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C-erb-B-2 oncoprotein and epidermal growth factor receptor in human hepatocellular carcinoma: An immunohistochemical study

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Summary. The aim of this study was to evaluate the immunohistochemical expression of epidermal growth factor (EGFR) and c-erb-B-2 oncoprotein in a series of 71 hepatocellular carcinomas as well as in the adjacent hepatic tissue and to assess any correlation with HBsAg expression. The total of the 71 hepatocellular carcinomas (HCCs) was classified into 17 low grade and 54 high grade cases with adjacent non-neoplastic liver parenchyma, observed in 14 and 28 cases respectively. Coexisting cirrhosis or fibrosis was noticed in the adjacent non-neoplastic parenchyma in 12 cases of low grade and 22 cases of high grade HCC. The immunohistochemical avidin-biotin-peroxidase complex (ABC) method was performed on formalin-fixed paraffin sections for the detection of EGFR, c-erb-B-2 oncoprotein and HBsAg using monoclonal antibodies. The expression of c-erb-B-2 was observed in 29.5% (21/71) of the HCCs showing no statistically significant correlation with histological grade. The c-erb-B-2 was also detected in the adjacent non-neoplastic parenchyma in 7/14 low grade HCCs, and in 9/28 high grade HCCs. No statistically significant differences in c-erb-B-2 oncoprotein expression were observed between the HCCs and the adjacent non-neoplastic parenchyma.

In addition, HBsAg was detected in 10/42 examined cases of HCC with adjacent non-neoplastic parenchyma, while only 4 cases of HCCs were simultaneously positive for c-erb-B-2 and HBsAg. EGFR was detected in only 3/71 cases of HCC, while the antigen was not detected at all in the adjacent non neoplastic parenchyma. HBsAg expression was not observed in any of the EGFR-positive HCCs.

Our results suggest that both c-erb-B-2-oncoprotein and EGFR do not seem to be predominantly involved in the transformation of hepatocytes to the malignant phenotype.

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Introduction

In recent years, the role of oncogenes in carcinogenesis has attracted increasing attention, because oncogene products appear to play a role in cell growth and proliferation. The activation of oncogene expression has been reported in association with a variety of human cancers (Bishop, 1981).

The epidermal growth-factor receptor (EGFR) and the c-erb-B-2 oncoprotein are members of the type 1 growth factor, receptor family (membrane receptors with a protein kinase activity) and are encoded by the c-erb-B-1 and c-erb-B-2 oncogenes respectively (Downward et al., 1984). EGFR is a transmembrane glycoprotein consisting of three domains, an extracellular EGF-binding domain, a transmembrane segment and a cytoplasmic domain with a tyrosine kinase activity (Carpenter, 1987). Increased expression and/or activity of EGFR has been reported in several types of tumours including squamous carcinomas from various sites (Ishitoya et al., 1989; Schneider et al., 1990a,b), gynaecological tumours (Owens et al., 1991), breast (Sainsbury et al., 1987) and bladder carcinomas (Neal et al., 1990) as well as gliomas (Liberman et al., 1985).

According to the literature, EGFR overexpression in breast, bladder, lung and ovarian carcinomas is associated with a poor prognosis (Sainsbury et al., 1987; Neal et al., 1990; Schneider et al., 1990a).

On the other hand, the c-erb-B-2 gene was first described as a result of DNA transfection studies in rat neuroglioblastomas and in man was mapped on chromosome 17 (Fukushige et al., 1986). The gene encodes a protein which has a structural and sequence similarity with the EGFR, suggesting that it is a receptor for an as yet unknown growth factor (Yamamoto et al.,

1986). Amplification and over-expression of this protein have been reported in a number of different human tumours, including breast (Walker et al., 1989), ovary (Haldane et al., 1990), bladder (Nakopoulou et al., 1992; Coombs et al., 1993), lung (Schneider et al., 1990b) and are associated with clinical outcome.

