Central genetic alterations common to all HCV-positive, HBV-positive and non-B, non-C hepatocellular carcinoma: A new approach to identify novel tumor markers

YUKINORI KUROKAWA[1,2], KIMI HONMA[3,4], ICHIRO TAKEMASA[2], SHOJI NAKAMORI[2], HIROKO KITA-MATSUO[1], MASAAKI MOTOORI[1,2], HIROAKI NAGANO[2], KEIZO DONO[2], TAKAHIRO OCHIYA[3], MORITO MONDEN[2] and KIKUYA KATO[1]

[1]Taisho Laboratory of Functional Genomics, Nara Institute of Science and Technology, Nara 630-0101; [2]Department of Surgery and Clinical Oncology, Graduate School of Medicine, Osaka University, Osaka 565-0871; [3]National Cancer Center Research Institute, Tokyo 104-0045; [4]Koken Bioscience Institute, Tokyo 115-0051, Japan

Received September 15, 2005; Accepted November 4, 2005

Abstract. Hepatocellular carcinoma (HCC) is a common malignancy, but the prognosis remains poor due to the lack of sensitive diagnostic markers. To gain insight into the central molecular features common to all types of HCC, and to identify novel diagnostic markers or therapeutic targets for HCC, we performed a gene expression profiling analysis using a high throughput RT-PCR system. After examining the mRNA expression of 3,072 genes in 204 (119 tumor and 85 non-tumor) liver samples, we identified differential gene expression between the HCV group (n=80), HBV group (n=19) and non-B, non-C group (n=20) with a principal component analysis and a correlation spectrum analysis. After selection of genes differentially expressed between tumor and non-tumor tissues ($p<0.01$) within each HCC group, a total of 51 differentially expressed genes (23 upregulated and 28 downregulated genes) were found to be common to the three HCC groups. Gene Ontology grouping analysis revealed that genes with functions related to cell proliferation or differentiation and genes encoding extracellular proteins were found to be significantly enriched in these 51 common genes. Using an Atelocollagen-based cell transfection array for functional analysis of eight upregulated genes, five (*CANX, FAM34A, PVRL2, LAMR1*, and *GBA*) significantly inhibited cellular apoptosis by two independent assays. In conclusion, we identified 51 differentially expressed genes, common to all HCC types. Among these genes, there was a high incidence of anti-apoptotic activity. This combination approach with the advanced statistical methods and the bioinformatical analysis may be useful for finding novel molecular targets for diagnosis and therapy.

Correspondence to: Dr Kikuya Kato, Research Institute, Osaka Medical Center for Cancer and Cardiovascular Diseases, 1-3-2 Nakamichi, Higashinari-ku, Osaka 537-8511, Japan
E-mail: katou-ki@mc.pref.osaka.jp

Abbreviations: HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HBV, hepatitis B virus; ATAC-PCR, adaptor-tagged competitive PCR; T, tumor; NT, non-tumor; PCA, principal component analysis; CR, correlation ratio; FDR, false discovery rate; GO, gene ontology

Key words: DNA microarray, PCR-array, gene expression profiling, apoptosis

Introduction

Hepatocellular carcinoma (HCC) contributes significantly to death from malignancy throughout the world, and the incidence of HCC is rising sharply, most likely due to the spread of the hepatitis C virus (HCV), particularly in Japan, Spain and Italy (1). Hepatitis B virus (HBV) also represents a major risk factor for HCC in East Asia where the prevalence of this infection is high (1). Although recent progress in diagnostic procedures and surgical techniques have resulted in considerably improved morbidity and mortality rates, the prognosis of HCC remains poor (2). Alpha-fetoprotein (AFP) is the only widely used molecular marker for the diagnosis of HCC, but it fails to identify early stages of HCC (3). Additional biochemical markers are sorely needed to accurately detect HCC during its early stages. Since HCC is one of the many tumors whose origins differ based on the milieu of epidemiological, clinical, and pathological risk factors under which it develops, the search for such markers is complicated by the high degree of hetero-geneity of HCC (4).

DNA microarray studies examining thousands of genes have recently clarified the genetic characteristics of HCV- or HBV-infected HCC to some degree (5-7), and we characterized the gene expression profiles of non-B, non-C HCC (8). In this report, we wished to identify molecular features common to the HCV group, the HBV group and the non-B, non-C group. The identified molecules represent genes intrinsic to HCC with little heterogeneity, and these are promising candidates for novel molecular targets for both diagnostic and therapeutic interventions for HCC (9,10).

We performed gene expression profiling using adaptor-tagged competitive PCR (ATAC-PCR) technology: a PCR-based array system (11). To avoid inflated results due to small sample size, we prepared more than 200 liver samples for this study. Strict statistical analysis allowed us to identify 51 genes common to all etiological groups, which revealed extremely distinct expression patterns between tumorous and non-tumorous livers. Bioinformatical Gene Ontology analysis was employed to examine the annotated functions and cellular characteristics of these common genes. Additionally, we analyzed the relationship of identified genes to apoptosis with hepatoma cell lines using an Atelocollagen-based cell transfection array (12-14).

Materials and methods

Tissues and patients. We obtained 204 liver samples (119 HCC, 85 non-tumorous liver) from 119 patients, with informed written consent, who underwent hepatic resection for HCC at Osaka University Hospital between January 1997 and September 2001. Tumor (T) and non-tumor (NT) tissue samples were enucleated separately from either the tumorous or non-tumorous part of the resected tissue. Serologically, of the 119 patients, 80 were HCV antibody-positive, and 16 were HBs antigen-positive. No patients were double-positive for HCV antibody and HBs antigen. Although three patients were double-negative for HCV antibody and HBs antigen, the presence of HBV mRNA was confirmed using the previously reported RT-PCR method (15). These patients were considered to have HBV-positive HCC for further analysis. For the remaining 20 non-B, non-C patients who were double-negative for HCV antibody and HBs antigen, the absence of viral genomes was confirmed by RT-PCR as reported to rule out possible cryptic HCV or HBV infection (15,16). In total, 80 cases were in the HCV group, 19 in the HBV group, and 20 in the non-B, non-C group. All aspects of our study protocol were approved by the Ethics Committee of Osaka University Medical School.

PCR-based array system. To select genes expressed in liver tissue, we constructed three cDNA libraries: one from a mixture of HCC and non-tumorous liver, one from normal liver, and one from metastatic liver cancer, as described (17). We designed PCR primers for ATAC-PCR reactions for a total of 2,774 genes from these EST collections. In total, we prepared 3,072 primers for ATAC-PCR; this total includes an additional 298 genes established in the literature. The specificity of this gene selection provides an advantage over more universal gene sets, such as those selected from the UniGene database, which include genes not expressed in liver tissue. The ATAC-PCR experimental procedure was performed as described (18,19). The complete list of genes and detailed protocols for the ATAC-PCR experimental procedure are available on our website (http://genome.mc.pref.osaka.jp/).

Statistical analysis of PCR-based array data. The relative expression level of each gene was calculated by calibrating against a standard mixture of more than 20 liver tissues including HCC and non-tumor samples, as described (8,19,20). Following conversion to a logarithmic scale (base 2), the data matrix was normalized to a mean of 0 by standardizing each sample. Principal component analysis (PCA) was performed using GeneMaths 2.0 software.

The correlation ratio (CR) of gene i is defined as follows (21):

$$(CR_i)^2 \equiv \frac{\sum_{c=1}^{C} n_c((\sum_{j=J_c} x_{i,j})/n_c - \bar{x}_i)^2}{\sum_{j=1}^{M}(x_{i,j} - \bar{x}_i)^2}$$

where n_c is the number of genes in a particular class, J_c; $x_{i,j}$ is the expression level of gene i with sample j; and x_i is the average of the expression levels of gene i. In drawing a *CR* curve, genes were sorted by *CR* value order, and the ratios of the original total dataset were compared with those of the permutated data.

Permutation testing, which involves 50,000 randomly permutating class labels to determine gene-class correlations, was used to determine statistical significance as described (22,23). To estimate the false discovery rate (FDR), which is the percentage of genes erroneously identified, we used software downloaded from http://faculty.washington.edu/~jstorey/qvalue (24). Gene Ontology (GO) grouping was performed according to the annotated function in biological process and cellular component with a Generic Gene Ontology Term Finder program downloaded from http://helix.princeton.edu/cgi-bin/GOTermFinder/GOTermFinder (25). This software can find significant GO terms shared among a list of selected genes, helping us to discover what these genes may have in common (26). Each gene annotation was designated by the Gene Ontology Consortium at the European Bioinformatics Institute.

Atelocollagen-based cell transfection array. HepG2 cells were grown in DMEM (Sigma, St. Louis, MO) supplemented with 10% FBS (Gibco-BRL), at 37˚C in a 5% CO_2 humidified atmosphere. Atelocollagen-based cell transfection array, which enables reverse transfection of cells by Atelocollagen-mediated gene transfer, was used for functional analysis of candidate genes (12-14). The full-length cDNAs were cloned downstream of the CMV promoter of the expression vector. The expression vector without an insert was used as the control. The plasmid vectors and Atelocollagen complexes were prepared according to the described method (13). An equal volume of Atelocollagen (0.016% in PBS at pH 7.4) and plasmid DNA solution (5 μg/ml) was combined and mixed for 20 min at 4˚C. The final concentration of Atelocollagen was 0.008%. The Atelocollagen/plasmid DNA complexes were then arrayed and precoated on 96-well plates (plasmid DNA 0.25 μg/50 μl/well). The cultured HepG2 cells were plated into the complex-prefixed 96-well plate and the effects of overexpressed genes were then observed.

In vitro growth and apoptosis assay. For growth assay, cells were seeded at a density of 5×10^3 cells per well in 100 μl of culture medium on Atelocollagen-based cell transfection array. Cell proliferation was assayed for 4 days with TetraColor One, a cell-proliferation assay reagent (Seikagaku Co., Tokyo, Japan), according to the recommended method; cells were then measured for absorbency in the well at 450 nm with a reference wavelength of 650 nm. For apoptosis assay, cells were seeded at a density of 1×10^4 cells per well in 100 μl of

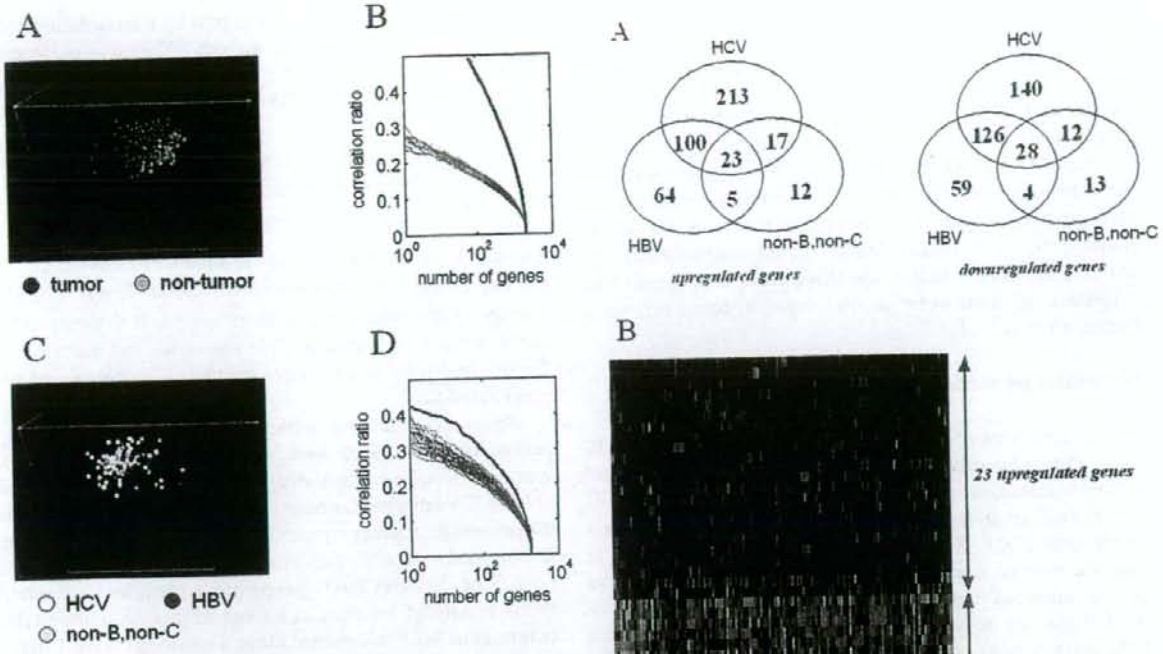

Figure 1. Expression profiling analyses of 204 liver samples (A and B, tumor vs. non-tumor) and 119 HCC samples (C and D, HCV group vs. HBV group vs. non-B, non-C group). (A and C) Principal component analyses. (B and D) Correlation spectrum analyses. Vertical axis represents the correlation ratio of the differences between tumor and non-tumor tissues (B) and between the three HCC groups (D). Horizontal axis, genes sorted by correlation ratios. Red, original data; blue, twenty trials of randomized data.

Figure 2. (A) The number of differentially expressed genes between tumor and non-tumor tissues with division of three HCC groups. Left, upregulated genes; right, downregulated genes. (B) Expression pattern of 51 genes commonly appearing in all three HCC groups.

culture medium on Atelocollagen-based cell transfection array, and, after three days, docetaxel, an anticancer agent, was added at a concentration of 10 nM for induction of apoptosis. For detection of apoptotic cells, Caspase-3/7 assay was performed using an Apo-ONE™ Homogeneous Caspase-3/7 assay kit (Promega). Condensation and fragmentation of cell nuclei was also evaluated by fluorescence microscopy following Hoechst DNA staining.

