation of ribavirin, even with full-dose PEG IFN-α-2a, reduced the SVR rate to 3%. This suggests that sufficient dosage during the early stages of therapy is required to achieve a high SVR rate with combination therapy. In our study, the SVR rate was also reduced in patients who received cumulative PEG IFN-α-2a and ribavirin doses of <60%, which was further decreased in patients who discontinued combination therapy. Therefore, it is important to alter the way adverse events of PEG IFN-α-2a and ribavirin therapy are managed to minimize the number of patients needing to reduce doses or discontinue therapy.

Conclusion

The attainment of an SVR following PEG IFN-α-2a plus ribavirin combination therapy was not influenced by any of the host-related factors evaluated in this analysis, although males aged ≥60 years tended to have a lower SVR rate. In contrast, younger age, male sex and lower baseline HCV RNA levels significantly increased the likelihood of achieving SVR with monotherapy. Dose reductions had a negative impact on SVR in elderly patients receiving combination therapy. Therefore, it is important to minimize PEG IFN-α-2a and ribavirin dose reductions by effectively managing treatment-related adverse events in elderly patients.

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ORIGINAL ARTICLE

Gene Expression in Fixed Tissues and Outcome in Hepatocellular Carcinoma

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ABSTRACT

BACKGROUND

It is a challenge to identify patients who, after undergoing potentially curative treatment for hepatocellular carcinoma, are at greatest risk for recurrence. Such high-risk patients could receive novel interventional measures. An obstacle to the development of genome-based predictors of outcome in patients with hepatocellular carcinoma has been the lack of a means to carry out genomewide expression profiling of fixed, as opposed to frozen, tissue.

METHODS

We aimed to demonstrate the feasibility of gene-expression profiling of more than 6000 human genes in formalin-fixed, paraffin-embedded tissues. We applied the method to tissues from 307 patients with hepatocellular carcinoma, from four series of patients, to discover and validate a gene-expression signature associated with survival.

RESULTS

The expression-profiling method for formalin-fixed, paraffin-embedded tissue was highly effective: samples from 90% of the patients yielded data of high quality, including samples that had been archived for more than 24 years. Gene-expression profiles of tumor tissue failed to yield a significant association with survival. In contrast, profiles of the surrounding nontumoral liver tissue were highly correlated with survival in a training set of tissue samples from 82 Japanese patients, and the signature was validated in tissues from an independent group of 225 patients from the United States and Europe ($P=0.04$).

CONCLUSIONS

We have demonstrated the feasibility of genomewide expression profiling of formalin-fixed, paraffin-embedded tissues and have shown that a reproducible gene-expression signature correlated with survival is present in liver tissue adjacent to the tumor in patients with hepatocellular carcinoma.

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IN DEVELOPING COUNTRIES, HEPATOCELLULAR carcinoma often comes to medical attention when the tumors are at an advanced stage and curative therapies are of limited benefit. In developed countries, however, at-risk populations of patients (e.g., those who are infected with hepatitis virus and have cirrhosis) are often under close surveillance; as a result, hepatocellular carcinoma is usually detected when the tumors are small and treatment is more likely to be successful.[1,2] Nevertheless, recurrences eventually occur in most patients.[1,2] Studies suggest that chemopreventive strategies may suppress recurrence and prolong survival,[1,3-6] although these findings are still uncertain. It would be ideal to treat only patients at greatest risk for recurrence. Several methods have been used to predict survival among patients with hepatocellular carcinoma, including the enumeration of anatomical and histopathological attributes (e.g., tumor multinodularity and vascular invasion), but these have become less useful as hepatocellular carcinoma is increasingly diagnosed at earlier stages.

A technical challenge facing the use of gene-expression profiling to predict the outcome of hepatocellular carcinoma has been the lack of suitable specimens from patients. Current methods of genomewide expression profiling require frozen tissue for analysis, whereas tissue banks with clinical outcome data generally have formalin-fixed, paraffin-embedded specimens. Even today, the vast majority of specimens are formalin-fixed; the collection of frozen tissues has yet to become routine clinical practice.

We tested a method for genomewide expression profiling of formalin-fixed, paraffin-embedded tissues. We applied the method to the analysis of the clinical outcome of hepatocellular carcinoma.

METHODS

PATIENTS AND SAMPLES

The training set consisted of tissue samples from 106 patients who were consecutively treated with surgery for primary hepatocellular carcinoma between 1990 and 2001 at Toranomon Hospital in Tokyo and for whom data on clinical outcomes (over a median follow-up period of 7.8 years) and formalin-fixed, paraffin-embedded blocks of tumor and adjacent tissue were available (Fig. 1). The validation set included tissue samples from 234 patients with hepatocellular carcinoma who consecutively underwent surgery between 1994 and 2005: 92 patients at the Mount Sinai School of Medicine in New York, 46 at Hospital Clínic Barcelona, and 96 at the National Cancer Institute of Milan (members of the HCC Genomic Consor-

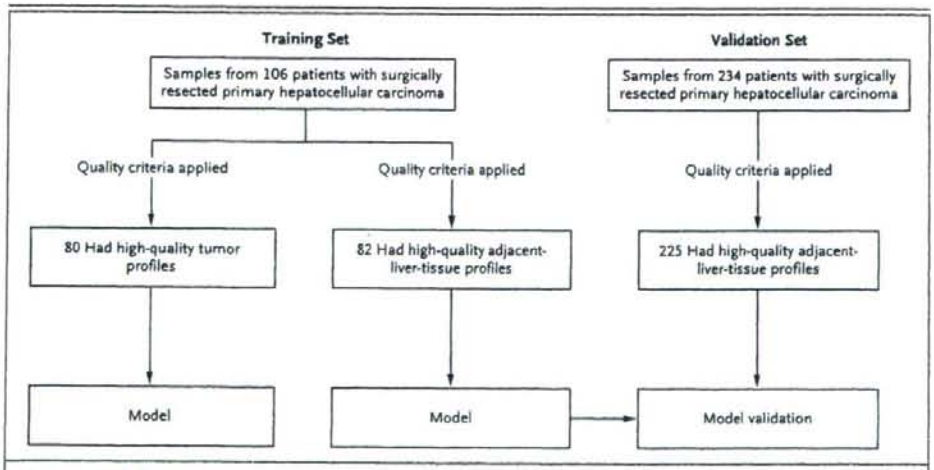


Figure 1. **Study Design.**
In the training set, tumor tissue and liver tissue adjacent to the tumor were profiled separately, and each was used to generate an outcome model. The model based on adjacent liver tissue was validated with the use of an independent validation set.

tium). Archived formalin-fixed, paraffin-embedded tissues obtained as part of routine clinical care were analyzed, with approval by the local institutional review boards granted on the condition that all samples be made anonymous. Formalin-fixed, paraffin-embedded blocks obtained at the time of resection were cut into three or four sections (each 10 μm thick), macrodissected to isolate tumor and adjacent liver tissue, and subjected to RNA extraction as described in the Supplementary Appendix (available with the full text of this article at www.nejm.org).

ANALYSIS OF GENE EXPRESSION

Gene-expression profiling was performed according to the complementary DNA–mediated annealing, selection, extension, and ligation (DASL) assay[7,8] (Illumina), and we selected 6100 transcriptionally informative genes for analysis (see the Supplementary Appendix). (Microarray data are available at www.ncbi.nlm.nih.gov/geo/, accession numbers GSE10143 and GPL5474.) Genes whose expression was associated with disease-specific survival and time to recurrence were selected with the use of the Cox score (see the Supplementary Appendix). The value of the signature was assessed on the basis of overall survival. Late recurrence was defined as tumor recurrence occurring more than 2 years after surgery.[9,10] Outcome association analysis was performed with the use of a nearest-neighbor–based method (see the Supplementary Appendix).