However, the role of oncogene activation in human hepatocarcinogenesis has not been fully investigated. Several experimental studies have demonstrated an increased expression of c-ras and c-myc in rat hepatic tumours and hepatoma cell cultures (Motoo et al., 1986; Fausto and Shank, 1987), but only a few studies have been done on oncogenes in human hepatocellular carcinomas (HCC) (Himeno et al., 1988; Brunt and Swanson, 1992). The epidemiological data and recent studies referring to HBV-DNA integration in HCCs leave little doubt that HBV infection is closely associated with HCC (Beasley, 1987; Matsubara, 1991). On the other hand, there is no well-established correlation between HBV integration in HCC and dominant expression of an activated oncogene (Lee et al., 1988).

The aim of this study was to evaluate the immunohistochemical expression of EGFR and c-erb-B-2 oncoprotein in a series of 71 HCC as well as in the adjacent hepatic tissue and to assess any correlation with HBsAg expression.

Materials and methods

Fifty-one surgical specimens and 20 needle biopsies were obtained from a total of 71 patients with HCC. Sixty-three patients were males and 8 were females with a mean age of 63.5 (49-93) and 80 (54-88) respectively. Sixteen liver specimens with chronic non-specific hepatitis were also included in our material and were used as controls. The histological type and grade of the HCC were assessed according to Edmondson and Steiner's (1954) criteria.

The total of the 71 HCC was classified into 17 low-grade cases (grades I and II) and 54 high-grade cases (grades III and IV) with adjacent non-neoplastic liver parenchyma observed in 14 and 28 cases respectively (Table 1).

The immunohistochemical avidin-biotin-peroxidase complex (ABC) method (Hsu et al., 1981) was performed on paraffin sections from formalin-fixed tissues for the detection of EGFR and c-erb-B-2 oncoprotein. In addition, the immunohistochemical expression of HBsAg was examined in 42 selected cases of HCC with adjacent non neoplastic tissue in order to ensure HBsAg positivity. A monoclonal antibody to HBsAg was used, kindly offered by Prof. S. Hadziyannis.

Deparaffinized and rehydrated paraffin sections were treated with methanol containing 0.3% H_2O_2 in order to block the endogenous peroxidase activity. Afterwards, the sections were incubated with the following antibodies:

1) normal rabbit or goat serum (1:20, Dakopatts Denmark) for 20 min.

2) primary antibodies: a) monoclonal mouse antibody to human $EGFR_1$ (1:80, Biomakor) overnight at 4 °C (tissue pretreatment with trypsin); b) monoclonal antibody 3 B_5 (c-erb-B-2 oncoprotein) raised against a synthesis peptide at the C-terminal end of the c-erb-B-2 gene product (1:20, Oncogene Science).

3) biotinylated rabbit to mouse IgG (1:300, Dakopatts, Denmark) for 30 min.

4) avidin-biotin-peroxidase complex (ABC) (Dakopatts, ABC kit, Denmark) for 20 min.

Sections were thoroughly rinsed with PBS (pH 7.4) between reaction steps. In all cases, antibody localization was performed with the 3,3'diaminobenzidine (DAB, Sigma Chem Co) reaction (6 mg DAB in 10 ml PBS at pH 7.4 to which 0.025 ml 30% H_2O_2 was added prior to use).

Finally, slides were counterstained with Mayer's haematoxylin, dehydrated in alcohol and mounted.

For each test, negative control studies were carried out in which rabbit serum was used instead of the primary antibody. Sections taken from known EGFR and c-erb-B-2 positive cases of breast carcinoma were used as positive controls.

The expression of EGFR and c-erb-B-2 oncoprotein was estimated with a semi-quantitative method. The statistical analysis of our results was performed using the chi-square test.

Results

The c-erb-B-2 oncoprotein was detected in 21 out of 71 cases of HCC using the monoclonal antibody $3B_5$. The antigen demonstrated a heterogeneous expression in the cytoplasmic membranes of the positive tumour cells and less frequently in their cytoplasm (Figs. 1, 2). The intensity of the membranous positive staining was greater than the cytoplasmic one, while the percentage of positive cells correlated strongly with the degree of the staining intensity. On the other hand, there was no statistically significant association ($p > 0.05$) between c-erb-B-2 expression and the histological grade of the positive tumours, since it was expressed in 7/17 low-grade and in 14/54 high-grade HCCs (Table 1). The non-neoplastic liver parenchyma, which was

Table 1. Immunohistochemical expression of c-erb-B-2 oncoprotein in hepatocellular carcinomas (HCC) and the adjacent non-neoplastic tissue.