Results

Expression features of HCC. After measuring the expression levels of 3,072 genes in 204 liver samples, we selected 1,812 genes with few missing values for further analysis. We first applied PCA, a statistical method for reducing the number of data dimensions, to present the relationship between tumor (T) and non-tumor (NT) tissues. Upon displaying the expression patterns of all 1,812 genes in three-dimensional space, we observed that T and NT were located separately, indicating distinct gene expression patterns (Fig. 1A). To statistically evaluate the correlation between the gene expression patterns of these two groups, we performed a correlation spectrum analysis. The correlation ratio (*CR*) is a statistical indicator of correlation of clinicopathological parameters. The *CR* value of the original dataset was much higher than that of any permutated dataset for any number of genes (Fig. 1B). Second, to estimate the differences in expression profiles among the 119 HCC samples from the three hepatitis groups (HCV associated, HBV associated and non-B, non-C associated), PCA and correlation spectrum analyses were performed using T:NT ratios. Although the expression difference between hepatitis virus groups was not clear after PCA (Fig. 1C), the *CR* curve of the original dataset was consistently higher than that of any permutated dataset (Fig. 1D). Thus, not only are gene expression patterns different between tumor and non-tumor liver tissues, but tumors arising in patients infected with HCV, HBV, or neither virus exhibit expression differences as well.

Genes differentially expressed between tumor and non-tumor tissues. To select genes differentially expressed between T and NT tissues, we performed random permutation testing. Because expression pattern heterogeneity was confirmed between the three hepatitis virus groups, permutation tests were performed after separating each HCC group. Using $p<0.01$ as a measure

Table I. Differentially expressed genes common to all types of HCC.

Up/down[a]	UniGene ID	Symbol	Gene name
Up	Hs.497674	*FAM34A*	Family with sequence similarity 34, member A (FAM34A)
Up	Hs.465224	*NARS*	Asparaginyl-tRNA synthetase
Up	Hs.333579	*HSPC152*	Hypothetical protein HSPC152
Up	Hs.308709	*GRP58*	Glucose regulated protein, 58 kDa
Up	Hs.511984	*GBA*	Glucosidase, ß
Up	Hs.326371	*PVRL2*	Poliovirus receptor-related 2
Up	Hs.433427	*RPS17*	Ribosomal protein S17
Up	Hs.374553	*LAMR1*	Laminin receptor 1
Up	Hs.304694	*GNB1*	Guanine nucleotide binding protein, ß polypeptide 1
Up	Hs.502756	*AHNAK*	AHNAK nucleoprotein (desmoyokin)
Up	Hs.405913	*GRCC10*	Likely ortholog of mouse gene rich cluster, C10 gene
Up	Hs.387905	*SPTAN1*	Spectrin, α, non-erythrocytic 1
Up	Hs.128065	*CTSC*	Cathepsin C
Up	Hs.284295	*NSE1*	Non-SMC (structural maintenance of chromosomes) element 1 protein
Up	Hs.523744	*COP1*	Constitutive photomorphogenic protein
Up	Hs.318567	*NDRG1*	N-myc downstream regulated gene 1
Up	Hs.310769	*HSPA5*	Heat shock 70 kDa protein 5
Up	Hs.111779	*SPARC*	Secreted protein, acidic, cysteine-rich
Up	Hs.521056	*ATP5J2*	ATP synthase, H^+ transporting, mitochondrial F0 complex, subunit f, isoform 2
Up	Hs.532399	*KIAA0663*	KIAA0663 gene product
Up	Hs.375108	*CD24*	CD24 antigen (small cell lung carcinoma cluster 4 antigen)
Up	Hs.188882	*NUDT3*	Nudix (nucleoside diphosphate linked moiety X)-type motif 3
Up	Hs.155560	*CANX*	Calnexin
Down	Hs.8109	*SMYD3*	SET and MYND domain containing 3
Down	Hs.380135	*FABP1*	Fatty acid binding protein 1, liver
Down	Hs.282557	*CP*	Ceruloplasmin (ferroxidase)
Down	Hs.74561	*A2M*	α-2-macroglobulin
Down	Hs.440409	*IGFBP3*	Insulin-like growth factor binding protein 3
Down	Hs.405156	*PPAP2B*	Phosphatidic acid phosphatase type 2B
Down	Hs.293636	*SHMT1*	Serine hydroxymethyltransferase 1
Down	Hs.82101	*PHLDA1*	Pleckstrin homology-like domain, family A, member 1
Down	Hs.93194	*APOA1*	Apolipoprotein A-I
Down	Hs.405946	*SOCS2*	Suppressor of cytokine signaling 2
Down	Hs.292477	*ETS2*	V-ets erythroblastosis virus E26 oncogene homolog 2
Down	Hs.418241	*MT2A*	Metallothionein 2A
Down	Hs.387367	*CYP39A1*	Cytochrome P450, family 39, subfamily A, polypeptide 1
Down	Hs.8821	*HAMP*	Hepcidin antimicrobial peptide
Down	Hs.418127	*CYP2C9*	Cytochrome P450, family 2, subfamily C, polypeptide 9
Down	Hs.117367	*SLC22A1*	Solute carrier family 22, member 1
Down	Hs.143436	*PLG*	Plasminogen
Down	Hs.81131	*GAMT*	Guanidinoacetate N-methyltransferase
Down	Hs.75431	*FGG*	Fibrinogen, γ polypeptide
Down	Hs.170953	-	EST
Down	Hs.356123	*KRT8*	Keratin 8
Down	Hs.458414	*IFITM1*	Interferon induced transmembrane protein 1
Down	Hs.524224	*C1R*	Complement component 1, r subcomponent
Down	Hs.300774	*FGB*	Fibrinogen, B ß polypeptide
Down	Hs.351593	*FGA*	Fibrinogen, A α polypeptide
Down	Hs.418497	*GC*	Group-specific component (vitamin D binding protein)
Down	Hs.534293	*SERPINA3*	Serine proteinase inhibitor, clade A, member 3
Down	Hs.435610	*WAC*	WW domain-containing adapter with a coiled-coil region

[a]Up- or down-regulation are defined as expression in HCC tissue compared to non-tumor tissue.

Table II. Gene Ontology categories enriched in biological process (A) and cellular component (B) for 51 common genes.

A. Biological process Gene Ontology term	p-value[a]	% FDR[b]	Genes annotated to the term
Copper ion homeostasis	0.0043	0.00	*CP, MT2A*
Blood coagulation	0.0050	0.00	*FGG, PLG, FGB, FGA*
Regulation of blood pressure	0.0062	0.00	*FGG, FGB, FGA*
Hemostasis	0.0067	0.00	*FGG, PLG, FGB, FGA*
Circulation	0.0087	0.00	*FGG, APOA1, FGB, FGA*
Regulation of body fluids	0.0094	0.00	*FGG, PLG, FGB, FGA*
Organismal physiological process	0.0152	0.25	*CTSC, PLG, HAMP, SERPINA3, CYP39A1, IFITM1, CD24, FGG, APOA1, FGB, SPARC, FGA, C1R*
Regulation of cell proliferation	0.0232	0.22	*FGG, PLG, FGB, FGA, IFITM1*
Lipid metabolism	0.0315	0.40	*FAM34A, SERPINA3, APOA1, PPAP2B, CYP39A1, FABP1, GBA*
Regulation of cellular process	0.0340	0.36	*FGG, PLG, NUDT3, SOCS2, FGB, IGFBP3, FGA, IFITM1*
Cell growth and/or maintenance	0.0352	0.33	*PLG, NUDT3, GC, SOCS2, SLC22A1, CP, PPAP2B, KRT8, IFITM1, FABP1, MT2A, FGG, APOA1, GRP58, FGB, A2M, IGFBP3, FGA, ETS2, GBA*
Positive regulation of cell differentiation	0.0388	0.31	*SOCS2, IGFBP3*

B. Cellular component Gene Ontology term	p-value[a]	% FDR[b]	Genes annotated to the term
Fibrinogen complex	0.0000	0.00	*FGG, FGB, FGA*
Extracellular region	0.0003	0.00	*HAMP, GC, SERPINA3, CP, FGG, APOA1, A2M, FGB, SPARC, IGFBP3, FGA, C1R*
Extracellular space	0.0089	0.00	*FGG, GC, FGB, CP, FGA*
Cytosolic small ribosomal subunit	0.0107	0.00	*RPS17, LAMR1*
Eukaryotic 48S initiation complex	0.0107	0.00	*RPS17, LAMR1*
Eukaryotic 43S preinitiation complex	0.0440	0.33	*RPS17, LAMR1*

[a]Corrected p-value and [b]false discovery rate (FDR) were calculated by a Generic Gene Ontology Term Finder program.

of significance, we selected 659, 409 and 114 significant genes in the HCV group, the HBV group and the non-B, non-C group, respectively. Considering the multiplicity of gene selection, we also evaluated the differentially expressed genes by FDR. The FDRs in the three HCC groups were 1.0%, 1.9% and 12.1%, respectively. After three random permutation tests, 23 upregulated and 28 downregulated genes were identified as common to all three groups (Fig. 2A, Table I).

Genes commonly identified among the three HCC groups. The expression patterns of 51 genes were similarly changed among the three HCC groups (Fig. 2B). The Gene Ontology annotations for these common genes were analyzed with the Generic GO Term Finder program with a p-value threshold of 0.05 (Table II). Twelve terms in the biological processes field and six terms in the cellular components field revealed significant p-values and low FDRs of <1%. As expected, many genes involved in cell proliferation/growth or cell differentiation were identified. Furthermore, the 51 common genes were also enriched for cellular components of the extracellular region/space. The identified genes are potential candidates for novel tumor markers or drug targets of HCC.

HepG2 in vitro assays for growth and apoptosis. We performed *in vitro* assays for growth and apoptosis to better understand the role of some of the commonly identified genes in HCC. Eight genes (*CANX, FAM34A, PVRL2, ATP5J2, LAMR1, GBA, AHNAK*, and *RPS17*) were selected from the 23 common upregulated genes for further analysis. These genes were cloned into expression vectors for use in an Atelocollagen-based cell transfection array of HepG2 cells. Although growth assays were performed for these eight genes,

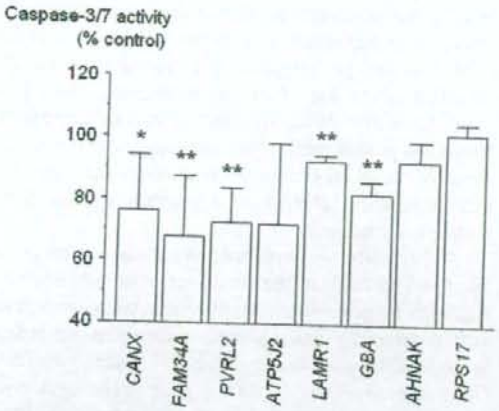

Figure 3. Caspase-3/7 activities of HepG2 cells measured using an Apo-ONE™ Homogeneous Caspase-3/7 assay kit. Vertical axis exhibits the percentage of Caspase-3/7 activity compared with control vector transfected cells. Data are expressed as the mean ± SD. Statistical difference in activity between each transfected cell and control vector cell was estimated by t-test. *$p<0.05$, **$p<0.01$.

no gene induced a significant change in cell proliferation (data not shown). To study the effects of these genes on apoptosis, we carried out Caspase-3/7 assays on transfected cells. Five (*CANX, FAM34A, PVRL2, LAMR1*, and *GBA*) of eight genes significantly inhibited apoptosis in transfected tumor cells (Fig. 3). We validated these results using a Hoechst staining assay, an independent confirmation of apoptosis. In all five genes, the number of cells with apoptotic nuclear fragmentation was clearly reduced in transfected cells compared with control vector transfected cells (Fig. 4). Thus, expression of these genes may play an important role in the inhibition of apoptosis in hepatoma cells.

Discussion

HCC typically develops following chronic liver inflammation caused by hepatitis viruses such as HCV and HBV (27). Some individuals who develop HCC are neither infected with HCV nor HBV, however, and these non-B, non-C cases can arise from various pathogenesis such as aflatoxin B1, alcoholic hepatitis, or nonalcoholic steatohepatitis (NASH) (28-30). Although structural alterations in many cancer-related genes are found in HCC (31), the high number of genes involved suggests that different etiological factors may affect different gene subsets within hepatocytes. Thus, distinct, but related, genetic pathways may be altered during hepatocarcinogenesis, possibly due to different initiators and promoters. Multiple studies linking hepatitis viruses and chemical carcinogens to hepatocarcinogenesis have provided clues for understanding this molecular system (32,33), but it is still unclear whether a single subset of genes is commonly modulated across all subsets of HCC. Genetic alterations common to all types of HCC would help elucidate a molecular mechanism underlying all human hepatocarcinogenesis independent of pathogenetic pathways. We wished to identify the genetic changes common to any types of HCC, which may lead to the discovery of novel diagnostic markers and therapeutic targets.