Table 1. **Characteristics of Patients in the Training Set and in the Validation Set, at the Time of Surgery.***

Characteristic	Training Set (N=82)	Validation Set (N=225)	P Value
Age — yr			<0.001
Median	59	66	
Interquartile range	52–64	57–71	
Male sex — no. (%)	64 (78)	173 (77)	0.88
HCV infection — no. (%)	60 (73)	104 (48)	<0.001
HBV infection — no. (%)	17 (21)	62 (29)	0.25
Alcohol use — no. (%)	3 (4)	19 (9)	0.22
Tumor diameter — cm			<0.001
Median	2.2	3.5	
Interquartile range	1.7–3.2	2.3–5.5	
Histopathological grade — no. (%)			
Well differentiated	18 (22)	34 (26)	0.68
Moderately differentiated	49 (60)	80 (60)	
Poorly differentiated	15 (18)	19 (14)	
Vascular invasion — no. (%)	4 (5)	74 (34)	<0.001
BCLC stage — no. (%)			
0	25 (30)	21 (9)	0.64†
A	50 (61)	186 (83)	<0.001‡
B	7 (9)	16 (7)	
Child–Pugh class A — no. (%)	72 (88)	204 (97)	0.52
Alpha-fetoprotein >100 ng/ml — no. (%)	53 (65)	53 (24)	0.14
Median follow-up — yr	7.8	2.2	—

* Some data were not available for all patients. The Barcelona Clinic Liver Cancer staging system (BCLC) ranks hepatocellular carcinoma in five stages, ranging from 0 (very early stage) to D (terminal stage). Histopathological grade was defined according to the International Union against Cancer (UICC) system. The Child–Pugh system classifies the severity of liver disease from A to C, with A representing the best liver function. HBV denotes hepatitis B virus, and HCV hepatitis C virus.

† P=0.64 for the pairwise comparison of stages 0 and A with stage B.

‡ P<0.001 for the multiple comparison of stage 0, stage A, and stage B.

STATISTICAL ANALYSIS

Functional annotation was performed by means of gene set enrichment analysis (GSEA, www.broad.mit.edu/gsea/).[11] Survival analyses were performed with the use of the log-rank test and Cox regression modeling. Subgroup analysis was performed on data from patients with a longer duration of follow-up (treated no later than 2004) and those with carcinoma classified as stage 0 or stage A according to the Barcelona Clinic Liver Cancer staging system (BCLC), which ranks hepatocellular carcinoma in five stages, ranging from 0 (very early stage) to D (terminal stage).[1,12] The hazard function for tumor recurrence was calculated as previously described.[10,13] All analyses were performed with the use of GenePattern[14] (www.broad.mit.edu/cancer/software/genepattern/) or the R statistical package (www.r-project.org). (See the Supplementary Appendix for details on the statistical analyses and methods of clonality analysis.)

RESULTS

VALIDATION OF THE PROFILING METHOD

We first sought a method that was suitable for gene-expression profiling of formalin-fixed, paraffin-embedded material. An approach has been reported for the analysis of several hundred transcripts based on DASL, a multiplex, locus-specific polymerase-chain-reaction (PCR) assay.[7,8] However, an unbiased discovery of diagnostic signatures requires a genomewide profiling method. Accordingly, we modified the DASL method for probe selection and analysis and performed a bioinformatic meta-analysis to identify 6000 transcripts that captured the majority of variance in gene expression across the human transcriptome (see the Supplementary Appendix). This 6000-gene DASL assay served as a potential tool for genomewide analysis of formalin-fixed, paraffin-embedded tissues. We found the assay to be highly reproducible (R^2>0.96 in replicate experiments), with an overall success rate of 90% among all the specimens, including formalin-fixed, paraffin-embedded tissue blocks collected up to 24 years ago (see the Supplementary Appendix). We found that representing each transcript with one probe only (as opposed to three, as previously reported[7,8]) resulted in little loss of assay performance (Fig. 1 in the Supplementary Appendix).

Figure 2 (facing page). **Survival Signatures and Survival Curves in the Training Set.**

Curves are shown for survival according to the association of the gene signature with survival, based on leave-one-out cross-validation testing (Panel A), and for overall survival according to the level of expression of the 186 signature genes (Panel B); of these, 113 were associated with a good prognosis and 73 with a poor prognosis. Panel C shows the expression pattern of the survival signature (comprising 186 genes). The 20 genes most closely associated with a poor prognosis are listed on the left, and the 20 most closely associated with a good prognosis on the right. Red indicates high expression; blue indicates low expression. Panel D shows representative photomicrographs of sections of liver tissue adjacent to tumor that were profiled in this study; there were no histologic correlates with survival. Staining was with hematoxylin and eosin.

