Correlation between Bcl-2 Overexpression and H-ras Mutation in Naturally Occurring Hepatocellular Proliferative Lesions of the B6C3F1 Mouse

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The correlation between the mutation at codon 61 of the H-ras gene and the expression of the Bcl-2 protein was investigated in naturally occurring hepatocellular proliferative lesions in B6C3F1 mice. Specimens of histologically diagnosed neoplastic or preneoplastic lesions of the liver, obtained from the control mice used for 2-year carcinogenicity studies, were examined by immunohistochemical techniques. All of 25 lesions confirmed to be hepatocellular carcinomas stained positive for the Bcl-2 protein. Three of 12 foci of cellular alterations, as well as 24 of 42 hepatocellular adenomas, stained weakly positive. Bcl-2 protein was expressed to a greater degree in hepatocellular carcinomas as opposed to adenomas and confirmed by Western blot analysis. Seven of 18 hepatocellular adenomas that stained positive for Bcl-2 and three of 16 hepatocellular adenomas that stained negative had a mutation at codon 61 of the H-ras gene. Overexpression of Bcl-2 protein is likely to enhance the malignant turnover of the neoplastic cells, following a mutation at codon 61 of the H-ras gene particularly. These findings suggest that Bcl-2 overexpression and the mutation at codon 61 of the H-ras gene may be critical factors in the development of naturally occurring hepatocellular tumors in B6C3F1 mice.

Key Words: Bcl-2; H-ras; B6C3F1 mouse; liver tumor.

The liver is one of the most common target organs of xenobiotic insult in chronic toxicity and carcinogenicity studies. Molecular alterations at the subcellular and genetic levels will become of paramount importance in the elucidation of mechanistic carcinogenic evaluations in experimental animals and their relevance to risk assessment in human populations. A primary concern and often a confounding factor of lifetime rodent bioassays currently being employed to detect carcinogenic potential is the high incidence of naturally occurring hepatocellular tumors in B6C3F1 mice, commonly used in these bioassays (Enomoto et al., 1990). Recent advances in the molecular biology and genetics of hepatocellular tumors revealed that the H-ras oncogene possessing mutation in codon 61 is activated in approximately 60% of naturally occurring hepatocellular tumors in the B6C3F1 mouse (Goldsworthy et al., 1998; Iida et al., 1997; Maronpot et al., 1995). Three types of alterations are commonly found on codon 61 in naturally occurring hepatocellular tumors: CAA to AAA, CGA, and CTA, in proportions of approximately 60:28:12, respectively. The specificity of chemicals exhibiting a preference for ras or non-ras pathways have been analyzed by comparing the frequency of H-ras activation in these tumors with that in tumors induced by a variety of chemicals (Goldsworthy et al., 1998; Maronpot et al., 1995).

Numerous reports on the inhibitory role of the bcl-2 oncogene in apoptosis have been published recently (Goldsworthy et al., 1996a,b; Reed, 1998; Williams, 1991; Wyllie, 1997). This gene was identified by cloning breakpoints of 14–18 translocations, t(14;18), a characteristic of human B-cell lymphomas (Tsujimoto et al., 1985). This translocation determines the juxtaposition of the bcl-2 gene on the immunoglobulin heavy chain locus (Bakhshi et al., 1985) resulting in deregulation of overexpression of the bcl-2 gene. The bcl-2 gene inhibits the release of cytochrome c and apoptosis-inducing factor (AIF) from the mitochondria, thereby preventing apoptotic death (Kluck et al., 1997; Yang et al., 1997). Prevention of induced or spontaneous apoptosis via bcl-2 may result in the selective survival of preneoplastic cells and ultimate neoplastic transformation. Lee (1997) reported that the Bcl-2 protein is overexpressed in diethylnitrosamine-induced hepatocellular tumors of B6C3F1 mice, and demonstrated the difference in Bcl-2 expression among histological phenotypes of hepatocellular tumors induced by the combined effects of diethylnitrosamine and phenobarbital using a 2-step initiator-promoter model. However, the role of the Bcl-2 protein on naturally occurring hepatocellular tumors in B6C3F1 mice has not been demonstrated. The present study was conducted to examine the role of Bcl-2 expression in the development of the naturally occurring hepatocellular tumors in B6C3F1 mice and to demonstrate the correlation between Bcl-2 expression and H-ras activation in carcinogenesis.
MATERIALS AND METHODS

