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# C-*erb*B-2, p53, N-*ras* EXPRESSION IN HEPATOCELLULAR CARCINOMA

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**To assess the relationship between C-*erb*B-2, p53, N-*ras* status at a premalignant stage and in HCC, the authors studied the imunohistological expression of these genes in HCC, liver cirrhosis and in the adjacent normal resected liver tissue, using monoclonal antibody to mutated p53 and activated C-*erb*B-2, N-*ras*. C-*erb*B-2 was expressed in 97.1% (35/36) of HCC and 100% (18/18) of hepatic cirrhosis, low level C-erbB-2 expression was observed in 2/14 (14.3%) of normal liver specimens; The positive incidence of overexpression of mutant p53 protein in HCC and hepatic cirrhosis were 55.6% (20/36) and 66.7% (10/18) respectively; 29 (76.5%) specimens of HCC and 16 (88.9%) of hepatic cirrhosis were positive for N-*ras* protein. The overexpression of the three oncogene proteins were significantly higher than that of normal liver tissues ($P<0.01$). These results indicated that activated C-*erb*B-2, N-*ras* and altered p53 genes may have a role in human HCC pathogenesis through promiting the development of HCC from hepatic cirrhosis and the progression of HCC.**

**Key words: C-*erb*B-2, p53, N-*ras*, Hepatocellular carcinoma, Immunohistochemistry.**

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer death throughout the world, despite major advance in cancer diagnosis and treatment in the past two decades, the etiology of HCC has improved only minimally. However, recent progress in the study of the molecular biology of cancer has contributed to our better understanding of its molecular pathogenesis, including that of hepatocellular carcinoma. Under such circumstances, it may be possible to discover the oncogenes and their associated genes, and explore their roles in the mechanisms of human HCC. The oncogene C-*erb*B-2 was identified by Shin et al. in 1981 in rats with chemically induced neuroblastomas.[1-3] Its human equivalent is located on chromosome 17 and encodes a membrane phosphoglycoprotein of 185 kilodalton. Although the function of this protein in unknown, it has tyrosine kinase activity and may act as a cell-growth-regulating receptor. Amplification and overexpression of the C-*erb*B-2 has been studied in patients with different tumors.[4,5] However, its etiological role in HCC has not been well established; Abnormalities of the p53 gene represent the most common genetic alterations in human cancer being implicated in tumorgenesis and tumor progression of both sporadic and inherited forms of malignancies.[6-15] p53, which is located on the short arm of chromosome 17, acts as a tumor suppressor gene negatively regulating the cell cycle and requiring loss of function mutations for tumor formation. The abnormal structure and expression of p53 in human HCC cell lines is common, as first reported by Bressac et al.; N-*ras* is located on the short arm of chromosome 1 and encoded P21 protein which possesses homological sequence with G protein and involved in the process of signal transduction. It has also been reported that

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the activation of N-*ras* may have a role in the pathogenesis of some tumors[16–20] Although some authors have studied the amplification and overexpression of p53, N-*ras* oncogene in the mentioned neoplasms, the results published were discordant, and their clinical value was not clearly defined. Therefore, we decided to investigate C-*erb*B-2, p53, N-*ras* oncogene expression in HCC by using immunohistochemical staining. It was hoped that such a study would give information about the cellular distribution of these oncogene protein in both normal and malignant hepatic tissues, and demonstrated the association of C-*erb*B-2, p53, N-*ras* expression, if any, with the expression of HBsAg, HBcAg.

## MATERIALS AND METHODS

### Patients

Sixty-eight patients enrolled in this study were evaluated at the department of pathology, Dalian Medical University during the year 1993–1995. Patients were grouped according to their histological diagnosis, as shown in Table 1. For the diagnosis of HBV infection, serum was assessed for HBV surface antigene (HBsAg) and HBV c/e antigene using an enzyme immunoassay (EIA). Hepatitis C virus (HCV) infection was assessed by measuring anti-HCV antibody using an EIA kit. The patients in each group had received no chemotherapy or radiation within one month before the biopsy or surgery. Tissue samples were obtained from beside liver biopsies or from surgical specimens in patients with liver tumors. The samples were fixed in formalin, embedded in paraffin, sectioned at 4 μm.