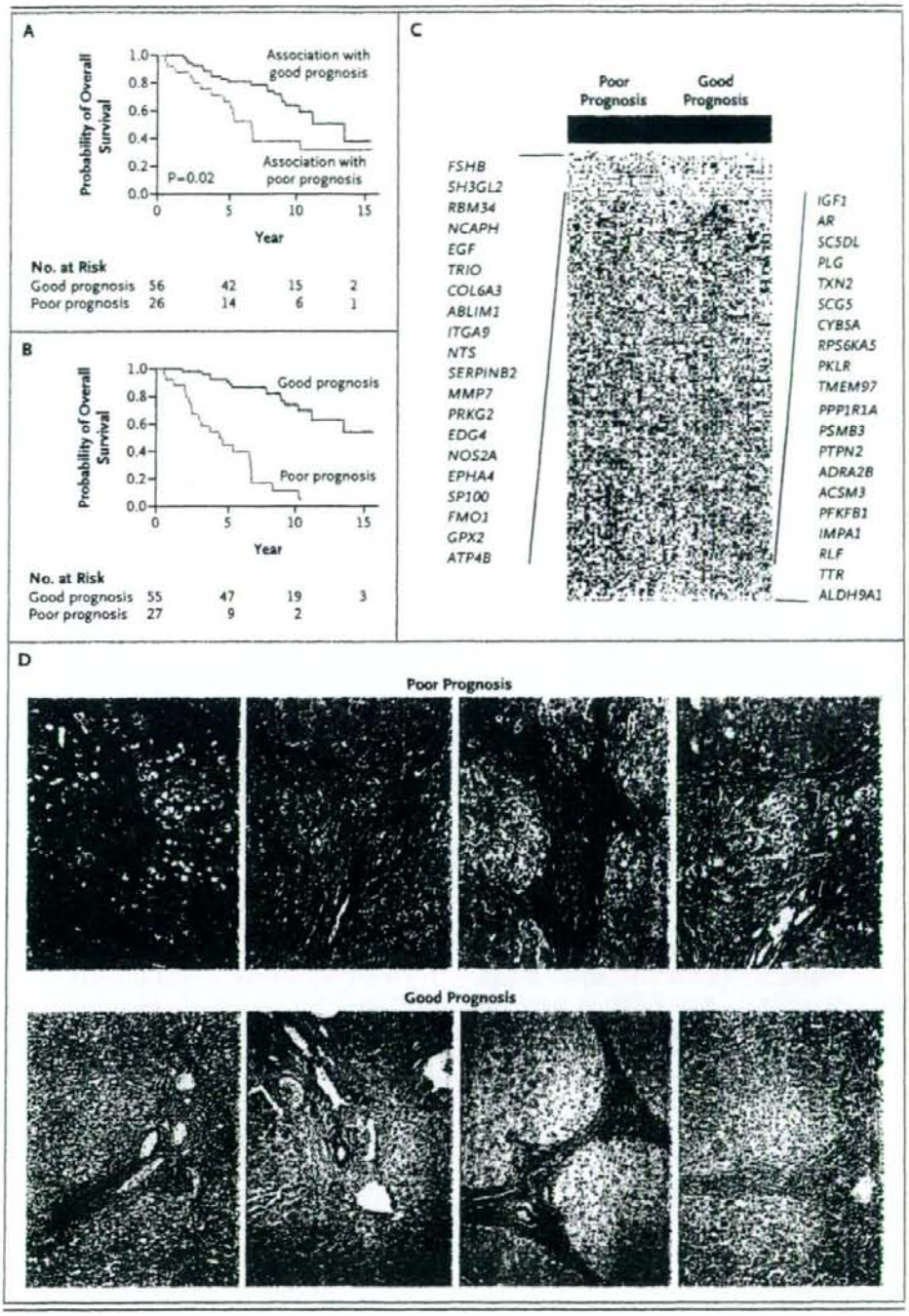
PATIENTS

Table 1 summarizes the clinical characteristics of the patients in the training and validation sets. All patients were treated with curative surgical resection, which was, in some cases, followed by second-line treatments at the time of recurrence.

By design, the training set included tissue samples from a large proportion of patients with very-early-stage hepatocellular carcinoma (BCLC stage 0), because these patients represent the greatest clinical challenge with respect to outcome prediction. Indeed, no clinical variables, either alone or in combination, were associated with survival among these patients (Table 1 in the Supplementary Appendix). Although there were no significant differences between the training set and validation set with respect to the number of patients with advanced-stage carcinoma (BCLC stage B) or the status of liver function, there was heterogeneity between the two sets with respect to certain tumor characteristics, such as diameter and type of viral infection (Table 1). Such heterogeneity may help to ensure that molecular predictors have real-world applicability across heterogeneous populations of patients.

PROFILES OF HEPATOCELLULAR CARCINOMA TUMORS

We first investigated whether gene-expression profiles of hepatocellular carcinoma tumors were associated with the clinical outcome. For each of the 106 patients in the training set, tumor-containing portions of the formalin-fixed paraffin-embedded blocks were macrodissected away from



surrounding liver tissue. Eighty tumors (75%) yielded high-quality gene-expression profiles (see the Supplementary Appendix). Using a leave-one-out cross-validation procedure and a nearest-neighbor–based algorithm, we failed to detect a significant gene-expression correlate of either tumor recurrence (P=0.22) or survival (P=0.70) (Fig. 2A in the Supplementary Appendix). Furthermore, a previously reported signature associated with survival among patients with hepatocellular carcinoma[15] was not associated with survival in our series of patients (P=0.76) (Fig. 2B in the Supplementary Appendix). This failure to identify an outcome-associated signature is unlikely to be due to a technical flaw of the formalin-fixed, paraffin-embedded DASL method, because we observed the same molecular-subclass structure in the formalin-fixed, paraffin-embedded samples as that observed in collections of frozen samples of hepatocellular carcinoma (Fig. 2B and 3B in the Supplementary Appendix). Although this result does not exclude the possibility of tumor-derived expression profiles as predictors of the outcome of hepatocellular carcinoma, the data suggest that at least in this training set, the outcome was largely related to other factors.

SURVIVAL SIGNATURE IN ADJACENT LIVER TISSUE

The lack of association between tumor-derived gene-expression profiles and survival led us to consider the pattern of recurrence of early-stage hepatocellular carcinoma. In contrast to advanced tumors, which tend to recur rapidly after resection, early-stage tumors, which are increasingly diagnosed in modern clinical practice, recur much later, generally more than 2 years after resection[9,10] (Fig. 4 in the Supplementary Appendix). This emerging pattern of late recurrence of hepatocellular carcinoma (due at least in part to the diagnosis of hepatocellular carcinoma at an early stage) has led to the notion that a late recurrence may not be an actual recurrence but rather a second primary tumor in an at-risk liver, presumably due to the carcinogenic effects of cirrhosis.[1,2,9] We therefore hypothesized that the surrounding liver tissue — not the tumor itself — might harbor a gene-expression signature associated with subsequent recurrence.

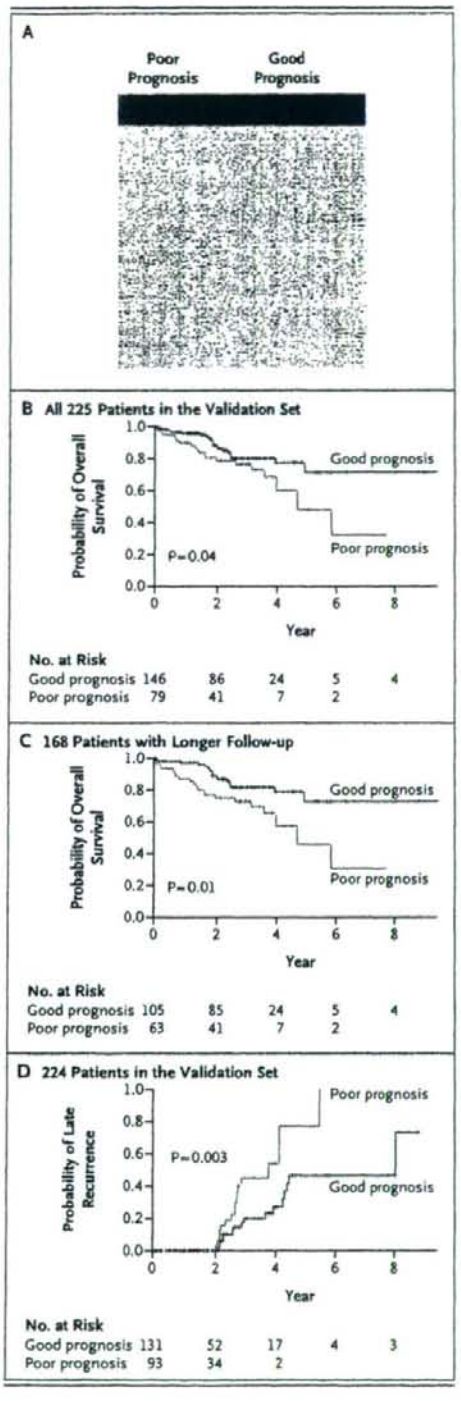
To test this hypothesis, we assessed the gene-expression profiles of the liver tissue surrounding the resected tumor in the 106 formalin-fixed, paraffin-embedded blocks that constituted the training set. Eighty-two samples (77%) yielded high-quality gene-expression profiles (see the Supplementary Appendix). Using a standard leave-one-out cross-validation procedure, we found the liver signature to be significantly correlated with survival (P=0.02) (Fig. 2A). The aggregate survival-correlated signature contained 186 genes (Fig. 2B and 2C, and Table 2 in the Supplementary Appendix) and was tested in the validation set. Using GSEA, which shows whether a defined set of genes has a significant association with a phenotype of interest, we found the good-prognosis signature to contain genes associated with normal liver function (Tables 2 and 3 in the Supplementary Appendix), including the plasma proteins C4, C5, C8, C9, and F9 and several drug-metabolizing enzymes: the alcohol dehydrogenases ADH5 and ADH6, the aldo–keto-reductases AKR1A1 and AKR1D1, the aldehyde dehydrogenase ALDH9A1, the cytochrome P450 CYP2B6, and hepatic lipase (LIPC). These findings are consistent with the association between impaired liver function and a poor outcome.[3] In addition, the poor-prognosis signature contained gene sets associated with inflammation, including those related to interferon signaling, activation of nuclear factor-κB, and signaling by tumor necrosis factor α. Histologic features of liver inflammation were not found to be associated with the outcome (Fig. 2D, and Table 4 and Fig. 5 in the Supplementary Appendix). Of particular interest, GSEA showed that the downstream targets of interleukin-6 were strongly associated with the poor-prognosis signature, which is consistent with the finding that disruption of interleukin-6 signaling protects mice from chemically induced hepatocellular carcinoma.[16]