Naturally occurring hepatocellular proliferative lesions including 12 foci of cellular alterations (FCA), 42 hepatocellular adenomas (HCA), and 25 hepatocellular carcinomas (HCC) were obtained from control B6C3F1 (C57BL/6 × C3H/He) mice (SLC Inc., Shizuoka, Japan) that were used in 2-year carcinogenicity studies conducted at the Biosafety Research Center, Foods, Drugs, and Pesticides (An-Pyo Center), Shizuoka during 1996 to 1998. Liver nodules obtained at necropsy were sectioned in two. One section was stored at −80°C for Western blot analysis. The second section wasfixed in 10% neutral buffered formalin, embedded in paraffin, and cut into 4 μm sections for histological and immunohistochemical staining and terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) reaction. One specimen from the latter section was stained with hematoxylin and eosin (H&E) for routine histological examinations. Other specimens were used for immunohistochemical staining of either Bcl-2 or PCNA and for TUNEL reaction for apoptosis analysis. Hepatocellular proliferative lesions of B6C3F1 mice were classified according to the report of Maronpot et al. (1987) into FCA, HCA, and HCC by H&E staining. Sections of liver tissue that appeared normal by visual inspection were used as normal control.

Immunohistochemistry

Immunohistochemical staining for Bcl-2 or PCNA was performed with an antigen retrieval system. Sections were autoclaved at 120°C for 5 min in distilled water for Bcl-2 or with a microwave oven in 10 mM citrate buffer (pH 6.0) for PCNA. Following cooling, the sections were incubated with primary antibodies overnight at 4°C. Rabbit anti-mouse Bcl-2 serum (PharMingen, San Diego, CA) was employed as the Bcl-2 antibody and was diluted 1:500 in 1% bovine serum albumin/phosphate-buffered saline (PBS). Sections were stained by the LSAB method (Labeled Streptavidin Biotin Kit, DAKO, Japan). Mouse monoclonal anti-PCNA (PC10; DAKO, Japan) was used as a PCNA antibody at a dilution of 1:200 and stained by the LSAB method. The color indicative of immune-reactivity was developed using diaminobenzidine (DAB) for 7 min. Slides were rinsed with distilled water, lightly counterstained with hematoxylin, and mounted. The primary antibody was omitted in negative control samples. The PCNA labeling index (LI) was calculated as the percentage of positive cells per 1000 cells in randomly selected fields.

TUNEL Method

The Apop Tag in situ Apoptosis Detection Kit-Peroxidase (Oncor, Gaithersburg, MD) was used to identify apoptosis by the method described by Gavrieli et al. (1992). Sections were deparaffinized with xylene, rinsed in ethanol, and digested with 20 μg/ml Protease K (Boeringer-Mannheim, Germany) for 15 min at room temperature and rinsed with distilled water. Endogenous peroxidase activity was blocked with 2% hydrogen peroxide in PBS for 5 min and rinsed with PBS. Sections were incubated in equilibration buffer for 15 min at room temperature and incubated in working strength TdT enzyme at 37°C for 1 h. Slides were washed in stop/wash buffer for 10 min, rinsed with PBS, incubated with anti-digoxigenin-peroxidase for 30 min at room temperature, and washed with PBS. Slides were developed with DAB, washed with distilled water, counterstained with methyl green, and mounted. Distilled water was used in place of the TdT enzyme, in negative controls. The apoptotic index (AI) was calculated as the percentage of positive cells per 1000 cells in randomly selected fields.

Western Blot Analysis

We used Western blotting to analyze Bcl-2 expression at the protein level. Total tissue lysates containing 25 μg of denatured protein/lane were run on 12% Ready Gel J (Bio-Rad, California) and electroblotted onto a nitrocellulose membrane (Hybond-ECL, Amersham Pharmacia, Uppsala, Sweden). The membrane was blocked with 0.5% non-fat dry milk in PBS with 0.1% Tween 20 (PBST) and incubated with rabbit anti-mouse Bcl-2 serum (PharMingen) diluted in PBST with 5% non-fat dry milk (1:1000 dilution) at 4°C overnight. After washing in PBST, the membrane was incubated with horseradish peroxidase-labeled anti-rabbit secondary antibodies (Amersham Pharmacia) diluted in PBST with 5% non-fat dry milk (1:2000 dilution) at 4°C overnight and washed again. Finally, the immunoreaction was visualized using enhanced chemiluminescence (Amersham Pharmacia).