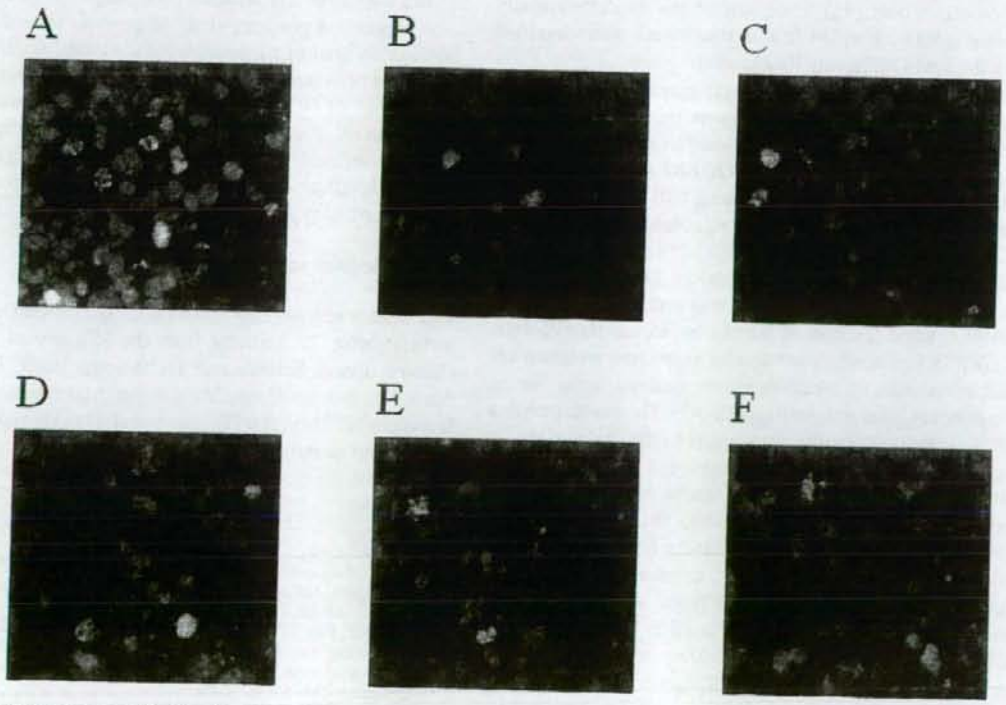

Figure 4. Hoechst staining of HepG2 cells. (A) Control vector transfected cells, (B) *CANX* transfected cells, (C) *FAM34A* transfected cells, (D) *PVRL2* transfected cells, (E) *LAMR1* transfected cells, and (F) *GBA* transfected cells.

Gene expression profiling is a powerful molecular technique wherein the expression levels of an entire mRNA population of a tissue are characterized. It allows for the increased understanding of global molecular abnormalities in various malignancies, and HCC has been previously studied using this approach (5-7). Previous studies identified differences in gene expression profiles underlying different causal viruses; however, few studies examined common gene expression profiles between HBV and HCV. Here, we performed gene expression profiling analysis with more than 200 liver samples using a PCR-based array system instead of DNA microarrays. Although DNA microarrays have contributed to the literature of gene expression profiling, they detect only a fraction of the changes in gene expression which can be detected by RT-PCR (34). We have therefore prepared a PCR-based technology which is a high throughput quantitative PCR method based on ATAC-PCR (11). The aforementioned benefits and the strength of this system for cancer research, established in previous work on not only HCC but also other cancers, makes this technique a powerful method of obtaining a better understanding of the molecular characteristics of cancer (35-37).

Two advanced statistical methods revealed distinct gene expression patterns between tumor and non-tumor tissues. We performed random permutation tests to select the differentially expressed genes between HCC and non-tumor samples by isolating causative hepatitis virus groups, because there was heterogeneity between the HCV, HBV, and non-B, non-C groups. We selected 659, 409 and 114 significant genes in the HCV, HBV and non-B, non-C groups, respectively. From these genes, we obtained 51 genes that were common to these three types of HCC. Comparing these common genes with the previous report (38), over half of the 51 differentially expressed genes identified in this study were also identified among the 1,648 differentially expressed genes.

Among these 51 genes, several cancer-related genes previously reported to be associated with HCC were identified. For example, a well known HCC-related gene, *insulin-like growth factor binding protein 3* (*IGFBP3*), was included. *IGFBP3* plays a key role in regulating cell proliferation and apoptosis, and it also plays an important inhibitory role in the development and/or growth of HCC (39,40). *GRP58* is a chaperone in the endoplasmic reticulum lumen, which was previously identified as one of several accessory proteins in the S100 cytosol fraction of human hepatoma Hep3B cells (41). *GRP58* was also reported to be associated with hepatocellular carcinoma by a cDNA library analysis using human liver cancer vascular endothelial cells (42). The overexpression of *CD24*, a sialoglycoprotein anchored to the cell surface by a glycosyl phosphatidylinositol linkage, and *SPARC*, a glycoprotein involved in extracellular matrix remodeling, were observed with other previous studies using human HCC (43,44). In contrast, *α-2-macroglobulin* (*A2M*), a protease inhibitor and cytokine transporter, or *plasminogen* (*PLG*), the zymogen in circulating blood from which plasmin is formed, are significantly underexpressed in human HCC and hepatoma cells (45-47). *Apolipoprotein A-I* (*APOA1*), the major apoprotein of high density lipoprotein (HDL) in the plasma, is a candidate for an HCC biomarker, because its expression level is significantly lower in serum samples from HCC patients compared to healthy controls (48). Furthermore, using the Gene Ontology program, genes with functions related to cell proliferation were found to be significantly enriched in the list of 51 common genes. Such genes are candidates for novel diagnostic markers because most traditional protein markers are associated with cell proliferation (49). We also identified several genes that encode extracellular proteins, which have a greater likelihood of acting as serological markers.

Additionally, we performed *in vitro* assays for growth and apoptosis to analyze the functions of some selected genes. Although no genes significantly altered cell growth, five of the eight examined genes inhibited apoptosis in two independent *in vitro* assays. Among these five genes, we identified *Glucosylceramidase* (*GBA*), a gene encoding a lysosomal membrane protein that cleaves the ß-glucosidic linkage of glycosylceramide, an intermediate in glycolipid metabolism. Although many studies have reported that mutations in this gene cause Gaucher disease, a lysosomal storage disease characterized by the accumulation of glucocerebrosides (50), the correlation between the *GBA* gene and HCC has not yet been identified. This gene also inhibited apoptosis in hepatoma cells. In fact, although an increased risk of malignancy in patients with Gaucher disease has been observed, the coexistence of Gaucher disease and hepatocellular carcinoma is extremely rare (51). In patients with Gaucher disease, the *GBA* mutation may induce not only abnormal storage of glucocerebrosides but also inhibition of hepatocarcinogenesis through the promotion of apoptosis. The regulation of apoptosis in hepatoma cells is poorly understood, and further studies are needed to identify the role of *GBA* and other genes in this process.

We used over 200 separate liver samples in this large scale gene expression profiling study to generate several important findings. A total of 51 genes were identified as differentially expressed between tumor and non-tumor tissues regardless of the etiology of HCC. It is thought that these genes may play significant roles in the development of cancer independent of hepatitis viruses. Such genes are potential targets for the rational development of new cancer drugs and for the early detection of all types of HCC.

Acknowledgements

This work was supported by Grant-in-Aid for the Development of Innovative Technology from the Ministry of Education, Culture, Sports, Science and Technology, Japan. The authors thank Ms. Noriko Ueno, Ms. Chiyuri Maruyama, Ms. Keiko Ikenaga, Ms. Mihoko Yoshino and Ms. Satoko Kinjo for their expert technical assistance.

References

1. Montalto G, Cervello M, Giannitrapani L, Dantona F, Terranova A and Castagnetta LA: Epidemiology, risk factors, and natural history of hepatocellular carcinoma. Ann NY Acad Sci 963: 13-20, 2002.
2. Poon RT, Fan ST, Lo CM, Ng IO, Liu CL, Lam CM and Wong J: Improving survival results after resection of hepatocellular carcinoma: a prospective study of 377 patients over 10 years. Ann Surg 234: 63-70, 2001.
3. Nguyen MH and Keeffe EB: Screening for hepatocellular carcinoma. J Clin Gastroenterol 35: S86-S91, 2002.

4. Thorgeirsson SS and Grisham JW: Molecular pathogenesis of human hepatocellular carcinoma. Nat Genet 31: 339-346, 2002.
5. Okabe H, Satoh S, Kato T, Kitahara O, Yanagawa R, Yamaoka Y, Tsunoda T, Furukawa Y and Nakamura Y: Genome-wide analysis of gene expression in human hepatocellular carcinomas using cDNA microarray: identification of genes involved in viral carcinogenesis and tumor progression. Cancer Res 61: 2129-2137, 2001.
6. Delpuech O, Trabut JB, Carnot F, Feuillard J, Brechot C and Kremsdorf D: Identification, using cDNA macroarray analysis, of distinct gene expression profiles associated with pathological and virological features of hepatocellular carcinoma. Oncogene 21: 2926-2937, 2002.
7. Iizuka N, Oka M, Yamada-Okabe H, *et al*: Comparison of gene expression profiles between hepatitis B virus- and hepatitis C virus-infected hepatocellular carcinoma by oligonucleotide microarray data on the basis of a supervised learning method. Cancer Res 62: 3939-3944, 2002.
8. Kurokawa Y, Matoba R, Takemasa I, *et al*: Molecular features of non-B, non-C hepatocellular carcinoma: a PCR-array gene expression profiling study. J Hepatol 39: 1004-1012, 2003.
9. Smith MW, Yue ZN, Geiss GK, Sadovnikova NY, Carter VS, Boix L, Lazaro CA, Rosenberg GB, Bumgarner RE, Fausto N, Bruix J and Katze MG: Identification of novel tumor markers in hepatitis C virus-associated hepatocellular carcinoma. Cancer Res 63: 859-864, 2003.
10. Petricoin EF III, Hackett JL, Lesko LJ, Puri RK, Gutman SI, Chumakov K, Woodcock J, Feigal DW Jr, Zoon KC and Sistare FD: Medical applications of microarray technologies: a regulatory science perspective. Nat Genet 32: 474-479, 2002.
11. Kato K: Adaptor-tagged competitive PCR: a novel method for measuring relative gene expression. Nucleic Acids Res 25: 4694-4696, 1997.
12. Ochiya T, Takahama Y, Nagahara S, Sumita Y, Hisada A, Itoh H, Nagai Y and Terada M: New delivery system for plasmid DNA *in vivo* using atelocollagen as a carrier material: the Minipellet. Nat Med 5: 707-710, 1999.
13. Honma K, Ochiya T, Nagahara S, Sano A, Yamamoto H, Hirai K, Aso Y and Terada M: Atelocollagen-based gene transfer in cells allows high-throughput screening of gene functions. Biochem Biophys Res Commun 289: 1075-1081, 2001.
14. Honma K, Miyata T and Ochiya T: The role of atelocollagen-based cell transfection array in high-throughput screening of gene functions and in drug discovery. Curr Drug Discovery Technologies 1: 287-294, 2004.
15. Yotsuyanagi H, Shintani Y, Moriya K, Fujie H, Tsutsumi T, Kato T, Nishioka K, Takayama T, Makuuchi M, Iino S, Kimura S and Koike K: Virologic analysis of non-B, non-C hepatocellular carcinoma in Japan: frequent involvement of hepatitis B virus. J Infect Dis 181: 1920-1928, 2000.
16. Schneeberger PM, Keur I, van der Vliet W, van Hoek K, Boswijk H, van Loon AM, van Dijk WC, Kauffmann RH, Quint W and van Doorn LJ: Hepatitis C virus infections in dialysis centers in the Netherlands: a national survey by serological and molecular methods. J Clin Microbiol 36: 1711-1715, 1998.
17. Matoba R, Kato K, Saito S, Kurooka C, Maruyama C, Sakakibara Y and Matsubara K: Gene expression in mouse cerebellum during its development. Gene 241: 125-131, 2000.
18. Matoba R, Kato K, Kurooka C, Maruyama C, Sakakibara Y and Matsubara K: Correlation between gene functions and developmental expression patterns in the mouse cerebellum. Eur J Neurosci 12: 1357-1371, 2000.
19. Kurokawa Y, Matoba R, Nakamori S, Takemasa I, Nagano H, Dono K, Umeshita K, Sakon M, Monden M and Kato K: PCR-array gene expression profiling of hepatocellular carcinoma. J Exp Clin Cancer Res 23: 135-141, 2004.
20. Kurokawa Y, Matoba R, Takemasa I, *et al*: Molecular-based prediction of early recurrence in hepatocellular carcinoma. J Hepatol 41: 284-291, 2004.
21. Muro S, Takemasa I, Oba S, *et al*: Identification of expressed genes linked to malignancy of human colorectal carcinoma by parametric clustering of quantitative expression data. Genome Biol 4: R21, 2003.
22. Golub TR, Slonim DK, Tamayo P, Huard C, Gaasenbeek M, Mesirov JP, Coller H, Loh ML, Downing JR, Caligiuri MA, Bloomfield CD and Lander ES: Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. Science 286: 531-537, 1999.
23. Pomeroy SL, Tamayo P, Gaasenbeek M, *et al*: Prediction of central nervous system embryonal tumour outcome based on gene expression. Nature 415: 436-442, 2002.
24. Storey JD and Tibshirani R: Statistical significance for genome-wide studies. Proc Natl Acad Sci USA 100: 9440-9445, 2003.
25. Boyle EI, Weng S, Gollub J, Jin H, Botstein D, Cherry JM and Sherlock G: GO::TermFinder - open source software for accessing Gene Ontology information and finding significantly enriched Gene Ontology terms associated with a list of genes. Bioinformatics 20: 3710-3715, 2004.
26. Patil MA, Chua MS, Pan KH, Lin R, Lih CJ, Cheung ST, Ho C, Li R, Fan ST, Cohen SN, Chen X and So S: An integrated data analysis approach to characterize genes highly expressed in hepatocellular carcinoma. Oncogene 24: 3737-3747, 2005.
27. Kasai Y, Takeda S and Takagi H: Pathogenesis of hepatocellular carcinoma: a review from the viewpoint of molecular analysis. Semin Surg Oncol 12: 155-159, 1996.
28. Wogan GN: Aflatoxins as risk factors for hepatocellular carcinoma in humans. Cancer Res 52: 2114s-2118s, 1992.
29. Mohamed AE, Kew MC and Groeneveld HT: Alcohol consumption as a risk factor for hepatocellular carcinoma in urban Southern African blacks. Int J Cancer 51: 537-541, 1992.
30. Bugianesi E, Leone N, Vanni E, Marchesini G, Brunello F, Carucci P, Musso A, De Paolis P, Capussotti L, Salizzoni M and Rizzetto M: Expanding the natural history of nonalcoholic steatohepatitis: from cryptogenic cirrhosis to hepatocellular carcinoma. Gastroenterology 123: 134-140, 2002.
31. Murakami Y, Hayashi K, Hirohashi S and Sekiya T: Aberrations of the tumor suppressor p53 and retinoblastoma genes in human hepatocellular carcinomas. Cancer Res 51: 5520-5525, 1991.
32. Andrisani OM and Barnabas S: The transcriptional function of the hepatitis B virus X protein and its role in hepatocarcinogenesis (Review). Int J Oncol 15: 373-379, 1999.
33. Bressac B, Kew M, Wands J and Ozturk M: Selective G to T mutations of p53 gene in hepatocellular carcinoma from Southern Africa. Nature 350: 429-431, 1991.
34. Holland MJ: Transcript abundance in yeast varies over six orders of magnitude. J Biol Chem 277: 14363-14366, 2002.
35. Iwao K, Matoba R, Ueno N, Ando A, Miyoshi Y, Matsubara K, Noguchi S and Kato K: Molecular classification of primary breast tumors possessing distinct prognostic properties. Hum Mol Genet 11: 199-206, 2002.
36. Kurokawa Y, Matoba R, Nagano H, Sakon M, Takemasa I, Nakamori S, Dono K, Umeshita K, Ueno N, Ishii S, Kato K and Monden M: Molecular prediction of response to 5-fluorouracil and interferon-alpha combination chemotherapy in advanced hepatocellular carcinoma. Clin Cancer Res 10: 6029-6038, 2004.
37. Iwao-Koizumi K, Matoba R, Ueno N, Kim SJ, Ando A, Miyoshi Y, Maeda E, Noguchi S and Kato K: Prediction of docetaxel response in human breast cancer by gene expression profiling. J Clin Oncol 23: 422-431, 2005.
38. Chen X, Cheung ST, So S, *et al*: Gene expression patterns in human liver cancers. Mol Biol Cell 13: 1929-1939, 2002.
39. Huynh H, Chow PK, Ooi LL and Soo KC: A possible role for insulin-like growth factor-binding protein-3 autocrine/paracrine loops in controlling hepatocellular carcinoma cell proliferation. Cell Growth Differ 13: 115-122, 2002.
40. Hanafusa T, Yumoto Y, Nouso K, *et al*: Reduced expression of insulin-like growth factor binding protein-3 and its promoter hypermethylation in human hepatocellular carcinoma. Cancer Lett 176: 149-158, 2002.
41. Ndubuisi MI, Guo GG, Fried VA, Etlinger JD and Sehgal PB: Cellular physiology of STAT3: where's the cytoplasmic monomer? J Biol Chem 274: 25499-25509, 1999.
42. Zhong X, Ran YL, Lou JN, Hu D, Yu L, Zhang YS, Zhou Z and Yang ZH: Construction of human liver cancer vascular endothelium cDNA expression library and screening of the endothelium-associated antigen genes. World J Gastroenterol 10: 1402-1408, 2004.
43. Huang LR and Hsu HC: Cloning and expression of CD24 gene in human hepatocellular carcinoma: a potential early tumor marker gene correlates with p53 mutation and tumor differentiation. Cancer Res 55: 4717-4721, 1995.
44. Le Bail B, Faouzi S, Boussarie L, Guirouilh J, Blanc JF, Carles J, Bioulac-Sage P, Balabaud C and Rosenbaum J: Osteonectin/SPARC is overexpressed in human hepatocellular carcinoma. J Pathol 189: 46-52, 1999.