VALIDATION OF THE LIVER-DERIVED SURVIVAL SIGNATURE

We next tested the 186-gene survival signature in an independent set of tissue samples from eligible patients at three treatment centers in the United States and Europe. Of the 234 samples in this validation set, 225 (96%) yielded gene-expression profiles of high quality (see the Supplementary Appendix). The survival signature (Fig. 3A) was associated with significant differences in survival among patients (P=0.04) (Fig. 3B), despite the modest duration of follow-up (median, 2.2 years). The separation of the survival curves was even more pronounced when, in a prespecified subgroup analysis, we limited our attention to the 168

Figure 3. **Survival Signatures and Survival Curves in the Validation Set.**

Panel A shows the expression pattern of the 186-gene survival signature. Red indicates a poor prognosis; blue indicates a good prognosis. Survival curves are shown for overall survival according to the level of expression of the 186 signature genes among all 225 patients whose tissue samples constituted the validation set (Panel B) and among the 168 patients with a longer duration of follow-up (treated no later than 2004) (Panel C). Panel D shows the probability of late recurrence according to the level of expression of the late-recurrence gene signature. Data are missing for one patient in Panel D.



patients with a longer duration of follow-up (median, 2.8 years; P=0.01) (Fig. 3C). These results support the validity of the survival signature and highlight the potential role of nontumoral liver tissue in predicting the outcome for patients with early hepatocellular carcinoma.

RECURRENCE-ASSOCIATED SIGNATURE

We performed a similar analysis using tumor recurrence as the clinical end point. A 132-gene late-recurrence signature defined in the training set was tested in the validation set. Whereas the recurrence signature did not show an association with recurrence within the first 2 years after surgery (a finding that was consistent with its development in association with late recurrence) (Fig. 6A and 6B in the Supplementary Appendix), it was significantly associated with late recurrence (P=0.003) (Fig. 3D). Not surprisingly, a nonparametric enrichment test indicated that the survival and late-recurrence signatures were closely associated (P<0.001) (Fig. 6C in the Supplementary Appendix).

MULTIVARIATE ANALYSIS

We next examined the signature in the context of the factors that are generally accepted as indicating a poor prognosis for patients with hepatocellular carcinoma (tumor multinodularity, the presence of microvascular invasion, and a high serum alpha-fetoprotein level[1,9]) in the validation set. These factors were associated with early recurrence (within 2 years after treatment) (Table 5 in the Supplementary Appendix). In contrast, multivariate analysis showed that the late-recurrence signature was the only independent prognostic variable for late recurrence (Table 2). Prespecified subgroup analyses showed that this associa-

Table 2. **Associations of Gene-Expression Signatures and Clinical Variables with Late Recurrence or Overall Survival, from Multivariate Analysis of the Validation Set.**

Variable	Hazard Ratio (95% CI)*	P Value
Late recurrence: late-recurrence signature	2.94 (1.39–6.20)	0.005
Overall survival		
All 225 patients		
Poor-prognosis signature	2.08 (1.03–4.18)	0.04
Alpha-fetoprotein >100 ng/ml	2.29 (1.14–4.61)	0.02
Vascular invasion	2.01 (1.01–3.99)	0.05
168 Patients with longer follow-up		
Poor-prognosis signature	2.56 (1.22–5.38)	0.01
Alpha-fetoprotein >100 ng/ml	2.01 (0.94–4.26)	0.07
Vascular invasion	2.20 (1.06–4.53)	0.03

* The hazard ratio was for late recurrence among patients with the late-recurrence gene signature as compared with those without the signature or for overall survival among patients with the poor-prognosis gene signature as compared with those without the signature, with alpha-fetoprotein levels of more than 100 ng per milliliter as compared with levels of 100 ng per milliliter or less, or with vascular invasion as compared with the absence thereof.

tion remained significant in both the subgroup of 168 patients with a longer period of follow-up and the subgroup of 207 patients with early-stage hepatocellular carcinomas (BCLC stage 0 or A) (Fig. 4, and Table 6 in the Supplementary Appendix). Similarly, the survival signature was independently associated with survival in multivariate analysis (Table 2), and this association persisted in the subgroup of patients with longer follow-up (Table 2).

These results indicate that clinical and histopathological factors are associated with early recurrence of hepatocellular carcinoma and that late recurrence is associated with the gene-expression signature of nontumoral liver tissue adjacent to the primary tumor. The latter finding is consistent with the notion that late recurrences are not actually recurrences but rather new primary tumors. In support of this view, we detected highly discordant patterns of gains and losses in gene-copy number (including in regions exhibiting loss of heterozygosity) between the primary and recurrent hepatocellular carcinoma tumors but did not detect such patterns in endometrial, ovarian, renal, or lymphoma tumors (Table 7 and Fig. 7 in the Supplementary Appendix). These results strongly suggest that the primary and recurrent hepatocellular carcinoma tumors arise from distinct clones.

DISCUSSION

The full potential of gene-expression profiling of cancer has been hindered in part by technical limitations — in particular, the requirement of frozen material for analysis. Although frozen tissues are increasingly being banked at tertiary care centers, the duration of clinical follow-up of these collections is usually short, and the vast majority of tumor-biopsy specimens and resections are performed outside of major research hospitals. There is therefore a need for methods that allow for the genomewide expression profiling of formalin-fixed tissue samples, which are routinely collected in the clinical setting. Such approaches have been described,[17] but their extensive validation has yet to be reported. We describe here a DASL-based method capable of profiling approximately 6000 human transcripts, and we have tested the method on more than 2000 formalin-fixed, paraffin-embedded blocks collected as long as 24 years ago. Through the assay of 6000 genes across the genome that show maximal variation in expression, this approach is expected to capture the bulk of transcriptional differences in any collection of samples. However, recent increases in array density support the analysis of all human genes on a single array (whole-genome DASL assay, Illumina).

The DASL-based discovery method that we describe here should be distinguished from candidate-gene profiling methods based on the reverse transcriptase (RT)-PCR assay, such as those used in the commercially available OncotypeDx test for determining the prognosis in patients with breast cancer.[18] Whereas standard RT-PCR methods can measure a small number of transcripts in formalin-fixed, paraffin-embedded samples, genomewide discovery studies are not feasible with the use of RT-PCR–based methods. In addition, we speculate that the use of formalin-fixed, paraffin-embedded tissue specimens will aid the transition from exploratory research to clinical implementation.

We applied the DASL profiling method to an increasingly important challenge in the care of patients with hepatocellular carcinoma. Tumors are often small at the time of diagnosis (owing to increased surveillance and advanced imaging in patients at risk), and existing prognostic factors are less informative for patients with small tumors than for those with larger tumors.