Extraction and Amplification of DNA from Paraffin-embedded Hepatocellular Tissue

DNA was obtained for oncogenic analysis from paraffin-embedded hepatocellular tissues. Tissue samples obtained from normal liver tissues and hepatocellular proliferative lesions, 12 FCA (3 Bcl-2-positive and 9 Bcl-2-negative), 34 HCA (18 Bcl-2-positive and 16 Bcl-2-negative) and 14 Bcl-2-positive HCC were deparaffinized with xylene and rinsed with ethanol. Sections from these specimens were collected with a sterile disposable scalpel and placed in 1.5 ml microcentrifuge tubes. DNA was extracted from each sample by the same method previously reported (Iida et al., 1999). The polymerase chain reaction (PCR) was carried out as described previously (Iida et al., 1997) to amplify a 116 bp fragment of exon 2 of the H-ras gene, which includes codon 61. An aliquot (10 μl) of DNA extracts was used for PCR in a PCR reaction mixture containing 50 μl reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 0.001% (v/v) gelatin, 200 μM dNTPs (dATP, dCTP, dGTP, and dTTP), 0.2 μM of each primer and 5 units of AmpliTaq (PE Biosystems, California). The sequences of primers 1 and 2 were 5′-GAG-ACATGTCTACTGGAATCTT-3′ (sense) and 5′-GTGTTGTGATTGCGC- AAATACACAGAGG-3′ (antisense), respectively. The mixture was covered with a layer of mineral oil (Sigma, St. Louis, MO) to prevent evaporation and transferred to the thermal cycler (TR-100; Taitec, Japan) for 38 cycles. Each cycle consisted of 30 seconds at 94°C, one min at 60°C, and 30 s at 72°C. Following this reaction, the mixture was incubated at 72°C for 8 min. Each amplification reaction containing DNA from normal liver tissue, positive controls for H-ras mutation, and controls without DNA template was run with all sets of reactions. DNA amplification was confirmed by electrophoresis on a 1.5% agarose gel containing (0.5 μg/ml) of ethidium bromide.

Non-RI SSCP

Non-radioisotope single strand conformation polymorphism (non-RI SSCP) technique, in combination with the silver staining method, was chosen to detect point mutations, since it is highly sensitive and simple. Aliquots of 10 μl of the PCR products were added to 45 μl of loading buffer (95% formamide, 20 mM EDTA, 0.05% BPB, 0.05% xylene cyanol), denatured by heating for 3 min at 80°C, and then chilled on ice. Aliquots of 3 μl of this mixture were electrophoresed by the Phast System (Amersham Pharmacia) using PhastGel Homogeneous 12.5 (Amersham Pharmacia) and PhastGel Native Buffer strips (Amersham Pharmacia). The Phast System was run under the following conditions: (i) pre-run, 15 mA, 200 V, 10 min; (ii) Sample application, 15 mA, 300 V, 2.5 W, 4°C. 2 Vh; and (iii) Run, 15 mA, 300 V, 2.5 W, 4°C, 200 Vh. The relationship between immunohistochemical staining for Bcl-2 and the mutation at codon 61 of the H-ras gene in HCA was analyzed using Fisher’s exact test, following classification of the patterns of H-ras mutations.

Direct DNA Sequencing

Representative samples were selected for direct sequencing to determine the nucleotide substitution detected by non-RI SSCP. Aliquots of 30 μl were purified following amplification using Ultradex DA (Millipore, Bedford, MA). Samples were sequenced using an ABI PRISM Big dye Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems) according to the manufacturer’s instructions. The sequencing primer used was a nested primer, 5′-GATGGCGAAATACACAGAGG-3′. An ABI PRISM 310 genetic analyzer (PE Biosystems) analyzed reaction products. All the mutations were confirmed by sequencing at least twice.
RESULTS

We examined Bcl-2 protein in hepatocellular proliferative lesions immunohistochemically. As shown in Table 1, of 12 FCA, 24 of 42 HCA and 25 of 25 HCC specimens were stained positive for Bcl-2 protein. Most of the FCA and HCA specimens showed weak, monochrome positive staining for Bcl-2. However, all 25 HCC specimens stained more clearly, but sporadically for Bcl-2, with small dots in multi-layered hepatocytes exhibiting trabecular structures (Table 1 and Fig. 1).