	TUMOR TISSUE (HCC)		NON-NEOPLASTIC ADJACENT TISSUE	
HCC	No.	Positive	No.	Positive
Low grade	17	7	14	7
High grade	54	14	28	9
TOTAL	71	21	42	16

observed adjacent to 14 low grade and 28 high grade HCCs was also found to be positive to c-erb-B-2 oncoprotein in 7 and 9 cases respectively (Table 1). The c-erb-B-2 oncoprotein was also expressed in 5/16 control liver specimens with chronic non-specific hepatitis B. According to our results, the expression of c-erb-B-2 oncoprotein in HCCs was not statistically different from the one observed in the adjacent non-neoplastic parenchyma ($p > 0.05$). Moreover, there was no significant difference in the antigen expression between HCCs and the control specimens with chronic non-specific hepatitis.

In addition, HBsAg was detected in 10 out of 42 (23.8%) examined cases with adjacent liver tissue. Only 4 HCC cases were simultaneously positive to c-erb-B-2 oncoprotein and HbsAg. The low frequency of HBsAg expression in our cases may be due to the inadequate adjacent hepatic tissue included in the examined cases as well as to the heterogeneous expression of HBsAg. In addition, HBsAg did not demonstrate a constant expression in the tumour cells of HCCs.

EGFR was detected in 3/71 cases of HCC, showing a heterogeneous membranous expression, while the antigen was not detected at all in the adjacent non-neoplastic parenchyma (Fig. 3). HBsAg expression was not observed in any of the EGFR-positive HCCs.

Discussion

HCC is one of the most common cancers, particularly in Asia and Africa (Munoz and Bosch, 1987; Colombo, 1992). After the demonstration of an etiological role for HBV infection in hepatocarcinogenesis (Beasley, 1987), interest surged even in low-incidence Western countries, particularly among molecular biologists and oncologists, in order to investigate the involvement of proto-oncogenes and suppressor genes in the formation of hepatic tumours.

According to the literature, several experimental studies have demonstrated an increased expression of c-myc and c-N-ras oncogenes in rat hepatic tumours

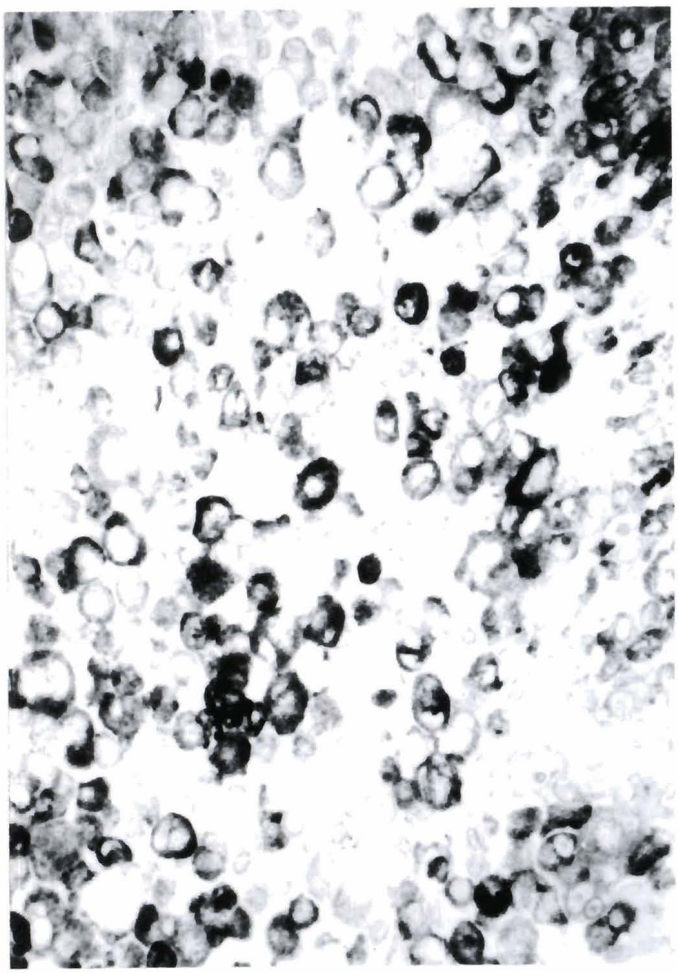

Fig. 1. Immunohistochemical expression of c-erb-B-2 oncoprotein in many tumour cells of HCC. ABC. x 300