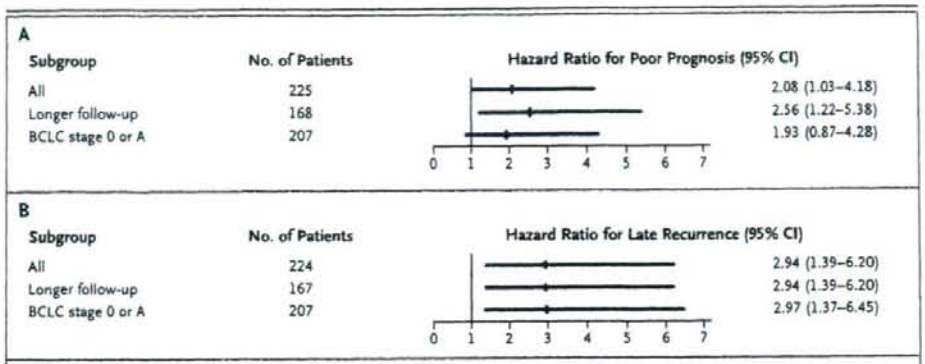


Figure 4. **Hazard Ratios for Poor Survival and Late Recurrence in Selected Subgroups of Patients in the Validation Set.** The hazard ratio was for poor survival among patients with the poor-prognosis gene signature (Panel A) or for late recurrence among patients with the late-recurrence gene signature (Panel B), as compared with those without the signature. BCLC denotes Barcelona Clinic Liver Cancer staging system, which ranks hepatocellular carcinoma in five stages, ranging from 0 (very early stage) to D (terminal stage). Data are missing for one patient in Panel B.

We did not observe a significant association between the expression profiles of the tumors themselves and the outcome for patients with surgically resected early hepatocellular carcinoma. In contrast, others have described tumor-derived prognostic signatures for hepatocellular carcinoma.[15,19] The populations of patients in those studies, however, tended to have more advanced disease. Our training set primarily exhibited a pattern of late recurrence that is typical of small tumors.[1,9] Accordingly, it is likely that early recurrence (reflecting locally invasive and incompletely resected tumor) is associated with molecular features of the primary tumor, but such features are not associated with late recurrences, which seem to result from new primary tumors arising in a damaged organ (the "field effect") rather than the proliferation of residual tumor cells derived from the original tumor.

Also supporting the concept that late recurrence of hepatocellular carcinoma represents new primary tumors in patients at risk, we found little correlation between the molecular characteristics of tumors resected at initial diagnosis and those from the same patients at the time of recurrence. In particular, the results of clonality analysis indicated that the late recurrences of hepatocellular carcinoma tended to derive from a different clone than the preceding primary tumors. In addition, the obvious measures of liver damage (e.g., the extent of cirrhosis and the Child–Pugh stage[20]) were not associated with survival in our study, given that we restricted our analysis to patients with preserved liver function.

Our findings indicate a field effect, in which environmental exposure (e.g., viral infection) leads to an increased potential for future malignant transformation. This has in general been overlooked by genomic approaches to studying cancer that have focused only on tumor cells. Our results suggest that a gene-expression signature can serve as a sensitive "readout" of the biologic state of the liver in at-risk patients. It is likely that the survival signature reflects the extent of liver damage and the presence or absence of a proinflammatory milieu, which is mediated in part by gene products involved in an inflammatory response. A heritable basis for the signature, although improbable, cannot be ruled out. Additional work is needed to fully understand the biologic basis of the signature.

Further clinical validation of the survival signature will be needed before it is introduced into clinical practice; our observation that the signature is associated with the outcome across heterogeneous populations of patients is encouraging. We envision the use of this test to identify the patients at highest risk for recurrence of hepatocellular carcinoma and to target intensive clinical follow-up or chemopreventive strategies in such patients.[21]

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INTERNAL MEDICINE

ORIGINAL ARTICLE

Natural Human Interferon β Plus Ribavirin Combination Therapy in Japanese Patients Infected with Hepatitis C Virus and a High Viral Load

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Abstract

Objective The aim of this pilot study was to determine the safety and efficacy of natural human interferon β (nIFNβ) plus ribavirin (RBV) in patients with chronic hepatitis C who did not respond to pegylated interferon alpha (PEG-IFN), with special emphasis on the incidence of mental disorders or refusal for fear of adverse effects.

Methods We studied 19 patients with HCV genotype 1b, 2a or 2b and a high viral load, including 8 patients with mental disorders. They were treated with nIFNβ-RBV. Eleven patients with HCV genotype 1b of these patients were treated with nIFNβ-RBV for 48 weeks (group A), and compared with 22 matched controls treated with PEG-IFN plus RBV for 48 weeks (group B). The other 8 patients with HCV genotype 2 were treated with nIFNβ-RBV for 24 weeks.

Results Six of 8 patients with mental disorders and 9 of 11 patients without mental disorders completed nIFNβ-RBV therapy; 1 patient with mental disorder dropped out due to exacerbation of depression, and 3 patients suspended the therapy due to insufficient response. The sustained virological response (SVR) was 27% (3/11) in group A and 41% (9/22) in group B ($p = 0.70$). During treatment, platelet count increased in group A but not in group B. SVR was 88% (7/8) in patients of genotype 2 and high viral load treated with nIFNβ plus RBV.

Conclusion nIFNβ-RBV therapy offers sufficient safety and efficacy for patients with mental disorders, and thus could represent an excellent second-line therapy for subpopulations that are not suitable for PEG-IFN-RBV.

Key words: hepatitis C virus, interferon β, ribavirin, depression

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Introduction

Pegylated interferon α (PEG-IFN) plus ribavirin (RBV) is the first line treatment for patients infected with hepatitis C virus (HCV) genotype 1, and high viral load, and can achieve sustained virological response (SVR) in 41-47% of these patients (1-3). However, such treatment causes adverse effects in some patients, such as mental disorders, apathy and laboratory abnormalities. Previous studies indicated that 10-16% of patients treated with PEG-IFN plus RBV for 48 weeks discontinued the therapy due to adverse effects, especially depression (1-3). Individuals with depression or previous history of interferon (IFN) α-induced mental disorders

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are not suitable candidates for IFNα therapy (4). Moreover, several patients reject the therapy for fear of depression arising as a side effect. IFNβ, a type I IFN that binds to the same cell surface receptor as IFNα, triggers distinct biological responses and elicits distinct patterns of gene expression within the same target cells (5-8). Three forms of human IFNβ are available (9): *1)* Natural human IFNβ (nIFNβ) is produced by human fibroblasts. *2)* Recombinant human IFNβ-1a (rhIFNβ-1a) is procured by mammalian cells and is identical to nIFNβ. *3)* Recombinant human IFNβ (rhIFNβ-1b) is produced by *Escherichia coli* in which cysteine at position 17 is substituted by serine. Previous reports showed that IFNβ has sufficient tolerability (10). Other reports indicated that IFNβ is effective in HCV eradication, although it seems that IFNβ monotherapy does not result in a satisfactory outcome in patients with HCV, particularly those infected with genotype 1 and have a high viral load (11, 12). Recent randomized trials demonstrated the efficacy of rhIFNβ-1a plus RBV (13, 14). However, there is little information regarding nIFNβ plus RBV (15, 16). Furthermore, the safety of IFNβ for patients with mental disorders has not been reported.

There is evidence to suggest that monitoring of early viral kinetics is useful for earlier identification of the likelihood of response to IFN therapy (17). Correlation viral kinetics and therapeutic outcome of PEG-IFN plus RBV has been investigated (18, 19), but not that of IFNβ plus RBV.