| Type | Bcl-2 | Immunohistochemistry | LI (%) |
|------|-------|----------------------|--------|
| FCA  | –     | 9/12 (75.0)          | 0.92 ± 0.15 |
|      | +     | 3/12 (25.0)          | 1.19 ± 0.12 |
| HCA  | –     | 18/42 (42.9)         | 3.56 ± 1.72 |
|      | +     | 24/42 (57.1)         | 4.11 ± 2.35 |
| HCC  | +     | 25/25 (100.0)**      | 4.00 ± 3.21 |

Note. +, positive; –, negative; FCA, foci of cellular alteration; HCA, hepatocellular adenoma; HCC, hepatocellular carcinoma; LI, PCNA labeling index. Nine FCA/Bcl-2-negative, 3 FCA/Bcl-2-positive, 12 HCA/Bcl-2-negative, 13 HCA/Bcl-2-positive, and 8 HCC/Bcl-2-positive specimens were analyzed by immuno-staining of anti-PCNA for cell proliferation. Bcl-2 immunohistochemistry, % in parentheses. The LI was calculated as the percentage of positive cells per 1000 cells in randomly selected fields under light microscopy; mean ± SD.

| Type          | Number | Bcl-2-positive (%) |
|---------------|--------|--------------------|
| Basophilic    | 8      | 5 (62.5)           |
| Clear type    | 26     | 14 (53.8)          |
| Eosinophilic  | 2      | 0 (0.0)            |
| Mixed type    | 37     | 31 (83.8)          |
| Vacuolic type | 6      | 2 (33.3)           |

Note. Histological phenotypes by H&E staining as basophilic, clear, eosinophilic, mixed and vacuolic appearances in hepatocellular proliferative lesions were compared with incidence of Bcl-2-positives between each histological phenotype. Hepatocellular proliferative lesions of B6C3F1 mice were classified by their cytological features and tinctorial properties in H&E stained sections.

Hepatocellular proliferative lesions stained for Bcl-2 were compared to each histological phenotype by H&E staining and were characterized as being either eosinophilic, basophilic, vacuolic, and/or mixed in appearance. As shown in Table 2, the percentage of Bcl-2-positive specimens in hepatocellular proliferative lesions examined was 63% for basophilic, 54% for clear, 84% for mixed, and 33% for vacuolic appearance. Therefore, the staining manner of Bcl-2 did not correlate with these phenotypes. The number of eosinophils is too small to evaluate correlation. Only one to a few hepatocytes in the central lobular zone were sometimes stained positive for Bcl-2 in normal liver tissue of mice at 109 weeks-of-age. Bcl-2 protein

FIG. 1. Staining of Bcl-2 in liver tissues. (A) Bcl-2 staining in naturally occurring hepatocellular adenoma of B6C3F1 mouse. Ad, hepatocellular adenoma; N, normal liver. Adenoma cells are positively stained, whereas normal hepatocytes are negative. (B) Bcl-2 staining in naturally occurring hepatocellular carcinoma of B6C3F1 mouse. Arrows point to the small dots in the positive area.
of 26 kDa was detected in HCA and HCC by Western blot analysis, as shown in Figure 2. The amount of Bcl-2 protein in HCC was more than twice the HCA levels measured by NIH’s Image. Western blot analysis showed that the normal tissue, which was stained positive for Bcl-2, contained a small amount of Bcl-2 protein.

Immunostaining of anti-PCNA for cell proliferation was conducted on both Bcl-2-positive and Bcl-2-negative sections. Nine FCA/Bcl-2-negative, 3 FCA/Bcl-2-positive, 12 HCA/Bcl-2-negative, 13 HCA/Bcl-2-positive, and 8 HCC/Bcl-2-positive specimens were analyzed. The PCNA LI (mean ± SD) was 0.92 ± 0.15 for FCA/Bcl-2-negative specimens, 1.19 ± 0.12 for FCA/Bcl-2-positive specimens, 3.56 ± 1.72 for HCA/Bcl-2-negative specimens, 4.11 ± 2.35 for HCA/Bcl-2-positive specimens, and 4.00 ± 3.21 for HCC/Bcl-2-positive specimens. There were no statistically significant differences between positive and negative specimens (Table 1).

The extent of apoptosis in the Bcl-2-positive and Bcl-2-negative sections that were used to determine PCNA LI was also examined by the TUNEL method. Since the AI was very low (0.1–0.5%) in all hepatocellular proliferative lesions examined, it was concluded that an adequate incidence for comparison did not exist (data not shown).