### Immunohistochemistry

Immunohistochemical staining for C-*erb*B-2, p53, N-*ras* (P21), HBsAg and HBcAg expression was performed by a peroxidase-labeled streptavidin-biotin technique. 4-micron paraffin tissue sections were mounted on aminoalkysilane-treated glass slides and were deparaffinizied in xylene and alchol and placed for 15 min. in alchol-$H_2O_2$ for blocking endogenous peroxidase, sections were treated with bovine serum albumin to prevent background staining. The immunohistochemical technique involved sequential application of diluted C-*erb*B-2 antibody (1:200, DAKO Corp. [Glostrup, Denmark]), diluted p53 antibody (1:100, DAKO Corp, [Glostrup, Denmark]), diluted P21 antibody (1:100, DAKO Corp, [Glostrup, Denmark]) or nondiluted ready-to-use HBsAg, HBcAg antibodies (a kind gift from Dr. Yokihiro Matsugama) overnight at 4 ℃. Slides were rinsed with phosphate buffer saline for 3 min. and incubated first with the biotinylated linked second antibody for 30 min. and then with the labeling reagent, peroxidase conjugated streptavidine for 30 min. After the slides were rinsed, the peroxidase label was demonstrated using diaminobenzidine in the presence of hydrogen peroxide. Sections were counterstained with a light hematoxylin, stained slides were mounted with a coverslip. Tissues from breast cancer known to overexpress C-*erb*B-2, P21, served as a positive control. The other positive control was tissue from a gastric cancer specimen with a known p53 mutation. Negative control serial sections for each sample were run using the same technique but omitting the primary antibody and adding the streptavidine-biotin complex.

*Table 1. Study population according to histological diagnosis*

| | Normal | Cirrhosis | HCC |
|---|---|---|---|
| No. of patients | 14 | 18 | 36 |
| Age (y) | 33 (19–42) | 47 (17–68) | 61 (29–84) |
| Sex (M/F) | 8/6 | 12/6 | 20/16 |
| Clinical diagnosis | HBV (1) | HBV (9) | HBV (20) |
| | HCV (1) | HCV (6) | HCV (12) |
| | NAD (12) | Alcholic (1) | Other cause (4) |
| | | Other cause (2) | |

NAD: No Abnormality Demonstrated. HBV: Hepatitis B Virus. HCV: Hepatitis C Virus.
HCC: Hepatocellular Carcinoma Some patients have more than one etiological factor.

## RESULTS

### Overexpression of C-*erb*B-2, p53, P21 Protein in Various Liver Tissues

Immunohistochemical staining for C-*erb*B-2 revealed membrane and cytoplasmic staining in positive cases. Results were graded as strong, weak and negative. Among the three groups, the liver cirrhosis cases stained the most strongly positive, HCC cases stained relatively strong, there were only 14.3% (2/14) of normal liver cases were weakly positive. Positive p53 staining was nuclear in all cases, with only a minimal cytoplasmic background in a few cases. Results were again graded as strong, weak and negative. 66.7% (12/18) of liver cirrhosis cases were strongly positive and 55.6% (20/36) of HCC cases were weakly positive. Fourteen cases of normal liver were negative for p53 staining; P21 overexpression was detected in membrane and cytoplasmic staining in positive cases. Liver cirrhosis cases were stained strongly positive for P21. Positive HCC cases is in the second place and negative staining for all normal liver specimens. Immunohistochemical staining for all HBcAg showed granular brown nuclear staining in most of HCC adjacent non-tumor tissues. HBsAg was expressed in the membrane or/and cytoplasmic in positive cases and negative for all normal liver specimens. Test for differences of C-*erb*B-2, p53, P21, HBsAg, HBcAg expression among the three groups were performed using $x^2$ method. The overexpression of C-*erb*B-2, p53, P21, HBsAg, HBcAg in HCC and liver cirrhosis groups were significantly higher than that of normal liver cases ($P<0.01$) (Table 2).

### The Relationship between Oncogene Protein Overexpression and HBV Antigene Expression

As shown in Table 3, no significant difference was observed when either of C-*erb*B-2, p53, P21 overexpression compared with each of HBsAg, HBcAg expression respectively ($P>0.05$).