45. Paradis V, Bieche I, Dargere D, Laurendeau I, Laurent C, Bioulac Sage P, Degott C, Belghiti J, Vidaud M and Bedossa P: Molecular profiling of hepatocellular carcinomas (HCC) using a large-scale real-time RT-PCR approach: determination of a molecular diagnostic index. Am J Pathol 163: 733-741, 2003.
46. Baffet G, Braciak TA, Fletcher RG, Gauldie J, Fey GH and Northemann W: Autocrine activity of interleukin 6 secreted by hepatocarcinoma cell lines. Mol Biol Med 8: 141-156, 1991.
47. Steel LF, Shumpert D, Trotter M, Seeholzer SH, Evans AA, London WT, Dwek R and Block TM: A strategy for the comparative analysis of serum proteomes for the discovery of biomarkers for hepatocellular carcinoma. Proteomics 3: 601-609, 2003.
48. Kinoshita M and Miyata M: Underexpression of mRNA in human hepatocellular carcinoma focusing on eight loci. Hepatology 36: 433-438, 2002.
49. Hui AM, Makuuchi M and Li X: Cell cycle regulators and human hepatocarcinogenesis. Hepatogastroenterology 45: 1635-1642, 1998.
50. Tsuji S, Choudary PV, Martin BM, Stubblefield BK, Mayor JA, Barranger JA and Ginns EI: A mutation in the human glucocerebrosidase gene in neuronopathic Gaucher's disease. N Engl J Med 316: 570-575, 1987.
51. Erjavec Z, Hollak CEM and Vries EGE: Hepatocellular carcinoma in a patient with Gaucher disease on enzyme supplementation therapy. Ann Oncol 10: 243, 1999.

Expression of type I interferon receptor as a predictor of clinical response to interferon-α therapy of gastrointestinal cancers

HIDEO OTA[1], HIROAKI NAGANO[1], YUICHIRO DOKI[1], MITSUGU SEKIMOTO[1], MOTOI KONDO[1], HIROSHI WADA[1], MASATO NAKAMURA[1], TAKEHIRO NODA[1], BAZARRAGCHAA DAMDINSUREN[1], SHIGERU MARUBASHI[1], ATSUSHI MIYAMOTO[1], YUTAKA TAKEDA[1], KEIZO DONO[1], KOJI UMESHITA[1], SHOJI NAKAMORI[1], KENICHI WAKASA[2], MASATO SAKON[1] and MORITO MONDEN[1]

[1]Department of Surgery, Graduate School of Medicine, Osaka University, 2-2 Yamadaoka E-2, Suita, Osaka 565-0871, [2]Department of Pathology, Osaka City University Hospital, 1-5-7 Asahi-cho, Abeno-ku, Osaka 545-0051, Japan

Received February 20, 2006; Accepted March 13, 2006

Abstract. Interferon (IFN) is used in the treatment of many malignancies and viral disorders. We recently reported a significant correlation between the efficacy of IFN-α combined with chemotherapy in the treatment of advanced hepatocellular carcinoma (HCC) and IFN-α/type I IFN receptor (IFNAR2) expression. It is possible that the expression of IFNAR2 in gastrointestinal cancerous tissue, apart from HCC, may predict the efficacy of IFN-α combination therapy. We investigated the expression of IFNAR2 in 100 gastrointestinal cancerous tissues. IFNAR2 expression was examined using immunohistochemistry, in surgically resected tissue samples (20 esophageal, 20 gastric, 20 colorectal, 20 cholangiocarcinoma, and 20 pancreatic samples). The expression rate of IFNAR2 was 35.0% (7/20), 25.0% (5/20), 20.0% (4/20), 45.0% (9/20), and 25.0% (5/20) in esophageal, gastric cancer, colorectal, cholangiocarcinoma and pancreatic cancer samples, respectively. In our previous report, the expression rate of IFNAR2 in HCC samples was 64.8% (59/91). Thus, the expression rates of IFNAR2 in the five types of gastrointestinal cancers tested here were low, compared with HCC. The clinical efficacy of IFN-α mono- or combination therapies in patients with gastrointestinal neoplasms is expected to be lower than in patients with HCC based on the expression level of IFNAR2.

Correspondence to: Dr Hiroaki Nagano, Department of Surgery, Graduate School of Medicine, Osaka University, Osaka 565-0871, Japan
E-mail: hnagano@surg2.med.osaka-u.ac.jp

Abbreviations: IFN, interferon; IFNAR2, type I interferon-α/ß receptor 2; HCC, hepatocellular carcinoma; 5-FU, 5-fluorouracil

Key words: gastrointestinal cancer, IFNAR2, IFN-α, therapy, prediction

Introduction

Interferon (IFN)-α is used in the treatment of several neoplasms and viral disorders such as chronic myeloid leukemia, hairy-cell leukemia, adult T cell leukemia, multiple myeloma, renal cell carcinoma, and chronic hepatitis B and C. For example, Kantarjian *et al* (1) reported that major cytogenetic responses occurred in 37% of 148 treated chronic myeloid leukemia patients. Furthermore, the myeloma trialists collaborative groups (2) demonstrated complete and partial responses to IFN monotherapy in 57.5% of patients with myeloma in meta-analysis study. IFN-α therapy for these diseases is associated with a good prognosis. Based on these findings, IFN-α therapy has been introduced in patients with colorectal cancer. However, Figlin *et al* (3) reported no responses in 18 patients with colorectal adenocarcinoma, as did Silgals *et al* (4) in 15 patients with colorectal cancer. However, Eggermont *et al* (5) reported a 10% response rate in 10 patients with colorectal cancer. From these reports, it is plausible that IFN-α monotherapy might be effective in patients with colorectal carcinoma. In addition, IFN-α has been used in combination with several anti-cancer agents to enhance the treatment effect. However, this treatment effect was not expected in gastrointestinal cancers and remains controversial, even in colorectal cancer.

IFN-α therapy has also been introduced in hepatocellular carcinoma (HCC). In several randomized controlled trials, IFN-α significantly improved survival in patients with unresectable HCC (6, 7). Moreover, combination therapy with IFN-α has been used for HCC with good efficiency (8-14). In a randomized control trial from Hong Kong, IFN-α treatment was associated with a significant increase in major and minor responses (22%) in comparison to doxorubicin (0%) (15). However, occasional dramatic responses were not seen in 5-fluorouracil (5-FU) monotherapy of patients with HCC (16-18).

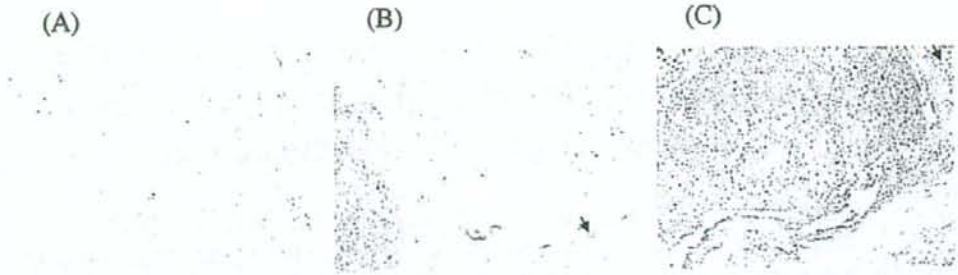

Figure 1. Immunohistochemistry of IFNAR2 expression in HCC tissues as control samples. Representative tissues were used as control samples. The intensity of IFNAR2 was scored on a scale from 0 to 2; 0, no or faint staining (A); 1, moderate staining (B); and 2, strong staining (C). The latter level of staining was used as an inner control within the sample, which was designated arbitrarily as intensity 1, because the epithelial cells of the bile ducts generally expressed moderate levels of IFNAR2.

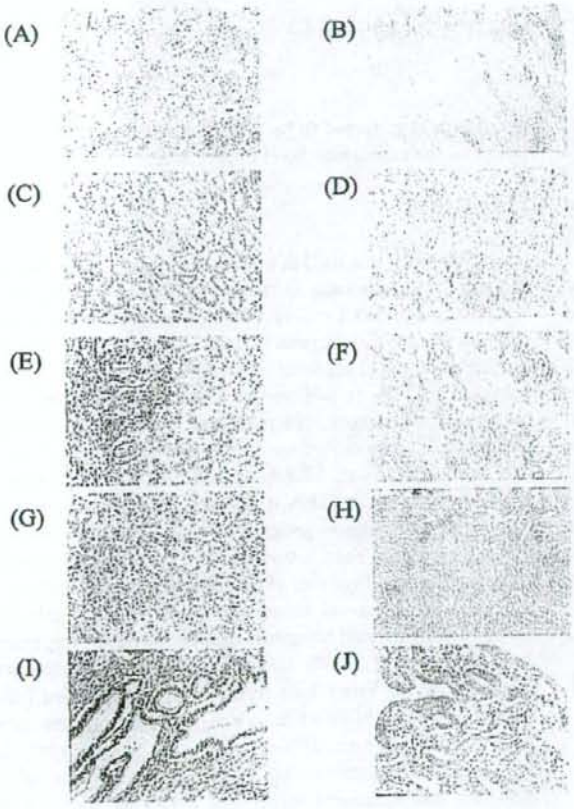

Figure 2. Immunohistochemical analysis of IFNAR2 expression in cancerous tissue. The intensity of IFNAR2 was scored from 0 to 2 simultaneously with representative tissues of staining intensity of 0 to 2 in HCC. (A, B) Esophageal cancers; intensity 0 (A) and intensity 2 (B). (C, D) Gastric cancers; intensity 0 (C) and intensity 1 (D). (E, F) Colorectal cancers; intensity 0 (E) and intensity 2 (F). (G, H) Cholangiocellular carcinomas; intensity 0, (G) and intensity 2 (H). (I, J) Pancreatic cancers; intensity 0 (I) and intensity 2 (J).

Based on these reports, the clinical use of IFN-α in combination with cytotoxic drugs, such as 5-FU, cisplatin, methotrexate, doxorubicin, may result in beneficial effects in HCC patients.

The mechanism of IFN-α therapy involves binding of IFN-α to type I IFN-receptors (IFNAR1 and IFNAR2 long forms), and binding to the promoter elements of type I IFN-induced genes to initiate their transcription (19-21), resulting in apoptosis. In these signal transduction pathways, IFN receptors play an important role in the treatment of several neoplastic disorders. It thus follows that IFN-α resistance is associated with a lack of IFN receptors. We recently reported that IFNAR2 expression significantly correlates with the effects of IFN-α/5-FU combination therapy in HCC (22). Therefore, the expression of IFNAR2 may be a useful predictive factor for the effect of IFN-α therapy.