Nine FCA/Bcl-2-negative, 3 FCA/Bcl-2-positive, 16 HCA/Bcl-2-negative, 18 HCA/Bcl-2-positive, and 14 HCC/Bcl-2-positive DNA samples were analyzed by SSCP as having a mutation at codon 61 in the H-ras gene. The H-ras gene with mutated codon 61 was detected in 3 cases of HCA/Bcl-2-negative, 7 cases of HCA/Bcl-2-positive, and 8 cases of HCC. However, no mutations of the H-ras gene were detected in any of 9 FCA/Bcl-2-negative or 3 FCA/Bcl-2-positive samples (Table 3 and Fig. 3). Mutations in hepatocellular tumors were confirmed by DNA sequencing. Three HCA/Bcl-2-negative, 4 HCA/Bcl-2-positive and 4 HCC/Bcl-2-positive, which showed the shift-positive results in non-RI PCR-SSCP, were found to contain C to A transversion at the first base of the codon 61 in the H-ras gene (see lanes 2, 3, and 4 in Fig. 3). In addition, 3 HCA/Bcl-2-positive and 4 HCC/Bcl-2-positive were found to contain A to G transition at the second base (see lane 5 in Fig. 3). Mutations at codon 61 in the H-ras gene were found in 7 (38.9%) of 18 HCA specimens that stained positive for Bcl-2, and in 3 (18.8%) of 16 HCA specimens stained negative for Bcl-2 (Table 3). The incidence of mutations at codon 61 in the H-ras gene was higher in Bcl-2-positive specimens than in Bcl-2-negative specimens.

DISCUSSION

Korsmeyer studied the role of the bcl-2 gene family in the regulation of programmed cell death and demonstrated, using transgenic mice, that an overexpression of the bcl-2 gene of the B-cell line resulted in prolongation of cell survival with subsequent progression to lymphoma formation (Korsmeyer,

![Image 2](Western blot analysis of the Bcl-2 protein in normal liver and naturally occurring liver tumors of B6C3F1 mice. Twenty-five μg of denatured protein was electrophoresed, transferred to a nitrocellulose membrane, and incubated with rabbit anti-mouse Bcl-2 serum, as described in Materials and Methods. The position of the Bcl-2 protein is indicated by an arrow of 26 kDa (lanes 2, 3, and 4). Lanes 1 and 2, normal liver; lane 3, hepatocellular adenoma; lane 4, hepatocellular carcinoma. Normal livers used in lanes 1 and 2 were obtained from different mice.)

![Image 3](Detection of mutation by non-RI SSCP in the H-ras exon 2 region in Bcl-2-positive, naturally occurring liver tumors of B6C3F1 mice. Three μl of denatured single-strand DNA were electrophoresed for SSCP analysis using the conditions described in Materials and Methods. The extra bands were detected in hepatocellular tumors (arrowheads, lanes 2, 3, 4, and 5). Lane 1, normal liver; lanes 2 and 3, hepatocellular adenoma containing mutated bands for the second exon of the H-ras oncogene; lanes 4 and 5, hepatocellular carcinoma containing mutated bands for the second exon of the H-ras oncogene; lane 6, positive control of C to A transversion at the first base of codon 61; lane 7, positive control of A to G transition at the second base of codon 61.)

![Table 3](Frequency and Spectrum of H-ras Codon 61 Mutations in Naturally Occurring Hepatocellular Proliferative Lesions of B6C3F1 Mice)

| Type        | Bcl-2 | H-ras mutations (%) | CAA → AAA | CAA → CGA |
|-------------|-------|---------------------|-----------|-----------|
| FCA         | –     | 0/9 (0.0)           | –         | –         |
| +           | 0/3   (0.0)         | –         | –         |
| HCA         | –     | 3/16 (18.8)         | 3         | 0         |
| +           | 7/18  (38.9)        | 4         | 3         |
| HCC         | +     | 8/14 (57.1)         | 4         | 4         |