*Table 2. C-erbB-2, p53, P21, HBsAg, HBcAg expression in various liver tissues*

| Group | C-*erb*B-2 | p53 | p21 | HBsAg | HBcAg |
|---|---|---|---|---|---|
| NL | 14.3% | 0% | 0% | 0% | 0% |
| LC | 100% | 66.7% | 88.9% | 83.3% | 61.1% |
| HCC | 97.1% | 55.6% | 76.5% | 77.8% | 46.9% |

NL: Normal Liver LC: Liver Cirrhosis HCC: Hepatocellular Carcinoma

*Table 3. The association of C-erbB-2, p53, P21 overexpression with HBsAg, HBcAg expression in HCC tissues*

| % | HBsAg | | HBcAg | |
|---|---|---|---|---|
| | - | - | - | - |
| C-*erb*B-2 (+) | 100% | 87.5% | 100% | 94.4% |
| p53 (+) | 64.3% | 25% | 70.5% | 42.1% |
| p21 (+) | 89.2% | 50% | 94.1% | 68.4% |

## DISCUSSION

Cancer has myriad causes, but many of them may have a common way — by damaging DNA. By no means or another, on the basis of circumstantial evidence of considerable variety, damage to diverse protooncogene has been implicated in the genesis of human tumors. The same genetic lesions have been found repeatedly in the DNA of human tumors, in original specimens as well as explanted cell lines.

With frequencies that seem beyond coincidence, these lesions have involved protooncogenes already identified by retroviral transduction, and the damage carried by the genes is of a sort we know to be pathogenic. Provocative corrections can be made between at least some of the genetic lesions and distinctive features of the tumors in which they are found.[21] The results in this report show that C-*erb*B-2, p53, N-*ras* genes are overexpressed in hepatocellular carcinoma and liver cirrhosis tissues. The development of primary tumors and HCC is a stepwise process that involves activation of oncogenes and loss of tumor suppressor genes function, leading to initiation, promotion and progression of tumors. The p53 tumor suppressor gene plays a major role in cell cycle control.[22,23] Alteration in p53 gene in HCC cells is well documented from studies reported during the last few years. In one study, these reports were grouped by geographical area, with one group containing studies from areas with a high incidence of HCC. Specific mutations at high rates in the p53 gene and an increase of the overall frequency in mutations along the whole p53 gene previously were reported in areas where HCC is endemic. Dietary aflatoxin and HBV and HCV infections probably contributed to the high incidence of HCC in a number of patients. The contribution of dietary aflatoxin to p53 mutation was suggested through its adduct formation targeting the p53 gene, including a G:C to T:A transversion mutation hot spot at condon 249. The contribution of HBV to the stabilization and overexpression of p53 was contributed to protein-protein interaction between one of the HBV encoded proteins, hepatitis X antigene, and p53.[24-26] The biochemical mechanisms by which the C-*erb*B-2, P21 protein may act the metabolic regulation by proteins that bind GTP in the manner of the familiar G protein and control of gene expression by influencing the biogenesis of mRNA.[27,28] Like early reports, we found a relationship between the overexpression of C-*erb*B-2, P21 and the tumorgenesis of HCC. These indicated that the activation of C-*erb*B-2, N-*ras* oncogene may have a role in HCC.

The hepatitis B virus (HBV) is an enveloped, hepatotropic DNA virus that causes acute and chronic liver cell injury and inflammation and Hepatocellular Carcinoma (HCC). More than 300 million people throughout the world are chronically infected by this virus. Served as its reservoir, and have a 200-fold greater risk of developing HCC than their noninfected counterparts. Although much is known about HBV structure, replication strategy, and life cycle, the pathogenesis mechanisms responsible for malignant transformation in HBV infection are not well understood. There are several alternative mechanisms whereby HBV might be directly involved in carcinogenesis, such as viral inactivation of cellular anti-oncogenes or the existence of an acutely transforming viral oncogene within the HBV genome, but it is very infrequent and hence does not explain the majority of HBV-induced HCC.[29,30] More and more evidence support the hypotheses that unregulated expression of HBV gene product (HBsAg, HBcAg, HBxAg, DNA polymerase) may contribute to the malignant transformation of the infected hepatocyte through their transcriptional trans-activating properties. To understand the functional mechanism of HBsAg, HBcAg in the pathogenesis of HCC. We have expressed the HBsAg, HBcAg expression in HCC tissue and investigated the association of HBsAg, HBcAg expression with C-*erb*B-2, p53, P21 oncogene protein expression. The results show that there is not close relationship among them. So, we can conclude that HBsAg, HBcAg may not be the transactivator and they may not have direct role in HCC. The molecular mechanism of tumorgenesis of HCC need to be further studied.

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