In the present study, we investigated the expression of IFNAR2 as a predictive marker for the therapeutic effect of IFN-α on the progression of gastrointestinal cancers.

Materials and methods

Tumor samples. One hundred surgically resected gastrointestinal cancer samples [20 esophageal, 20 gastric cancer, 20 colorectal (including 9 patients with colorectal liver metastasis), 20 cholangiocarcinoma, and 20 pancreatic] were used in this study. All patients underwent surgery at the Department of Surgery, Osaka University Hospital, between December 1997 and December 2004. Expression of the IFN-receptor was examined by immunohistochemistry. The study protocol was approved by the Human Ethics Review Committee of Osaka University.

Immunohistochemistry. Immunohistochemistry was carried out using the method described in our previous report (23). Tissue sections (4-μm thick) were deparaffinized in xylene and heat antigen retrieval was performed as described previously (23). The slides were then processed for immunohistochemistry on the TechMate Horizon automated staining system (Dako, Glostrup, Denmark) (24), using the EnVision+ peroxidase kit (Dako) (25). In the step of primary antibody reaction, the slides were incubated with the IFNAR2 antibody (final concentration: 2.5 mg/ml) (Otsuka Pharmaceutical Co., Tokushima, Japan) overnight at 4˚C. For negative controls, non-immunized rabbit IgG (Vector Laboratories, Burlingame, CA) or Tris buffered saline (TBS) was used as a substitute for the primary antibody to verify the possibility of false positive

Table I. Expression of IFNAR2 in gastrointestinal cancers.

	Number of cases	Intensity			Expression rate	Strong expression rate
		0	1	2		
Esophageal cancer	20	13	6	1	35.0%	5.0%
Gastric cancer	20	15	5	0	25.0%	0%
Colorectal cancer	20	16	3	1	20.0%	5.0%
Cholangiolar carcinoma	20	11	8	1	45.0%	5.0%
Pancreatic cancer	20	15	3	2	25.0%	10.0%
Hepatocellular carcinoma[a]	91	32	35	24	64.8%	26.4%

[a]As previously reported (23).

Table II. Randomized control trials of 5-FU and interferon-α in colorectal cancer.

Author	Regimen	Dose of 5-fluorouracil	Dose of IFN-α	Response rate
Hill *et al* (33)	5-FU/LV/IFN-α	750 mg/m^2 i.v. Day 1-5	10 MU/m^2 i.m. 3 times/week	19% (10/52)
	5-FU alone	750 mg/m^2 i.v. Day 1-5	-	30% (16/54)
Kosmidis *et al* (34)	5-FU/LV/IFN-α	450 mg/m^2 i.v. Day 1-5	5 MU/m^2 i.m. 3 times/week	9.8% (5/53)
	5-FU/LV	450 mg/m^2 i.v. Day 1-5	-	7.8% (4/53)
Palmeri *et al* (35)	5-FU/IFN-α	750 mg/m^2 i.v. Day 1-5	3 MU/m^2 i.m. 3 times/week	25% (25/101)
	5-FU alone	750 mg/m^2 i.v. Day 1-5	-	21% (22/104)
Hausminger *et al* (36)	5-FU/LV/IFN-α	500 mg/m^2 i.v. Day 1-5	7 MU/m^2 i.m. 3 times/week	36% (38/107)
	5-FU/LV	500 mg/m^2 i.v. Day 1-5	-	25% (28/112)
Colucci *et al* (37)	5-FU/LV/IFN-α	350 mg/m^2 i.v. Day 1-5	3 MU/m^2 sc. 5 consecutive days/week	24% (25/103)
	5-FU/LV	350 mg/m^2 i.v. Day 1-5	-	23% (23/101)

5-FU, 5-fluorouracil; LV, leucovorin; IFN, interferon.

responses from non-specific binding of IgG or from the secondary antibody. In addition, absorption tests were performed on tissue sections. The intensity of IFNAR2 was scored in a scale from 0 to 2, arbitrarily in 91 HCC samples as reported previously (23). HCC samples including one of no staining (Fig. 1A), one of moderate staining (Fig. 1B) and one of strong staining (Fig. 1C) were used as control samples. All 100 samples with gastrointestinal cancer, except HCC, were evaluated by comparing with these three representative control samples. IFNAR2 expression was often heterogeneous. The histological or immunohistological type that constituted the major volume of the tumor was selected as the representative type. Staining was repeated at least twice to avoid possible technical errors and identical results were obtained. All slides were interpreted by an investigator blinded to the clinical and pathological parameters.

Results

All 100 samples with gastrointestinal cancers, except HCC, were evaluated by comparison with tissue samples of no staining (Fig. 1A), moderate staining (Fig. 1B), and strong staining in HCC (Fig. 1C) as control samples. In the 20 samples of esophageal cancer, strong expression of IFNAR2 was observed in 5.0% (1/20) of the samples; 1 strongly, 6 moderately, and 13 without or faintly stained (Fig. 2A and B). In the 20 samples of gastric cancer, strong expression of IFNAR2 was observed in 0% (0/20) of the samples; 5 moderately and 15 without or faintly stained (Fig. 2C and D). In the 20 samples of colorectal cancer (including 9 colorectal liver metastasis samples), strong expression of IFNAR2 was observed in 5.0% (1/20) of samples; 1 strongly, 3 moderately, and 16 without or faintly stained (Fig. 2E and F). In the 20 samples of cholangiocellular carcinoma, strong expression of IFNAR2 was observed in 5.0% (1/20) of samples; 1 strongly, 8 moderately, and 11 without or faintly stained (Fig. 2G and H). In the 20 samples of pancreatic cancer, strong expression of IFNAR2 was observed in 10.0% (2/20) of samples; 2 strongly, 3 moderately, and 15 without or faintly stained (Fig. 2I and J) (Table I). In comparison, we have previously reported that the expression of IFNAR2 in HCC was observed in 26.4% (24/91) of the samples; 24 strongly, 35 moderately, and 32 without or faintly stained (23).

Table III. Interferon-α therapy in gastrointestinal cancers.

Cancer	Author/(Refs.)	Dose of IFN-α	Dose of 5-fluorouracil	Doses of other drugs	One term (weeks)	Response %
Colorectal cancer	Figlin *et al* (3)	3 MU/day i.m. 5 days/week	-	-	2	0 (0/18)
	Silgals *et al* (4)	30-50 MU/m^2 i.v. 5 days/week	-	-	2-3	0 (0/15)
	Eggermont *et al* (5)	20 MU/m^2 i.m. twice a week	-	-	12	10 (1/10)
	Wadler *et al* (26)	9 MU/m^2 sc. three times/week	750 mg/m^2 i.v. Day 1-5, continuous	-	-	76 (13/17)
	Wadler *et al* (27)	9 MU/m^2 i.m. three times/week	750 mg/m^2 i.v. Day 1-5, continuous	-	4	63 (23/32)
	Wadler *et al* (28)	15-18 MU/m^2 i.m. Day 1,3,5	750 mg/m^2 i.v. Day 1-5, continuous	-	-	42 (15/36)
	Hill *et al* (33)	10 MU/m^2 i.m. 3 times/week	750 mg/m^2 i.v. Day 1-5	-	2	19 (10/52)
	Kosmidis *et al* (34)	5 MU/m^2 i.m. 3 times/week	450 mg/m^2 i.v. Day 1-2/week	Leucovorin (200 mg/m^2 i.v)	8	9.8 (5/53)
	Palmeri *et al* (35)	3 MU/m^2 i.m. Day 1,3,5/week	750 mg/m^2 i.v. Day 1-5	-	4	25 (25/101)
	Hausminger *et al* (36)	7 MU/m^2 sc. 3 times/week	500 mg/m^2 i.v. Day 1-5	Leucovorin (100 mg/m^2 i.v)	10	36 (38/107)
	Colucci *et al* (37)	3 MU/m^2 sc. 5 consecutive days/week	350 mg/m^2 i.v. Day 1-5	Leucovorin (200 mg/m^2 i.v)	3	24 (25/103)
Esophageal cancer	Kelsen *et al* (39)	9 MU/m^2 sc. 3 times/week	750 mg/m^2 i.v. Day 1-5 continuous, Day 12 bolus	-	at least 1 month	27 (10/37)
	Wadler *et al* (40)	9 MU/m^2 sc. 3 times/week	750 mg/m^2 i.v, Day 1-5/week	-	6	25 (5/20)
	Ilson *et al* (41)	3 MU/m^2 sc. Day 1-2/week	750 mg/m^2 i.v. Day 1-5	CDDP (100 mg/m^2 i.v. Day 1)	4	50 (13/26)
	Wadler *et al* (42)	10 MU/m^2 sc. 3 times/week	750 mg/m^2 i.v, Day 1-5/week	CDDP (100 mg/m^2 i.v. Day 1)	4	65 (15/23)
Gastric cancer	Lee *et al* (43)	5 MU/m^2 i.m. Day 1-7/week	750 mg/m^2 i.v. Day 2-6	-	4	30.7 (4/13)
	Hudes *et al* (44)	5 MU/m^2 i.m. Day 1-7/week	370 mg/m^2 i.v. Day 2-6	Leucovorin (500 mg/m^2 i.v. Day 2-6)	4	12.5 (3/24)
	Wadler *et al* (45)	9 MU/m^2 i.m. 3 times/week	2600 mg/m^2 i.v, continuous. Day 1,8,15,22,29,36	Hydroxyurea (4300 mg/m^2 iv. Day 1,8,15,22,29,36)	6	37 (11/31)
Pancreatic cancer	Bernard *et al* (47)	6 MU/m^2 i.m. Day 1,8,15,22	500 mg/m2 i.v. Day 1,8,15,22	Folinic acid (500 mg/m^2 i.v. Day 1,8,15,22)	4	14.0 (8/57)
	Wadler *et al* (45)	9 MU/m^2 i.m. 3 times/week	2600 mg/m2 i.v, continuous. Day 1,8,15,22,29,36	Hydroxyurea (4300 mg/m^2 iv. Day 1,8,15,22,29,36)	6	4.7 (1/21)
	Wagener *et al* (46)	3 MU/m^2 s.c. Day 1-5/week	1000 mg/m^2 i.v. continuous, Day 1-5/week	CDDP (100 mg/m^2 i.v. Day 1/week)	4	13.3 (2/15)
Hepatocellular carcinoma	Lai *et al* (6)	5 MU/m^2 i.m. 3 times/week	-	-	1	31 (11/35)
	Yuen *et al* (7)	10 or 30 or 50 MU/m^2 TAI	-	-	8-12	64.3 (9/14)
	Patt *et al* (14)	5 MU/m^2 sc. 3 times/week	750 mg/m^2 i.a. Day 1-5/week	-	4	21 (6/28)
	Urabe *et al* (8)	3 MU/m^2 sc. Day 1,3,5/week	750 mg/m^2 i.a. weekly	CDDP (75 mg/m^2 i.a. , every 2 weeks) MTX (30 mg/m^2 i.a. every 4 weeks) leucovorin (30 mg/m^2 i.v. every 4 weeks)	4	47 (7/15)
	Leung *et al* (10)	5 MU/m^2 sc. Day 1-4/week	400 mg/m^2 i.v. Day 1-4	CDDP (20 mg/m^2 i.v. Day 1-4, every week), Doxorubicin (40 mg/m^2 i.v. Day 1)	3	26 (13/50)
	Chung *et al* (11)	5 MU/m^2 i.m. 3 times/week	-	CDDP (2 mg/kg continuous i.a. every 8 weeks)	8	33 (6/18)
	Sakon *et al* (13)	5 MU/m^2 sc. 3 times/week	300 mg/m^2 i.a. Day 1-5/week	-	4	63 (8/13)
	Patt *et al* (9)	4 MU/m^2 sc. 3 times/week	200 mg/m^2 i.v. Day 1-21	-	4	25 (9/36)

IFN, interferon; 5-FU, 5-fluorouracil; CDDP, cisplatin; MTX, methotrexate.

Discussion

IFN-α had been proposed to increase the efficacy of 5-FU in many single arm trials of IFN-α/5-FU combination therapy for colorectal carcinoma. For example, Wadler *et al* (26) reported that response rates were 76% (13/17) in untreated patients with advanced colorectal cancer. In 1990, they indicated that response rates were 63% in 32 untreated patients with advanced colorectal cancer (27). This combination therapy produced objective responses in 15 of 36 (42%) patients with colorectal carcinoma in an Eastern Cooperative Oncology Group (ECOG) study (28). In contrast, 5-FU monotherapy induced objective remissions in only 3 to 25% of patients with few complete responders, few durable remissions, and no improvement in overall survival (29-32). These results suggest that IFN-α/5-FU combination therapy may generate beneficial effects in colorectal cancer patients (Table II).

However, it was not clear whether IFN-α increases the efficiency of 5-FU in the treatment of colorectal cancer. Therefore, many randomized controlled trials comparing the combination of 5-FU and IFN-α to 5-FU alone have been performed. Hill *et al* (33) indicated that IFN adds no benefit to

5-FU in terms of response rates and survival and significantly increases toxicity in patients with advanced colorectal cancer. Kosmidis *et al* (34) reported that the addition of IFN-α2b to the combination of 5-FU and folinic acid contributes to decreased survival. Palmeri *et al* (35) noted significantly longer survival in patients who achieved a complete response after IFN-α therapy; however, overall survival was not affected. Hausminger *et al* (36) reported that the addition of IFN to 5-FU/Leucovorin (LV), in schedules and doses used in the present study, did not provide any clinical benefit over 5-FU/LV. Colucci *et al* (37) reported that no differences in the objective response rate, median duration of response, time to progression, and median survival [comparison between combination of levofolinic acid (l-FA)/5-FU and combination of l-FA/5-FU/IFN]. In a meta-analysis of these randomized control trials, IFN-α was not found to increase the efficiency of 5-FU in advanced colon cancer (38). Moreover, many therapies combined with IFN-α have been introduced for other gastrointestinal cancers. However, in common with colorectal cancer, the clinical use of IFN-α for other gastrointestinal cancers is doubtful (39-47) (Table III).