The present pilot study included 19 patients who had not received PEG-IFN or IFNα for their mental disorders or refused PEG-IFN for fear of adverse effects. The objective of this study was to assess the safety and efficacy of nIFNβ plus RBV in Japanese patients infected with HCV genotype 1b or 2 and high viral load. In addition, we assessed viral kinetics in patients infected with HCV genotype 1b with high viral load treated with nIFNβ plus RBV.

Patients and Methods

Study population

Nineteen HCV-infected Japanese patients were enrolled in this trial between 2001 and 2006 at Toranomon Hospital, Tokyo. The enrollment criteria were HCV genotype 1b, 2a or 2b confirmed by polymerase chain reaction (PCR) ; serum HCV RNA levels >100,000 international units (IU)/mL on quantitative PCR assay (defined as "high" viral load, Amplicor HCV Monitor version 2.0, Roche Diagnostics, Tokyo, Japan) ; No treatment with corticosteroids, immunosuppressants, or antiviral agents within 6 months prior to this trial; negativity for hepatitis B surface antigen (HBsAg), as determined by radioimmunoassay; hemoglobin concentration >12.0 g/dL; neutrophil count >1,500/μL; platelet count >60,000/μL; serum creatinine <1.5 times above the upper limit of normal; and body weight between 40 and 100 kg. The exclusion criteria were liver cancer or severe liver failure; pregnant or breastfeeding women; past history of hypersensitivity reactions to IFN or ribavirin. Psychiatric exclusion criteria included preexisting severe depression and suicidal ideation and/or attempt.

Eleven patients with HCV genotype 1b treated with nIFNβ plus RBV were defined as group A. To compare the clinical efficacy of the treatment, we retrospectively selected 22 patients treated with PEG-IFN plus RBV, matched 1:2 with patients of group A for genotype, sex, age, and response to previous IFNα or IFNα plus RBV (control group; group B). Patients of group B were selected from among 407 patients of Toranomon Hospital.

Study protocol

The study protocol was approved by the Human Ethics Review Committee of Toranomon Hospital and a signed consent form was obtained from each subject. Treatment was provided for 48 weeks to HCV genotype 1b, and for 24 weeks to HCV genotype 2a or 2b, with subsequent 24-week follow-up period.

nIFNβ group: 11 patients with HCV genotype 1b (group A), were treated with nIFNβ (Feron, Toray Industries Inc., Tokyo) intravenously at a dose of 6 million units (MU) daily for 2-8 weeks, followed by three times a week for 40-46 weeks (total 48 weeks). In group A, nIFNβ was administrated daily for 2 weeks to 6 patients, for 4 weeks to 1 patient and for 8 weeks to 4 patients. Eight patients with HCV genotype 2 were treated with nIFNβ intravenously at a dose of 6 MU daily for 2-8 weeks and then three times a week for 16-22 weeks (total 24 weeks).

Control group: 22 patients with HCV genotype 1b (group B) were treated with PEG-IFNα2b (Schering-Plough, Osaka, Japan) subcutaneously at a dose of 1.5 μg/kg weekly for 48 weeks.

Each patient was treated with oral RBV (Schering-Plough) at a total dose of 600-1,000 mg twice daily for 48 weeks for HCV genotype 1b and for 24 weeks for HCV genotype 2. The dose was adjusted according to body weight (600 mg for patients weighing ≤60 kg, 800 mg for those between 60 and 80 kg, and 1,000 mg for patients weighing between 80 and 100 kg). Both nIFNβ or PEG-IFNα2b and RBV were concurrently initiated.

Serum samples were collected from the patients at 0, 2 days, and 2, 4, 12 weeks, at the end of therapy, and 24 weeks after the end of therapy. HCV RNA in serum was quantified at each point by a quantitative reverse-transcription polymerase chain reaction (PCR) assay (Cobas Amplicor HCV Monitor version 2.0 using the 10-fold dilution method, Roche) with a low detection limit of 5 KIU/mL. When HCV RNA was undetectable by quantitative PCR assay, it was assessed using qualitative detection assay (Amplicor HCV, Roche) with a low detection limit of 50 IU/mL. End-of-treatment response (ETR) was defined as no detectable serum HCV RNA at the end of treatment. SVR was defined as no detectable serum HCV RNA at 24 weeks after the end of treatment.

Table 1. **Clinical Characteristics of Chronic Hepatitis C Patients with High Viral Load Treated with Combination Therapy of nIFNβ Plus Ribavirin**

n	19
Age (years)*	60 (33-73)
Gender (male/female)	9/10
Leukocytes (/μL)*	4600 (2300-7500)
hemoglobin (g/dL)*	14.3 (10.5-15.9)
Platelets ($\times 10^3$/μL)*	19 (6.8-25.4)
Alanine aminotransferase (IU/L)*	60 (24-726)
Genotype (1b/2a/2b)	11/4/4
HCV-RNA (KIU/mL)*	1100 (400-5000)
Histology (F: 1/2/3/4/ND)	3/6/1/1/8
Interferon: naive/retreatment	9/10
Previous IFN therapy	
Virological response : yes/no	6/4
Monotherapy / combination therapy	9/1

*Data represent the median (range) values.

nIFNβ, natural human interferon β; IFN, interferon

Liver fibrosis classified as: F1, periportal expansion; F2, portoportal septa; F3, portocentral linkage or bridging fibrosis; F4, cirrhosis; ND, not done.

Dose reduction of IFN and RBV

nIFNβ was reduced from 6 MU to 3 MU when neutrophil count decreased to <750/μL or platelet count to <30,000/μL. Furthermore, the dose of PEG-IFNα2b was reduced from 1.5 to 1.0-0.5 μg/kg/week if neutrophil count decreased to <750/μL or platelet count to <80,000/μL. RBV was reduced in a stepwise fashion by 200 mg/day when hemoglobin concentration decreased to 10 g/dL. Further dose reduction or discontinuation of these drugs was applied for ongoing hematological adverse effects or other unendurable adverse effects such as mental disorder, flu-like syndrome, and gastrointestinal symptoms.

Statistical analysis

Treatment outcome was analyzed on intention-to-treat basis. Mann-Whitney U test or Fisher's exact probability test was used for comparison between groups. All p values for statistical tests were two-tailed and those <0.05 were considered to denote a significant difference. Statistical analyses were performed using the SPSS software (SPSS Inc., Chicago, IL).

Results

Clinical background

The nIFNβ group consisted of 19 patients; 5 (26%) patients developed depression or had a history of depression, 3 (16%) had a history of IFNα-induced depression, 7 (37%) refused PEG-IFN for fear of adverse effects such as depression, 3 (16%) were older than 65 years, and 1 (5%) suffered from severe fatigue associated with previous IFNα therapy.

