Amplification of BCR Protein Associated with Oncogenesis in Human Hepatocellular Carcinoma

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The BCR gene is located on human chromosome 22. The normal cellular BCR gene encodes a 160,000-dalton phosphoprotein associated with a serine/threonine kinase activity. The BCR protein is involved in signal transduction. We investigated the expression of the BCR protein in hepatocellular carcinoma (HCC), surrounding noncancerous liver tissue, liver cirrhosis (LC), chronic hepatitis (CH), and normal liver with immunohistochemistry and a western blot analysis. BCR immunoreactivity was detected using a monoclonal antibody. In normal liver, and both CH and LC without association of HCC, the immunoreactivity of the BCR protein was minimal. In contrast, 73% (22 of 30) of noncancerous liver tissue adjacent to the HCC and 40% (12 of 30) of HCC expressed BCR protein; this difference was statistically significant ($P < 0.01$). The expression of the BCR protein expression correlated with the degree of histological differentiation of HCC ($P < 0.05$). In addition, the amplification of BCR protein in noncancerous cells was supported by the detection of specific protein using a western blot analysis. In two cases, the expression of BCR protein occurred only in overtly malignant HCC cells. As a result, the expression of the BCR protein may be associated with oncogenesis in human HCC.

**KEY WORDS:** BCR protein; hepatocellular carcinoma; oncogenesis; oncogenic products; oncogenic precursor.

Primary liver cancer is the third most common cause of cancer death, following gastric and lung carcinoma, in Japan. It occurs at a rate of 16.5 per 100,000 Japanese (1). Approximately 90% of the liver carcinoma cases seen in Japan are hepatocellular carcinoma (HCC). Most of these cases have a strong etiological association with either chronic hepatitis B or hepatitis C virus infection (2, 3). More than 85% of HCC cases are clinically preceded by chronic hepatitis or liver cirrhosis (4). This implies that liver cirrhosis may be precancerous condition. Pathological studies have demonstrated the development of well-differentiated HCC from areas of adenomatous hyperplasia.

A suggestion that HCC arises *ab initio* has been proposed recently (5, 6). Several studies have demonstrated various alternations in protein expression in various carcinoma. These have been reported to be a result of altered oncogene or the tumor suppressor gene expression.

Chronic myelogenous leukemia (CML) has three stages of progression: a chronic phase, an accelerated phase, and a blastic phase. This sequence is similar to the course of liver diseases such as chronic hepatitis (CH), liver cirrhosis (LC), and hepatocellular carcinoma.
noma (HCC). The Philadelphia (Ph) chromosome results from a reciprocal translocation between chromosomes 9 and 22 and occurs in more than 95% of patients with chronic myelogenous leukemia (CML). The BCR gene is located on human chromosome 22. It is the site of the breakpoint used in the generation of the Philadelphia chromosome translocation (19;22; q34;q11) found in CML and acute lymphocytic leukemia (ALL) (7–10). The normal BCR gene encodes for a 160-kDa phosphoprotein associated with a serine/threonine kinase activity, which is localized in the cytoplasm (11). The cellular BCR gene encodes two major mRNA species of 4.5 kb and 6.7 kb, which appear to differ in their untranslated regions (12, 13). The BCR protein is relatively constant in many tissues and cell lines, suggesting that it has an important role in general cell metabolism rather than having a tissue-specific function (14). A major phosphoprotein species of approximately 160 kDa (p160c-BCR) has been defined as the product of the BCR mRNA (15–17). The BCR protein has plural function domains and may function in intracellular signaling pathways (11, 18–21).

In order to gain insight into the physiological function of the BCR protein in connection with liver oncogenesis, we studied the distribution of its expression in HCC, noncancerous tissue of liver with HCC, LC, CH, and normal liver. The availability of a highly specific monoclonal antibody against the BCR gene product permitted us to carry out immunohistochemistry on sections of paraffin-embedded tissue and western blotting with protein isolated from these tissues. Herein we show that the expression of BCR protein is associated with oncogenesis in human hepatocellular carcinoma.