Several mechanisms for the anti-cancer effects of IFN-α have been proposed and can be direct and/or indirect anti-tumor effects. The direct anti-tumor effects include cell damage (48), upregulation of cancer antigens (49), and delayed action on the cell cycle (50). In contrast, indirect anti-tumor actions include activation of natural killer cells (51), T cells (52), and macrophages (53). IFN-α induces cyclin-dependent kinase inhibitors involved in G1/G0 arrest (54). IFN-α may also exert its anti-tumor effect indirectly via the immune system since it is known to augment T-cell cytotoxicity (55,56). Recently, we demonstrated that the modulation of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptor-mediated cytotoxic pathway might contribute to the anti-HCC effect of IFN-α/5-FU combination therapy (57). Furthermore, IFN-α induces apoptosis of various cancer cells (58). Another possible mechanism of action is via its anti-angiogenesis activity (59).

Based on these anti-tumor mechanisms of IFN-α, IFN-α suppressed the proliferation of all IFNAR2-positive cancer cell lines *in vitro* through mechanisms related to apoptosis or inhibition of the cell cycle (60). The importance of IFNAR2 expression for the anti-cancer effect of IFN-α injection was also reported in our recent studies (61,62). These findings suggest that the anti-neoplastic effects of IFN-α are mediated through IFNAR2. Recently, we found that the expression of IFNAR2 significantly correlates with IFN-α/5-FU combination therapy effects (22) and IFNAR2-induced signal transduction was useful for molecular prediction of the response to IFN-α/ 5-FU combination therapy in advanced HCC (63). Therefore, this significant relationship demonstrated in HCC should be also present in other gastrointestinal cancers. Accordingly, we investigated IFNAR2 expression in various gastrointestinal cancers using immunohistochemistry, as described in our previous report (23). The expression rate of IFNAR2 was not high in gastrointestinal cancers, in comparison with our previous results in HCC. We previously reported a strong expression rate of IFNAR2, at 26.4% (35/91), in HCC (23). The results of the present study indicate that the strong expression rate of IFNAR2 in gastrointestinal cancers, excluding HCC, was within 10% and lower than that in HCC. Based on these results, we believe that the efficiency of combination therapy with IFN-α in these cancers is expected be lower than that in HCC.

These results were compatible with those reported by Ambrus *et al* (64). They reported that all patients had increased levels of free interferon receptor-α/ß type-I in the circulation, with the highest levels reported in patients with adenocarcinoma. High IFN inhibitory activity in patients with cancer may be a significant factor in their increased susceptibility to progressive disease. These soluble forms can be both agonists and antagonists, depending on their concentration (65), and high levels of circulating soluble IFN receptors may block the anti-proliferative activity of IFN-α in adenocarcinomas.

In summary, we demonstrated that IFNAR2 expression rates in esophageal, gastric, colorectal, and pancreatic cancers and cholangiocarcinoma were lower than in HCC. Compared with our recent investigation in HCC, the clinical efficacy of IFN-α in combination therapies with other cytotoxic drugs in these gastrointestinal cancers is expected to be lower than that in HCC. To increase the treatment efficacy of IFN for gastrointestinal cancers other than HCC, other treatment modalities should be included, such as IFNAR2 gene transfection (66) or IFN-based chemoradiation (67).

Acknowledgements

The authors thank Dr Yasukazu Ohmoto from the First Institute of New Drug Research, and Otsuka Pharmaceutical Co., Ltd., for providing anti-human IFNAR2 antibody (OCT4813). The authors gratefully acknowledge Mrs. Satomi Yamane for her excellent technical assistance. This work was supported by a Grant-in-Aid for cancer research from the Ministry of Education, Culture and Science of Japan.

References

1. Kantarjian HM, O'Brien S, Cortes JE, Shan J, Giles FJ, Rios MB, Faderl SH, Wierda WG, Ferrajoli A, Verstovsek S, Keating MJ, Freireich EJ and Talpaz M: Complete cytogenetic and molecular responses to interferon-alpha-based therapy for chronic myelogenous leukemia are associated with excellent long-term prognosis. Cancer 97: 1033-1041, 2003.
2. Interferon as therapy for multiple myeloma: an individual patient data overview of 24 randomized trials and 4012 patients. Br J Haematol 113: 1020-1034, 2001.
3. Figlin RA, Callaghan M and Sarna G: Phase II trial of alpha (human leukocyte) interferon administered daily in adenocarcinoma of the colon/rectum. Cancer Treat Rep 67: 493-494, 1983.
4. Silgals RM, Ahlgren JD, Neefe JR, Rothman J, Rudnick S, Galicky FP and Schein PS: A Phase II trial of high-dose intravenous interferon alpha-2 in advanced colorectal cancer. Cancer 54: 2257-2261, 1984.
5. Eggermont AM, Weimar W, Tank B, Dekkers-Bijma AM, Marquet RL, Lameris JS, Westbroek DL and Jeekel J: Clinical and immunological evaluation of 20 patients with advanced colorectal cancer treated with high dose recombinant leukocyte interferon-alpha A (rIFN alpha A). Cancer Immunol Immunother 21: 81-84, 1986.
6. Lai CL, Lau JY, Wu PC, Ngan H, Chung HT, Mitchell SJ, Corbett TJ, Chow AW and Lin HJ: Recombinant interferon-alpha in inoperable hepatocellular carcinoma: a randomized controlled trial. Hepatology 17: 389-394, 1993.

7. Yuen MF, Ooi CG, Hui CK, Wong WM, Wong BC, Chan AO and Lai CL: A pilot study of transcatheter arterial interferon embolization for patients with hepatocellular carcinoma. Cancer 97: 2776-2782, 2003.
8. Urabe T, Kaneko S, Matsushita E, Unoura M and Kobayashi K: Clinical pilot study of intrahepatic arterial chemotherapy with methotrexate, 5-fluorouracil, cisplatin and subcutaneous interferon-alpha-2b for patients with locally advanced hepatocellular carcinoma. Oncology 55: 39-47, 1998.
9. Patt YZ, Hassan MM, Lozano RD, Brown TD, Vauthey JN, Curley SA and Ellis LM: Phase II trial of systemic continuous fluorouracil and subcutaneous recombinant interferon Alfa-2b for treatment of hepatocellular carcinoma. J Clin Oncol 21: 421-427, 2003.
10. Leung TW, Patt YZ, Lau WY, Ho SK, Yu SC, Chan AT, Mok TS, Yeo W, Liew CT, Leung NW, Tang AM and Johnson PJ: Complete pathological remission is possible with systemic combination chemotherapy for inoperable hepatocellular carcinoma. Clin Cancer Res 5: 1676-1681, 1999.
11. Chung YH, Song IH, Song BC, Lee GC, Koh MS, Yoon HK, Lee YS, Sung KB and Suh DJ: Combined therapy consisting of intraarterial cisplatin infusion and systemic interferon-alpha for hepatocellular carcinoma patients with major portal vein thrombosis or distant metastasis. Cancer 88: 1986-1991, 2000.
12. Miyamoto A, Umeshita K, Sakon M, Nagano H, Eguchi H, Kishimoto S, Dono K, Nakamori S, Gotoh M and Monden M: Advanced hepatocellular carcinoma with distant metastases, successfully treated by a combination therapy of alpha-interferon and oral tegafur/uracil. J Gastroenterol Hepatol 15: 1447-1451, 2000.
13. Sakon M, Nagano H, Dono K, Nakamori S, Umeshita K, Yamada A, Kawata S, Imai Y, Iijima S and Monden M: Combined intraarterial 5-fluorouracil and subcutaneous interferon-alpha therapy for advanced hepatocellular carcinoma with tumor thrombi in the major portal branches. Cancer 94: 435-442, 2002.
14. Patt YZ, Yoffe B, Charnsangavej C, Pazdur R, Fischer H, Cleary K, Roh M, Smith R, Noonan CA and Levin B: Low serum alpha-fetoprotein level in patients with hepatocellular carcinoma as a predictor of response to 5-FU and interferon-alpha-2b. Cancer 72: 2574-2582, 1993.
15. Lai CL, Wu PC, Lok AS, Lin HJ, Ngan H, Lau JY, Chung HT, Ng MM, Yeoh EK and Arnold M: Recombinant alpha 2 interferon is superior to doxorubicin for inoperable hepatocellular carcinoma: a prospective randomised trial. Br J Cancer 60: 928-933, 1989.
16. Link JS, Bateman JR, Paroly WS, Durkin WJ and Peters RL: 5-Flourouracil in hepatocellular carcinoma: report of twenty-one cases. Cancer 39: 1936-1939, 1977.
17. Doci R, Bignami P, Bozzetti F, Bonfanti G, Audisio R, Colombo M and Gennari L: Intrahepatic chemotherapy for unresectable hepatocellular carcinoma. Cancer 61: 1983-1987, 1988.
18. Tokyo Liver Cancer Chemotherapy Study Group: Phase II study of co-administration of uracil and tegafur (UFT) in hepatocellular carcinoma. Jpn J Clin Oncol 15: 559-562, 1985.
19. Taniguchi T: Cytokine signaling through nonreceptor protein tyrosine kinases. Science 268: 251-255, 1995.
20. Pestka S: The interferon receptors. Semin Oncol 24: S9-S40, 1997.
21. Hoey T and Schindler U: STAT structure and function in signaling. Curr Opin Genet Dev 8: 582-587, 1998.
22. Ota H, Nagano H, Sakon M, Eguchi H, Kondo M, Yamamoto T, Nakamura M, Damdinsuren B, Wada H, Marubashi S, Miyamoto A, Dono K, Umeshita K, Nakamori S, Wakasa K and Monden M: Treatment of hepatocellular carcinoma with major portal vein thrombosis by combined therapy with subcutaneous interferon-alpha and intra-arterial 5-fluorouracil; role of type 1 interferon receptor expression. Br J Cancer 93: 557-564, 2005.
23. Kondo M, Nagano H, Sakon M, Yamamoto H, Morimoto O, Arai I, Miyamoto A, Eguchi H, Dono K, Nakamori S, Umeshita K, Wakasa K, Ohmoto Y and Monden M: Expression of interferon alpha/beta receptor in human hepatocellular carcinoma. Int J Oncol 17: 83-88, 2000.
24. Ciaparrone M, Yamamoto H, Yao Y, Sgambato A, Cattoretti G, Tomita N, Monden T, Rotterdam H and Weinstein IB: Localization and expression of p27KIP1 in multistage colorectal carcinogenesis. Cancer Res 58: 114-122, 1998.
25. Short BG, Zimmerman DM and Schwartz LW: Automated double labeling of proliferation and apoptosis in glutathione S-transferase-positive hepatocytes in rats. J Histochem Cytochem 45: 1299-1305, 1997.
26. Wadler S, Schwartz EL, Goldman M, Lyver A, Rader M, Zimmerman M, Itri L, Weinberg V and Wiernik PH: Fluorouracil and recombinant alfa-2a-interferon: an active regimen against advanced colorectal carcinoma. J Clin Oncol 7: 1769-1775, 1989.
27. Wadler S, Goldman M, Lyver A and Wiernik PH: Phase I trial of 5-fluorouracil and recombinant alpha 2a-interferon in patients with advanced colorectal carcinoma. Cancer Res 50: 2056-2059, 1990.
28. Wadler S, Lembersky B, Atkins M, Kirkwood J and Petrelli N: Phase II trial of fluorouracil and recombinant interferon alfa-2a in patients with advanced colorectal carcinoma: an Eastern Cooperative Oncology Group study. J Clin Oncol 9: 1806-1810, 1991.
29. Comparison of antimetabolites in the treatment of breast and colon cancer. JAMA 200: 770-778, 1967.
30. Carter SK: Editorial: Large-bowel cancer - The current status of treatment. J Natl Cancer Inst 56: 3-10, 1976.
31. Davis HL: Chemotherapy of large bowel cancer. Cancer 50: 2638-2646, 1982.
32. Ansfield F, Klotz J, Nealon T, Ramirez G, Minton J, Hill G, Wilson W, Davis H Jr and Cornell G: A phase III study comparing the clinical utility of four regimens of 5-fluorouracil: a preliminary report. Cancer 39: 34-40, 1977.
33. Hill M, Norman A, Cunningham D, Findlay M, Nicolson V, Hill A, Iveson A, Evans C, Joffe J, Nicolson M, *et al*: Royal Marsden phase III trial of fluorouracil with or without interferon alfa-2b in advanced colorectal cancer. J Clin Oncol 13: 1297-1302, 1995.
34. Kosmidis PA, Tsavaris N, Skarlos D, Theocharis D, Samantas E, Pavlidis N, Briassoulis E and Fountzilas G: Fluorouracil and leucovorin with or without interferon alfa-2b in advanced colorectal cancer: analysis of a prospective randomized phase III trial. Hellenic Cooperative Oncology Group. J Clin Oncol 14: 2682-2687, 1996.
35. Palmeri S, Meli M, Danova M, Bernardo G, Leonardi V, Dastoli G, Rausa L, Russo A, Filippelli G, Palmieri G, Della Vittoria Scarpati M, Lo Russo V, Di Lauro L, Colucci G, Bruni G, Piazzi M, Gebbia N and Spada S: 5-Fluorouracil plus interferon alpha-2a compared to 5-fluorouracil alone in the treatment of advanced colon carcinoma: a multicentric randomized study. J Cancer Res Clin Oncol 124: 191-198, 1998.
36. Hausmaninger H, Moser R, Samonigg H, Mlineritsch B, Schmidt H, Pecherstorfer M, Fridrik M, Kopf C, Nitsche D, Kaider A and Ludwig H: Biochemical modulation of 5-fluorouracil by leucovorin with or without interferon-alpha-2c in patients with advanced colorectal cancer: final results of a randomised phase III study. Eur J Cancer 35: 380-385, 1999.
37. Colucci G, Maiello E, Gebbia V, Giuliani F, Serravezza G, Lelli G, Leo S, Filippelli G, Nicolella G and Brandi M: 5-Fluorouracil and levofolinic acid with or without recombinant interferon-2b in patients with advanced colorectal carcinoma: a randomized multicenter study with stratification for tumor burden and liver involvement by the Southern Italy Oncology Group. Cancer 85: 535-545, 1999.
38. Thirion P, Piedbois P, Buyse M, O'Dwyer PJ, Cunningham D, Man A, Greco FA, Colucci G, Kohne CH, Di Constanzo F, Piga A, Palmeri S, Dufour P, Cassano A, Pajkos G, Pensel RA, Aykan NF, Marsh J and Seymour MT: Alpha-interferon does not increase the efficacy of 5-fluorouracil in advanced colorectal cancer. Br J Cancer 84: 611-620, 2001.
39. Kelsen D, Lovett D, Wong J, Saltz L, Buckley M, Murray P, Heelan R and Lightdale C: Interferon alfa-2a and fluorouracil in the treatment of patients with advanced esophageal cancer. J Clin Oncol 10: 269-274, 1992.
40. Wadler S, Fell S, Haynes H, Katz HJ, Rozenblit A, Kaleya R and Wiernik PH: Treatment of carcinoma of the esophagus with 5-fluorouracil and recombinant alfa-2a-interferon. Cancer 71: 1726-1730, 1993.
41. Ilson DH, Sirott M, Saltz L, Heelan R, Huang Y, Keresztes R and Kelsen DP: A phase II trial of interferon alpha-2A, 5-fluorouracil, and cisplatin in patients with advanced esophageal carcinoma. Cancer 75: 2197-2202, 1995.
42. Wadler S, Haynes H, Beitler JJ, Hu X, Fell S, Camacho M, Levine B and Wiernik PH: Phase II clinical trial with 5-fluorouracil, recombinant interferon-alpha-2b, and cisplatin for patients with metastatic or regionally advanced carcinoma of the esophagus. Cancer 78: 30-34, 1996.
43. Lee KH, Lee JS, Suh C, Lee YS, Min YI, Ahn SH, Park KC, Kim SK and Kim SH: Combination of 5-fluorouracil and recombinant interferon alpha-2B in advanced gastric cancer. A phase I study. Am J Clin Oncol 15: 141-145, 1992.