Note. +, positive; –, negative; FCA, foci of cellular alteration; HCA, hepatocellular adenoma; HCC, hepatocellular carcinoma. DNA was isolated from hepatocellular tumors of B6C3F1 mice and a portion of the exon 2 of the H-ras gene was amplified by PCR. Mutations present in codon 61 were identified by direct sequencing of the amplified DNA fragments.)
Having an interest in the role of Bcl-2 in naturally occurring hepatocellular tumors, in the present study we examined the expression of Bcl-2 during tumor formation. In this study, all HCC specimens stained positive for Bcl-2 via immunohistochemical staining. Three of 12 FCA and 24 of 42 HCA specimens also stained positive in the pathological evaluations conducted on naturally occurring hepatocellular tumors in B6C3F1 mice (Table 1). Bcl-2 is widely used as a marker protein to distinguish benign hepatocellular adenomas or dysplasia from malignant hepatocellular carcinomas in humans (Zhao et al., 1994). However, the immunohistochemical results of this study showed diffuse, but weak staining of Bcl-2 in murine FCA or HCA. These results suggest that Bcl-2 staining may not be applicable for differentiating these types of proliferative lesions from one another in naturally occurring hepatocellular tumors in B6C3F1 mice. Clearly visible, stained small dots evident in the hepatocytes of HCC (Fig. 1B) appear to correspond to the presence of Bcl-2 proteins attached to the outer mitochondrial membrane, endoplasmic reticulum, and perinuclear membranes. Lee (1997) reported that basophilic hepatocellular proliferative lesions specifically stained positive for Bcl-2, and all eosinophilic tumors stained negative for Bcl-2, when the lesions were induced by the treatment with diethylnitrosamine alone and combined treatment with diethylnitrosamine and phenobarbital. However, the results of this study demonstrate that there is no apparent specificity of staining for Bcl-2 on histological phenotypes of naturally occurring hepatocellular tumors in B6C3F1 mice. These differences suggest that the mechanism of development of hepatocellular tumors is different between induced and naturally occurring tumors in B6C3F1 mice.

The Al values in the majority of the hepatocellular proliferative lesions were low in this study. Goldsworthy et al. (1996a,b) also reported that apoptosis occurs infrequently in hepatocellular tumors in B6C3F1 mice (0.01–0.1% in females). Although the specificity of Bcl-2 expression and a definitive correlation of Bcl-2 protein with PCNA (LI) were not demonstrated in HCA, marked and increased expression of Bcl-2 in HCC demonstrates its usefulness in the evaluation of the malignant behavior of the naturally occurring hepatocellular tumors in B6C3F1 mice.

Mutation at codon 61 of the H-ras gene was detected in 7 (38.9%) of 18 HCA that stained positive for Bcl-2 immunohistochemically, but in only 3 (18.8%) of 16 HCA stained negative for Bcl-2, suggesting a higher expression of Bcl-2 in HCA with H-ras mutation. These results suggest a cooperative synergistic effect between ras oncogenes and cell growth-regulatory factors, including Bcl-2, in the development of naturally occurring hepatocellular tumors in B6C3F1 mice. Kinoshita et al. (1995) proposed that H-ras gene mutations cause overexpression of the Bcl-2 protein and homologous proteins such as Bcl-xL, resulting in suppression of cell death in hematopoietic cells. Weinberg (1989) reported that ras-oncogene activation occurs during the cellular growth phase of benign tumors or preneoplastic lesions, resulting in the formation of large adenomas that may progress to malignant tumors. Overexpression of Bcl-2 was observed in cell lines that contain the human c-H-ras oncogene with an activating point mutation on codon 61 (Osanai et al., 1997). These observations suggest that activating point mutations in H-ras gene may induce Bcl-2 expression, which might result in the selective survival of the initiated hepatocytes via inhibition of apoptosis and the accumulation of alterations in other tumor-related genes.

A mutation at codon 61 in the H-ras gene was not detected by non-RI SSCP or DNA sequencing in 11 of 18 HCA that had stained positive for Bcl-2 immunohistochemically. Furthermore, although all the HCC stained positive for Bcl-2, 6 (47.1%) of 14 HCC contained no mutation at codon 61. These results suggest that in naturally occurring hepatocellular proliferative lesions factors other than the mutation at codon 61 in the H-ras gene lead to the enhanced expression of the Bcl-2 protein, and that the overexpression of Bcl-2 is more important for the development of malignancy in the naturally occurring hepatocellular carcinoma in the B6C3F1 mouse than H-ras activation by the mutation at codon 61.

Data supporting the correlation between Bcl-2 overexpression and the H-ras gene mutation will shed light on the molecular basis of cooperation between endogenous mutational effects of oncogenes and the regulatory factors of cellular growth as interactive and inter-related steps in the neoplastic turnover in hepatocytes. Further studies using molecular and morphologically specific markers will be required to elucidate the pathogenesis of naturally occurring hepatocellular tumors commonly found in the aged B6C3F1 mice.

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