In all patients who developed depression during treatment or had previous history of depression, depression was diagnosed by psychiatrists at our hospital. Table 1 shows the clinical features of 19 patients treated with nIFNβ plus RBV. Ten (53%) patients had been treated with IFN previously. Among the retreatment patients, 4 (40%) were nonresponders to previous IFN therapy. Table 2 shows the clinical features of 11 patients with HCV genotype 1 and high viral load treated with IFNβ plus RBV (case; group A) and 22 patients treated with PEG-IFN (control; group B) groups. There were no significant differences between the two groups in HCV-RNA level, fibrosis score, and laboratory pa-

Table 2. **Comparison of Clinical Features of Patients with Genotype 1b and High Viral Load Treated with nIFNβ Plus Ribavirin and PEG-IFN Plus Ribavirin**

	Group A (IFNβ+RBV)	Group B (PEG-IFN+RBV)	p value
n	11	22	
Age (years)*	57 (36-67)	54 (29-67)	matched
Sex (male/female)	7/4	14/8	matched
Interferon: naive/retreatment	4/7	8/14	matched
Previous IFN therapy			
Virological response: yes/no	4/3	8/6	matched
Monotherapy/IFN+RBV	6/1	12/2	matched
HCV-RNA (KIU/mL)*	1300 (530-3400)	2200 (370-4500)	0.26
Leukocytes (/μL)*	4700 (2300-6000)	4350 (2800-7100)	0.65
Hemoglobin (g/dL)*	14.7 (12.8-15.9)	14.5 (12.8-16.4)	0.88
Platelets ($\times 10^3$/μL)*	18.8 (9-24.9)	15.6 (9-25)	0.47
Alanine aminotransferase (IU/L)*	60 (40-156)	69 (28-237)	0.29
γ-glutamyl transpeptidase (IU/L)*	43 (15-106)	43 (20-244)	0.38
LDL-C (mg/dL)*	105 (52-162)	109 (50-162)	0.70
ICG-R(15) (%)*	11 (5-16)	12 (8-45)	0.052
RBV dose/body weight (mg/kg)*	11.8 (11.1-13.3)	11.1 (2.7-14)	0.063
Histology (F: 1/2/3/4/ND)	2/3/1/1/4	11/5/4/0/2	0.23

nIFNβ, natural human interferon β; PEG-IFN, pegylated interferon α;

*Data represent the median (range) values.

IFN, interferon; RBV, ribavirin; LDL-C, low density lipoprotein cholesterol

ICG-R(15), indocyanine green retention rate at 15 minutes

Liver fibrosis classified as: F1, periportal expansion; F2, portoportal septa; F3, portocentral linkage or bridging fibrosis; F4, cirrhosis; ND, not done.

rameters shown in Table 2.

Safety profile of nIFNβ plus RBV therapy

Table 3 shows the clinical features of 8 patients (genotype 1b; n=5, genotype 2; n=3) who developed mental disorders. One patient (12.5%) received antidepressant, and 2 patients (25%) received anti-anxiety drugs at the start of the treatment. During the therapy, those 3 patients did not need additional drugs. On the other hand, 1 patient (12.5%) received anti-anxiety drugs but such treatment was discontinued due to exacerbation of depression at 32 weeks after the start of therapy, and 1 patient (12.5%) received antidepressant during the therapy. The remaining 3 patients (37.5%) did not need anti-anxiety drugs/antidepressants. Among the remaining 11 patients with no history of mental disorder, 1 patient (9%) received anti-anxiety drugs during the therapy.

nIFNβ dose reduction was necessary in 1 (5.3%) patient due to the development of neutropenia. RBV dose reduction was applied in 9 (47%) patients, due to anemia (n=8) and extensive skin eruption (n=1). Therapy was suspended in 3 (16%) patients due to insufficient response to the therapy.

In the control group (n=22), treatment was discontinued in 2 (9%) patients due to adverse effects (fatigue and depression). Eight (36%) patients required PEG-IFN dose re-

Table 3. **Clinical Features of Patients with Mental Disorders Treated with nIFNβ Plus Ribavirin**

Patient No.	Gender	Age	Genotype	Mental disorder	Prescription at start of therapy	Prescription during therapy	nIFNβ plus ribavirin	Therapy duration (W)	Virological response
1	F	60	1b	depression	none	Anti-anxiety drug	Dropped out due to depression	32	NR
2	M	46	1b	depression	antidepressant	keeping on	Dropped out due to insufficient response	24	NR
3	M	49	1b	depression	none	antidepressant	completed	48	NR
4	M	40	1b	IFN-Induced	none	none	completed	48	SVR
5	M	60	1b	IFN-Induced	none	none	completed	48	NR
6	F	57	2a	depression	antianxiety drug	keeping on	completed	24	SVR
7	F	63	2a	IFN-Induced	antianxiety drug	keeping on	completed	24	SVR
8	F	60	2b	depression	none	none	completed	24	SVR

nIFNβ, natural human interferon β; IFN, interferon; SVR, sustained viral response; NR, non-responder

Table 4. **Virological Response of Patients with Genotype 1b and High Viral Load Treated with nIFNβ Plus Ribavirin and PEG-IFN Plus Ribavirin**

	Group A (nIFNβ+RBV)	Group B (PEG-IFN+RBV)	p value
End-of-treatment response	5/11 (45%)	15/22 (68%)	0.270
Sustained virological response	3/11 (27%)	9/22 (41%)	0.703

nIFNβ, natural human interferon β; PEG-IFN, pegylated interferon α; RBV, ribavirin

duction due to fatigue (n=2), dizziness (n=1), neutropenia (n=4) and thrombocytopenia (n=1). RBV dose reduction was applied in 12 (55%) patients due to anemia. Therapy was suspended in 2 (9%) patients at 24 weeks after commencement due to insufficient response.

Efficacy of IFNβ plus RBV therapy

Table 4 shows the ETR and SVR rates of groups A and B. ETR and SVR were achieved in 45% (5/11) and 27% (3/11) patients of group A (nIFNβ plus RBV) and in 68% (15/22) and 41% (9/22) patients of group B (PEG-IFN plus RBV), respectively. Differences between groups A and B were not significant ($p = 0.27$ and 0.70, respectively). In patients of genotype 2 and high viral load, ETR and SVR to nIFNβ plus RBV therapy were achieved in 88% (7/8) and 88% (7/8) patients, respectively.

Profile of leukocyte count, hemoglobin concentration and platelet count

Figure 1 shows profile of leukocyte count, hemoglobin concentration, platelet count of groups A and B. These parameters were assessed at week 2, week 4, and every 4 weeks until week 48. In groups A and B, the average leukocyte count, hemoglobin concentration and platelet count at baseline were 4,540/mm^3 and 4,590/mm^3, 14.4 g/dL and 14.6 g/dL, 17.4×10^4/mm^3 and 16.1×10^4/mm^3, respectively. There were no significant differences in leukocyte count and hemoglobin concentration between groups A and B at each time point. However, the dynamics of platelet count was different between the two groups. In group A, the platelet count decreased to 15.1×10^4/mm^3 at week 2, but increased above baseline after week 4. In groups A and B, the platelet count was significantly different at week 4, 8, 12, 16, 20, 24, 40, 44 and 48, respectively.

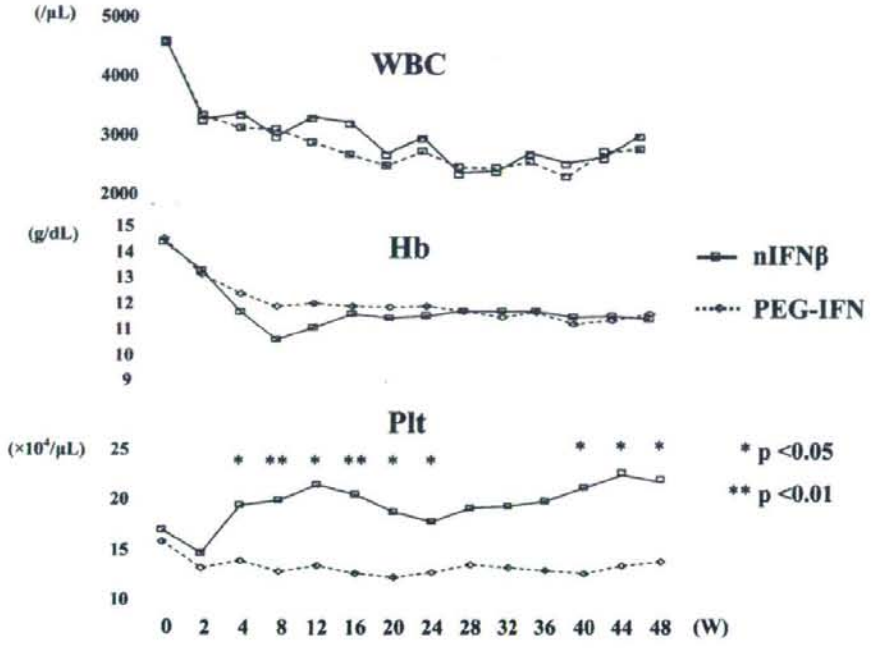

Figure 1. **Mean leukocyte count, hemoglobin concentration and platelet count during the 48 weeks of treatment of patients with chronic HCV infection, genotype 1b and high viral load. Solid lines: nIFNβ plus RBV group (group A), broken lines: PEG-IFN plus RBV group (group B). P values by Mann-Whitney U test.**