44. Hudes GR, Lipsitz S, Grem J, Morrisey M, Weiner L, Kugler JW and Benson A III: A phase II study of 5-fluorouracil, leucovorin, and interferon-alpha in the treatment of patients with metastatic or recurrent gastric carcinoma: an Eastern Cooperative Oncology Group study (E5292). Cancer 85: 290-294, 1999.
45. Wadler S, Damle S, Haynes H, Kaleya R, Schechner R, Berkenblit R, Ladner RD and Murgo A: Phase II/pharmacodynamic trial of dose-intensive, weekly parenteral hydroxyurea and fluorouracil administered with interferon alfa-2a in patients with refractory malignancies of the gastrointestinal tract. J Clin Oncol 17: 1771-1778, 1999.
46. Wagener DJ, Wils JA, Kok TC, Planting A, Couvreur ML and Baron B: Results of a randomised phase II study of cisplatin plus 5-fluorouracil versus cisplatin plus 5-fluorouracil with alpha-interferon in metastatic pancreatic cancer: an EORTC gastrointestinal tract cancer group trial. Eur J Cancer 38: 648-653, 2002.
47. Bernhard H, Jager-Arand E, Bernhard G, Heike M, Klein O, Riemann JF, Meyer zum Buschenfelde KH, Dippold W and Knuth A: Treatment of advanced pancreatic cancer with 5-fluorouracil, folinic acid and interferon alpha-2A: results of a phase II trial. Br J Cancer 71: 102-105, 1995.
48. Grander D, Xu B and Einhorn S: Cytotoxic effect of interferon on primary malignant tumour cells. Studies in various malignancies. Eur J Cancer 29A: 1940-1943, 1993.
49. Guadagni F, Schlom J, Johnston WW, Szpak CA, Goldstein D, Smalley R, Simpson JF, Borden EC, Pestka S and Greiner JW: Selective interferon-induced enhancement of tumor-associated antigens on a spectrum of freshly isolated human adenocarcinoma cells. J Natl Cancer Inst 81: 502-512, 1989.
50. Kimchi A: Cytokine triggered molecular pathways that control cell cycle arrest. J Cell Biochem 50: 1-9, 1992.
51. Ortaldo JR, Mantovani A, Hobbs D, Rubinstein M, Pestka S and Herberman RB: Effects of several species of human leukocyte interferon on cytotoxic activity of NK cells and monocytes. Int J Cancer 31: 285-289, 1983.
52. Brinkmann V, Geiger T, Alkan S and Heusser CH: Interferon alpha increases the frequency of interferon gamma-producing human CD4+ T cells. J Exp Med 178: 1655-1663, 1993.
53. Uno K, Shimizu S, Ido M, Naito K, Inaba K, Oku T, Kishida T and Muramatsu S: Direct and indirect effects of interferon on *in vivo* murine tumor cell growth. Cancer Res 45: 1320-1327, 1985.
54. Sangfelt O, Erickson S, Castro J, Heiden T, Gustafsson A, Einhorn S and Grander D: Molecular mechanisms underlying interferon-alpha-induced G0/G1 arrest: CKI-mediated regulation of G1 Cdk-complexes and activation of pocket proteins. Oncogene 18: 2798-2810, 1999.
55. Trinchieri G, Santoli D and Koprowski H: Spontaneous cell-mediated cytotoxicity in humans: role of interferon and immunoglobulins. J Immunol 120: 1849-1855, 1978.
56. Lindahl P, Leary P and Gresser I: Enhancement by interferon of the specific cytotoxicity of sensitized lymphocytes. Proc Natl Acad Sci USA 69: 721-725, 1972.
57. Yamamoto T, Nagano H, Sakon M, Wada H, Eguchi H, Kondo M, Damdinsuren B, Ota H, Nakamura M, Marubashi S, Miyamoto A, Dono K, Umeshita K, Nakamori S, Yagita H and Monden M: Partial contribution of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)/TRAIL receptor pathway to antitumor effects of interferon-alpha/5-fluorouracil against hepatocellular carcinoma. Clin Cancer Res 10: 7884-7895, 2004.
58. Horowitz RW, Heerdt BG, Hu X, Schwartz EL and Wadler S: Combination therapy with 5-fluorouracil and IFN-alpha2a induces a nonrandom increase in DNA fragments of less than 3 megabases in HT29 colon carcinoma cells. Clin Cancer Res 3: 1317-1322, 1997.
59. Folkman J and Ingber D: Inhibition of angiogenesis. Semin Cancer Biol 3: 89-96, 1992.
60. Yano H, Iemura A, Haramaki M, Ogasawara S, Takayama A, Akiba J and Kojiro M: Interferon alfa receptor expression and growth inhibition by interferon alfa in human liver cancer cell lines. Hepatology 29: 1708-1717, 1999.
61. Kondo M, Nagano H, Wada H, Damdinsuren B, Yamamoto H, Hiraoka N, Eguchi H, Miyamoto A, Yamamoto T, Ota H, Nakamura M, Marubashi S, Dono K, Umeshita K, Nakamori S, Sakon M and Monden M: Combination of IFN-alpha and 5-fluorouracil induces apoptosis through IFN-alpha/beta receptor in human hepatocellular carcinoma cells. Clin Cancer Res 11: 1277-1286, 2005.
62. Eguchi H, Nagano H, Yamamoto H, Miyamoto A, Kondo M, Dono K, Nakamori S, Umeshita K, Sakon M and Monden M: Augmentation of antitumor activity of 5-fluorouracil by interferon alpha is associated with up-regulation of p27Kip1 in human hepatocellular carcinoma cells. Clin Cancer Res 6: 2881-2890, 2000.
63. Kurokawa Y, Matoba R, Nagano H, Sakon M, Takemasa I, Nakamori S, Dono K, Umeshita K, Ueno N, Ishii S, Kato K and Monden M: Molecular prediction of response to 5-fluorouracil and interferon-alpha combination chemotherapy in advanced hepatocellular carcinoma. Clin Cancer Res 10: 6029-6038, 2004.
64. Ambrus JL Sr, Dembinski W, Ambrus JL Jr, Sykes DE, Akhter S, Kulaylat MN, Islam A and Chadha KC: Free interferon-alpha/beta receptors in the circulation of patients with adenocarcinoma. Cancer 98: 2730-2733, 2003.
65. Hardy MP, Owczarek CM, Trajanovska S, Liu X, Kola I and Hertzog PJ: The soluble murine type I interferon receptor Ifnar-2 is present in serum, is independently regulated, and has both agonistic and antagonistic properties. Blood 97: 473-482, 2001.
66. Wagner TC, Velichko S, Chesney SK, Biroc S, Harde D, Vogel D and Croze E: Interferon receptor expression regulates the antiproliferative effects of interferons on cancer cells and solid tumors. Int J Cancer 111: 32-42, 2004.
67. Takaoka A, Hayakawa S, Yanai H, Stoiber D, Negishi H, Kikuchi H, Sasaki S, Imai K, Shibue T, Honda K and Taniguchi T: Integration of interferon-alpha/beta signalling to p53 responses in tumour suppression and antiviral defence. Nature 424: 516-523, 2003.

Available online at www.sciencedirect.com

ScienceDirect

Hepatology Research 35 (2006) 289–295

HEPATOLOGY RESEARCH

http://www.elsevier.com/locate/hepres

Vitamin K2 has growth inhibition effect against hepatocellular carcinoma cell lines but does not enhance anti-tumor effect of combination treatment of interferon-α and fluorouracil *in vitro*☆

Masato Nakamura, Hiroaki Nagano*, Takehiro Noda, Hiroshi Wada, Hideo Ota, Bazarragchaa Damdinsuren, Shigeru Marubashi, Atsushi Miyamoto, Yutaka Takeda, Yuichiro Doki, Koji Umeshita, Keizo Dono, Masato Sakon, Morito Monden

Department of Surgery, Graduate School of Medicine, Osaka University, 2-2 Yamadaoka E-2, Suita, 565-0871 Osaka, Japan

Received 1 November 2005; received in revised form 4 April 2006; accepted 28 April 2006
Available online 12 June 2006

Abstract

Several studies have recently reported the efficacy of combination therapy of interferon (IFN) α and 5-fluorouracil (5-FU) for hepatocellular carcinoma (HCC). However, the clinical effect of this treatment was not complete. The new therapeutic modality should be necessary to rise up this clinical response rate. Recently, the anti-tumor effect of Vitamin K2 has been reported in terms of decreased recurrence rate of HCC patients. The aim of this study was to explore the additive or synergistic effect of Vitamin K2 to combined therapy of interferon (IFN) α and 5-fluorouracil (5-FU) against hepatocellular carcinoma (HCCs). The study was conducted using three hepatoma cell lines (PLC/PRF/5, Hep3B and HepG2). The 3-(4-5-dimethylthiazol-2-yl)-2, 5-dyphenyl tetrazolium bromide (MTT) assay (48 h) revealed anti-tumor effect of IFNα and 5-FU. Cell growth assay (3–7 days) showed growth inhibitory effect of Vitamin K2 on three cell lines after day 5. But additional effect of combination treatment of Vitamin K2 and IFNα/5-FU was not observed in any time course from 48 h to 7 days. Cell cycles were assessed with flowcytometry. Although either Vitamin K2 or IFNα/5-FU alone has the influence to the cell cycles, no significant change was shown in the combination of Vitamin K2 and IFNα/5-FU. In conclusion, Vitamin K2 itself has potentially growth inhibitory effect for HCC cell lines, but does not enhance the anti-tumor effect of IFNα and 5-FU.

Keywords: Vitamin K; Interferon; 5-FU; HCC

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common solid tumors worldwide [1]. The prognosis of HCC is still poor, in particular the growth of tumors with macroscopic tumor thrombus in the major branches of portal vein (Vp3-4) is extremely aggressive, and most of these patients die within several months after the diagnosis [2]. Conventional therapeutic modalities such as transcatheter arterial embolization, radiofrequency ablation and microwave coagulation therapy are not recommended when portal vein tumor thrombus (PVTT) is present because of low efficacy and potentially complications [3,4]. In addition, it has been reported that chemotherapeutic drugs are not effective in the view of tumor regression and prolonging survival against HCC [2,5].

Recently, we and others observed clinical anti-tumor effects of combination therapy of interferon-alpha (IFNα) and 5-fluorouracil (5-FU) for advanced HCC with PVTT [6–10]. This combination chemotherapy demonstrated almost 50% of response rate and obviously prolonged the survival of far advanced HCC patients with PVTT, even though either IFNα or 5-FU alone had no efficacy against HCC [10–12]. We also reported the *in vitro* anti-tumor effect about the synergicity of IFNα and 5-FU [13–15].

☆ *Grant support*: This work was supported by a grant-in-aid for cancer research from the Ministry of Education, Culture and Science of Japan.
* Corresponding author. Tel.: +81 6 6879 3251; fax: +81 6 6879 3259.
E-mail address: hnagano@surg2.med.osaka-u.ac.jp (H. Nagano).