MATERIALS AND METHODS

Samples. Thirty HCCs and their corresponding noncancerous liver tissues were obtained from 30 patients either at surgery, by ultrasound-guided liver biopsy, or at autopsy. The tissue samples were stored at −80°C until protein, DNA, and RNA isolation could be carried out. These tissues were fixed in 10% buffered formalin and embedded in paraffin wax. Several sections approximately 5 μm thick were prepared from each block. After it was deparaffinized, one section from each block was used for conventional histologic staining with hematoxylin and eosin. Seven LC specimens and 10 CH specimens were obtained from 17 patients who underwent laparoscopic liver biopsy. As a normal control, one normal adult liver and one liver from a stillbirth fetus (26 weeks of gestation) were obtained at autopsy. The tissues were treated as described for the study tissues.

Immunohistochemistry. Immunohistochemical observations were performed on adjacent deparaffinized sections using the peroxidase-labeled streptavidin–biotin technique with a monoclonal antibody specific for the BCR protein; anti-BCR (Ab-2) dilution 1:100, OSI, New York) (22–26). The peroxidase-labeled streptavidin–biotin technique was performed using a Histofine SAB-PO kit purchased from Nichirei (Seikagaku Kogyo Co., Tokyo, Japan). After endogenous peroxidase activity was blocked using 3% hydrogen peroxide, the specimens were treated as follows. They were exposed to 10% nonimmunized rabbit serum in phosphate-buffered saline (PBS) for 20 min, and primary antibodies were applied to the sections for 2 hr at 4°C. The sections were then incubated with biotinylated rabbit antimouse immunoglobulins for 2 hr, followed by incubation with horseradish peroxidase-labeled streptavidin for 15 min at room temperature. Reaction products were visualized by immersion of the section in complete Karnovsky solution (0.01% dianisobenzidine in Tris buffer containing 0.05% hydrogen peroxide and 0.01% sodium azide). The sections were counterstained with 3% methyl green. Negative control examinations were performed with the omission of the primary antisera.

Western Blot Analysis. The snap-frozen tissue specimens were thawed at room temperature, and 20 mg of each was excised, cut into minute fragments, and weighed on an analytical balance. Protein was extracted from snap-frozen HCC samples and their corresponding noncancerous liver samples. Tissue fragments were then suspended in 10 vol of a buffer containing 2% SDS, 100 nmol/liter dithiothreitol, and 60 nmol/liter Tris, pH 6.8. The resulting suspension was boiled for 10 min and passed several times through a 25-gauge hypodermic needle to shear genomic DNA. The total protein content of each lysate was determined colorimetrically using reagents obtained commercially (Bio-Rad Laboratories, Richmond, California). Equal amounts of protein (100 μg) were loaded into each lane for western blot analysis. SDS–polyacrylamide gel electrophoresis was carried out using standard methods (27). After electrophoresis, the proteins were transferred electrophoretically onto a nitrocellulose membrane. The BCR protein was detected using anti-BCR at a dilution of 1:100 with a detection system similar to that used for immunohistochemistry.

Southern Blot Analysis and Northern Blot Analysis. High-molecular-weight DNA was extracted from the carcinoma and the corresponding noncancerous liver by digestion with proteinase K containing SDS and phenol–chloroform extraction. Southern blot analysis was performed as follows: In brief, 10 μg DNA was digested with the appropriate restriction enzymes, separated by electrophoresis on 0.7% agarose gels, and transferred to nylon filters (28). The filters were hybridized under stringent conditions with 32P-labeled c-myc cDNA probes. After hybridization, the filters were washed and exposed to XAR-5 film (Eastman Kodak Co., Rochester, New York).

Total RNA was extracted from the carcinoma and its corresponding noncancerous liver. Frozen tissue were homogenized with a polytron homogenizer and total RNA was purified by the acid guanidinium thiocyanate–phenol–chloroform extraction (AGPC) method (29). Total RNA samples (20 μg) were electrophoresed in 1.2% agarose gels containing 0.25 mol/liter formaldehyde and then trans-