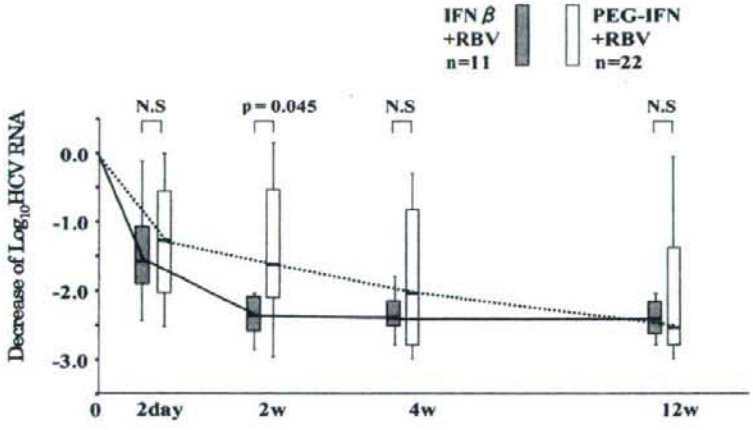

Figure 2. **Log changes in viral load from baseline during the initial 12 weeks of treatment in patients with chronic HCV infection, genotype 1b and high viral load. Gray-boxes and solid lines: nIFNβ plus RBV group (group A), light gray filled boxes and broken lines: PEG-IFN plus RBV group (group B). Bars within the boxes indicate the median value of log changes in viral load. The boxes denote the 25 to 75 percentiles, the lower and upper bars the 10 and 90 percentiles, respectively.**

Early viral kinetics

Figure 2 shows the early viral kinetics in groups A and B. Viral load was assessed at day 2, week 2, week 4 and week 12. At week 2, the median decrease in log viral load in group A was more than in group B (median, -2.34 logs vs. -1.80 logs, respectively; p = 0.045). However, the median decrease in log viral load was not significant between groups

A and B at other points.

Discussion

The frequent occurrence of psychiatric illness as a side effect of IFNα is well known; the reported incidences of IFNα- and IFNβ-induced depression range from 22 to 35% (1-3) and 10 to 21% (20-23), respectively. In general, however, IFNβ is a safe drug, but there is little or no information on its effects on patients with chronic HCV infection and depression or those with IFNα-induced depression.

In the present study, among 8 patients who developed mental disorders, only one patient stopped the therapy due to exacerbation of depression. One patient received antidepressant medication, 2 patients received anti-anxiety drugs at the start of the treatment. Moreover, during the therapy, 1 patient received anti-anxiety drugs and another one received an anti-depressant. The remaining 3 patients did not need any medications. Recently, Schaefer et al (24) reported that among 22 psychiatric patients treated with PEG-IFN plus RBV, 3 (13.6%) required antidepressants at the start of the treatment, 15 (68.2%) received antidepressants during anti-HCV therapy, and 1 (4.5%) discontinued PEG-IFN plus RBV treatment due to psychiatric disorders. The results of the above study and those of the present study indicate that the dropout rate due to psychiatric illness of patients treated with IFNβ is similar to that in patients on PEG-IFN. However, in our study, weaker anti-anxiety drugs were used during IFNβ therapy compared with the above report, suggesting that IFNβ-induced mental disorders are milder than those induced by PEG-IFN. However, the sample size of the present study is too small to make a firm conclusion and further studies are needed to specifically compare these two agents.

In the present study, the platelet count during administration of IFNβ plus RBV increased above baseline after week 4. A previous study reported that platelet count did not increase above the baseline during administration of nIFNβ monotherapy (21). In our study, at week 4, 64% (7/11) of patients were switched to 3 times a week administration. The increased platelet count after week 4 might be due to this switching and thus related to the study protocol. Interestingly, another previous study reported that platelet count increased after the time of daily administration of IFNα, but not above the baseline value (25). Such an increase of the platelet count above baseline after week 4 may be specific to only nIFNβ plus RBV combination therapy. Although the exact mechanism is not clear at this stage, nIFNβ plus RBV therapy may be useful for patients with thrombocytopenia who are not suitable for PEG-IFN plus RBV therapy. Further studies are required to clarify the mechanism of increased platelet count during nIFNβ plus RBV therapy.

The reported SVR rate of patients with genotype 1b and high viral load treated with nIFNβ monotherapy ranges from 0 to 11% (11, 12). Recent trials of rhIFNβ-1a plus RBV reported improved SVR rate. For example, the SVR reported by Chan et al (14) was 32.7% (18/55) in their patients with genotype 1b and high viral load treated with 44 μg rhIFNβ-1a (equivalent to 12 MU) 3 times weekly plus RBV for 24 weeks. Furthermore, the SVR reported by Pellicano et al (13) was 12.1% (4/33) in their patients with genotype 1 (viral titer was not mentioned) treated with 22 μg rhIFNβ-1a daily plus RBV for 24 weeks. In the present study, the SVR rate in our patients with genotype 1b and high viral load treated with 6 MU nIFNβ plus RBV for 48 weeks was 27% (3/11). This result ranked well with the above previous reports. We performed case-control study in patients treated with PEG-IFN plus RBV and the SVR rate was 41% (9/22). Although the SVR rate of the case group was lower than that of the control group, the difference was not statistically significant, suggesting that nIFNβ plus RBV combination therapy for 48 weeks is also efficacious.

The viral load of the case group decreased rapidly at week 2; the log drop was greater than that of the control group. However, after week 2, there were no differences in the rate of drop of viral load between the two groups at weeks 4 and 12. In the case group, nIFNβ was administrated daily for 2 weeks in 6 patients, for 4 weeks in 1 patient and for 8 weeks in 4 patients. These viral kinetics suggest that daily administration of nIFNβ plus RBV is more effective against HCV than PEG-IFN plus RBV, and that three times a week nIFNβ administration regimen might be less effective than PEG-IFN plus RBV. The fact that none of the patients dropped out in our study even during the 8-week daily administration of nIFNβ, suggests good tolerability of the combination therapy. Prolongation of daily administration might improve treatment outcome of nIFNβ plus RBV. However, our results, based on a small sample size, need to be confirmed in another large-scale study, including determination of the most appropriate duration of daily administration.

In conclusion, nIFNβ plus RBV therapy carries sufficient tolerability and efficacy in patients with mental disorders. nIFNβ plus RBV could be considered an efficacious and safe second-line therapy for subpopulations of patients who are not eligible for PEG-IFN plus RBV. Further studies in a larger group of will be necessary.

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