1386-6346/$ – see front matter © 2006 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.hepres.2006.04.014

Otherwise, several studies have been reported that Vitamin K2 inhibits growth of hepatocellular carcinoma cell lines *in vitro* [16–19]. Vitamin K2 is usually used as treatment for osteoporosis with no severe adverse effects and the safety was established. In clinical, prospective study showed Vitamin K2 decreased recurrence rate of HCC patients with high protein induced by Vitamin K absence II (PIVKA-II), especially recurrence with PVTT [20]. PIVKA-II is an abnormal prothrombin that lacks γ-carboxy residues and therefore cannot become an active clotting enzyme [21].

In this study, we examined additive or synergic effect of Vitamin K2 to enhance the IFNα/5-FU combined therapy against HCC cells *in vitro*, to explore the possibility of new clinical treatment modification of combined IFNα/5-FU.

2. Materials and methods

2.1. Cells

Human HCC cell lines (PLC/PRF/5 and HepG2) were obtained from Japan Cancer Research Resources Bank (JCRB) (Osaka, Japan) and Human HCC cell line Hep3B was obtained from Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). These cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin at 37 °C in a humidified incubator with 5% CO_2 in air.

2.2. Reagents

Purified human IFNα was obtained from Otsuka Pharmaceutical Co. (Tokushima, Japan), and 5-FU was supplied from Kyowa Hakko Co. (Tokyo, Japan). Vitamin K2 (menaquinone 4) was supplied from Eisai Chemical Co. (Ibaraki, Japan).

2.3. Cell growth assay

Cell growth was assessed by the 3-(4-, 5-dimethylthiazol-2-yl)-2, 5-dyphenyl tetrazolium bromide (MTT) assay (48 h) and growth inhibitory assay (3–7 days). About MTT assay, briefly, 3×10^3 cells were seeded to a 96-well plate in 100 μl of medium and left overnight to adhere. Several concentrations of drugs in 100 μl volumes were added, and cells were incubated for 48 h. After treatment, 10 μl of MTT solution

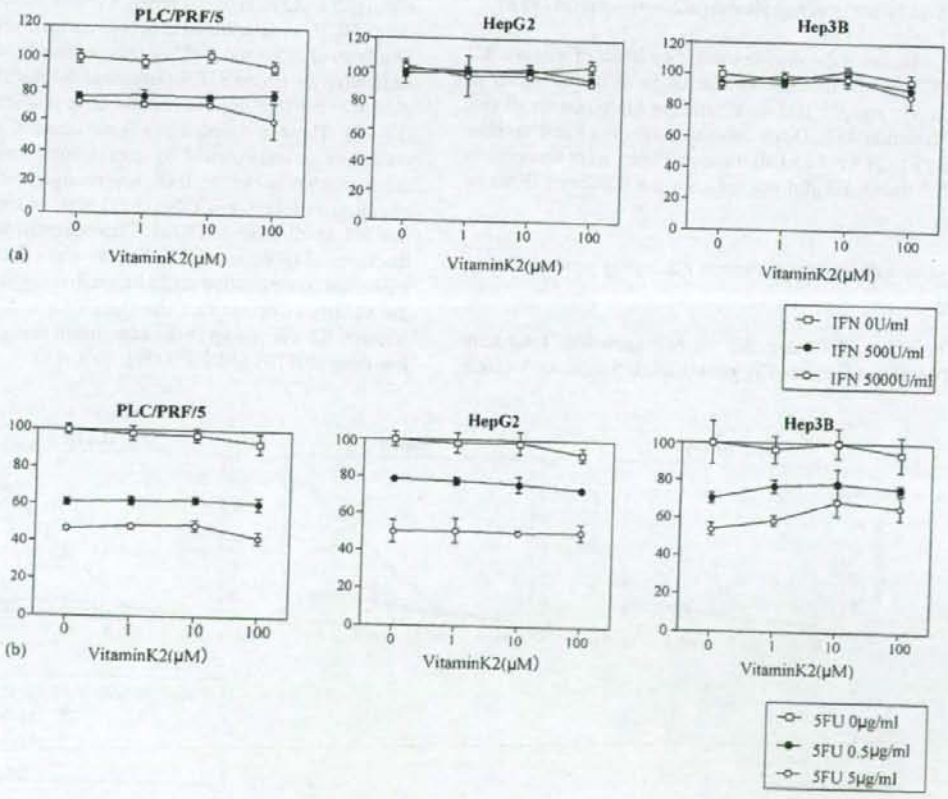

Fig. 1. Additive effect of Vitamin K2 on anti-tumor effect of IFNα (a) and 5-FU (b) in short term by MTT assay. Data represent mean ± S.D. of at least triplicate samples. Similar results were observed in three independent experiments. Vitamin K2 did not enhance the anti-tumor effect of IFNα and 5-FU.

was added to each well and incubated another 4 h at 37 °C. Then 100 μl of acid-isopropanol was added, and after 24 h at 4 °C, reduced MTT was measured spectrophotomechanically in a dual beam microtiter plate reader at 570 nm with a 650-nm reference. About growth inhibitory assay, cells were seeded onto six-well plates at a density of 5×10^4 cells/well, and drugs were added on the next day. After 3, 5 and 7 days from stimulation, cell numbers were calculated with hemocytometer by trypan blue dye exclusion. In each assay, even dose of ethanol was added to control group because Vitamin K2 is fat-soluble vitamin and needs 0.1% ethanol to solute water.

2.4. Cell cycle assay

The cell cycle distribution was analyzed by flow cytometry using the fluorescent dye propidium iodide. The proportion of nuclei in each cell cycle phase was determined using MODIFIT DNA analysis software (Becton Dickinson).

3. Results

3.1. Anti-tumor effect of Vitamin K2 in short term (48 h)

We calculated the growth inhibitory effect of Vitamin K2 in MTT assay with dose escalation in 48 h. As shown in Fig. 1, Vitamin K2 had no significant inhibition in all cell growth within 48 h. IFNα showed decrease of cell number in only PLC/PRF/5 and all three cell lines were sensitive to 5-FU. Vitamin K2 did not enhance the effects of IFNα or 5-FU.

3.2. Anti-tumor effect of Vitamin K2 in long term (3–7 days)

The effect of Vitamin K2 on cell growth in long term (3–7 days) was evaluated by growth inhibition assay. Vitamin K2 inhibited cell growth of all three HCC cell lines in dose dependent manner (Fig. 2). The growth inhibitory effect was apparently after 5 days. From these data, the Vitamin K2 concentrations causing 50% cell growth inhibitions (IC50) were determined (Table 1).

Table 1
IC50 of each cells on day 3, 5 and 7

	3 days	5 days	7 days
IC50 PLC/PRF/5	1372	20.2	8.1
IC50 Hep3B	111.6	7.2	0.36
IC50 HepG2	401.4	15.1	13.5

3.3. Combined treatment of Vitamin K2 and IFNα/5-FU in long term

Because Vitamin K2 has neither significant growth inhibition nor enhancement the effect of IFNα/5-FU within 48 h, the experiment about the combined treatment of Vitamin K2 and IFNα/5-FU was performed only long term (3–7 days). In all three cell lines, cells were stimulated with Vitamin K2 and/or IFNα/5-FU, to investigate the additive or synergistic effect in the situation of combined. Vitamin K2 was used with dose of IC50 as determined in previous experiment (Table 1). The doses of IFNα and 5-FU were determined as 500 units/ml and 0.5 μg/ml, because IFNα enhanced the biochemical modulation of 5-FU in this concentration, as previous our report [13–15]. These concentrations were identical with that in plasma of patients treated by continuous infusion in clinical. As shown in Fig. 3a, there was no significant difference of cell growth between IFNα/5-FU with and without Vitamin K2 on all three cell lines. Consequently, we decreased the doses of IFNα and 5-FU, because the effect of IFNα/5-FU in first concentration might be much stronger and masked the additive effect. In fact, the significant additive effect of Vitamin K2 was shown in the experiment using medium and low dose of IFNα and 5-FU (Fig. 3b and c).

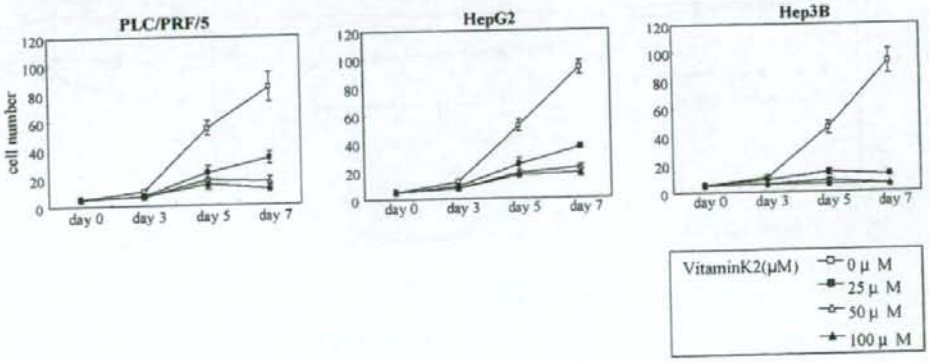

Fig. 2. Anti-tumor effect of Vitamin K2 in long term by growth inhibitory assay. Data represent mean ± S.D. of at least triplicate samples. Similar results were observed in three independent experiments. Data represent mean ± S.D. of at least triplicate samples.

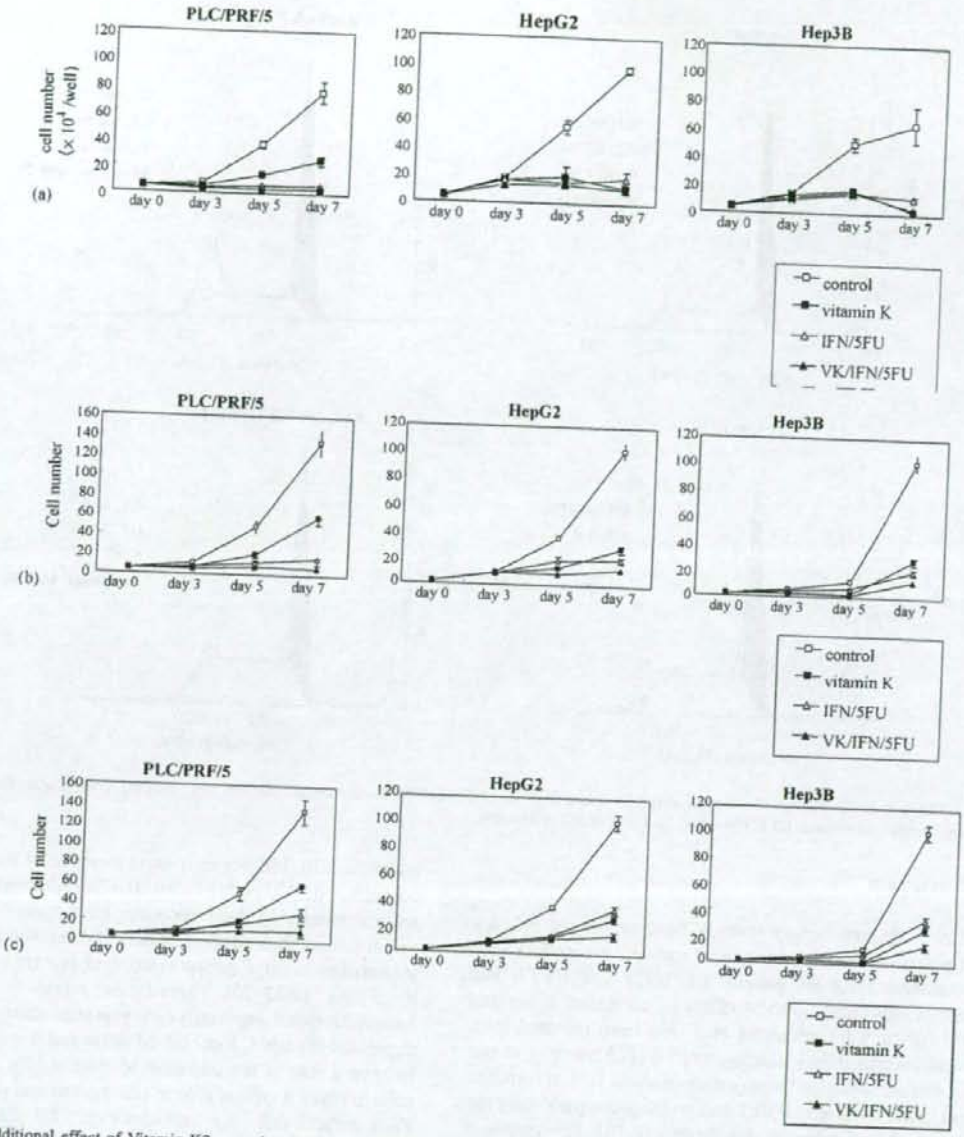

Fig. 3. Additional effect of Vitamin K2 on anti-tumor effect of IFNα/5-FU in long term by growth inhibitory assay. Doses of IFN and 5-FU was fixed as 500 U/ml and 0.5 μg/ml. Data represent mean ± S.D. of at least triplicate samples. There was no significant difference between IFNα/5-FU with and without Vitamin K2.

3.4. Cell cycle assay of Vitamin K2 and combination of IFNα/5-FU

The anti-tumor effect of Vitamin K2 and IFNα/5-FU caused by the cell cycle arrest has been reported. We investigated additive or synergistic effect in cell cycle regulation with combined treatment [13,16–19]. The representative data of Hep3B after 5 days treatment was shown in Fig. 4. Vitamin K2 stimulation had little influence to cell cycle, while IFN/5-FU induces increasing of S phase (54.1% versus control 38.4%) and decreasing of G1 phase (44.1% versus control 55.6%). Combination of Vitamin K2 and IFN/5-FU showed faint synergistic effect (S phase 57.1%, G1 phase 39.9%) (Fig. 4). Apoptotic cells were not increased with combination with Vitamin K2 (control 3.3%, Vitamin K2 0%, IFN/5-FU 13.3% and Vitamin K2 and IFN/5-FU 14.3%).