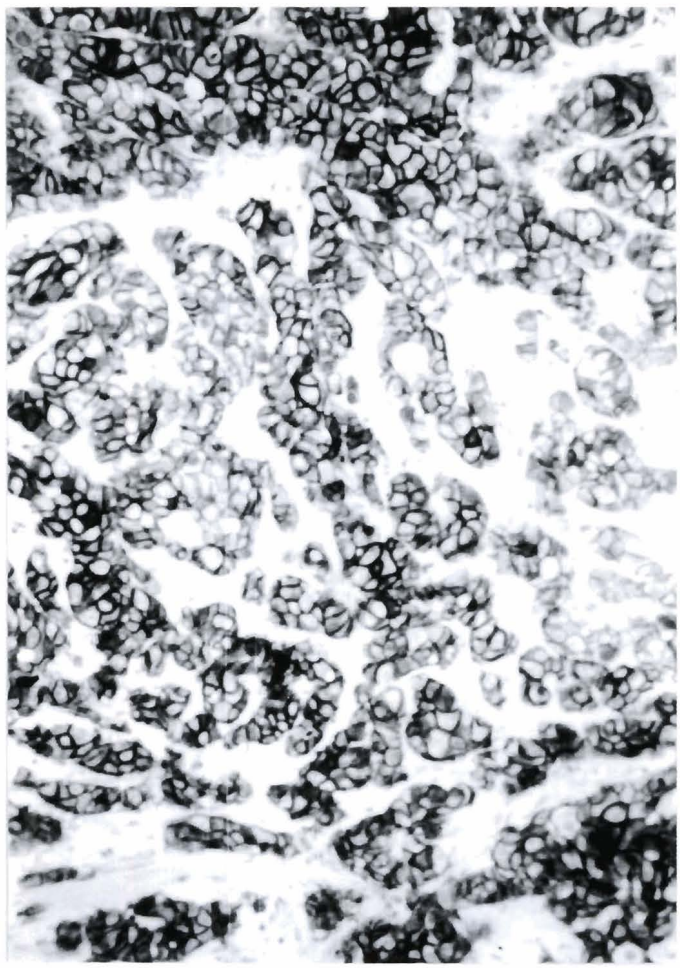

Fig. 2. Immunohistochemical expression of c-erb-B-2 oncoprotein along the cytoplasmic membranes in the majority of tumour cells in HCC. ABC. x 150

(Fausto and Shank, 1987; Lee et al., 1990) as well as in human HCCs (Zhang et al., 1990). Among the dominant transforming oncogenes studied, c-fos, c-fms, c-Ha-ras, c-sis, c-erb(A+B) also show elevated expression in certain stages of foetal liver development, hepatoma cell lines and HCCs, while c-K-ras, rel, myb, sis, mar, scr and bas are not usually expressed (Motoo et al., 1986; Zhang et al., 1987). Recent studies suggest that alterations in suppressor genes, like p53 may be important events in the transformation of hepatocytes to the malignant phenotype (Murakami et al., 1991).

Conflicting data are reported in the literature regarding c-erb-B-2 expression in human HCCs. Previous studies have demonstrated a lack of c-erb-B-2 expression in HCCs (Mori et al., 1987; Zhou et al., 1987). In contrast to the above results, the present study shows that c-erb-B-2 oncoprotein was expressed in 29.5% of the human HCCs examined. According to the statistical analysis, there was no correlation between c-erb-B-2 expression and tumour differentiation. This finding has not been previously emphasized and indicates that c-erb-B-2 expression does not seem to have a prognostic significance for HCCs as reported for other malignancies (Walker et al., 1989; Haldane et al., 1990). Moreover, the present study shows that c-erb-B-2 was expressed with the same frequency in both neoplastic and non-neoplastic surounding liver parenchyma. Our results are in agreement with those reported by Himeno et al. (1988) and Brunt and Swanson (1992). Himeno et al. (1988) did not find an increased expression of c-erb-B-2 in the HCCs since there was no significant difference in m-RNA levels of c-erb-B-2 among HCC, cirrhosis and chronic hepatitis groups. In addition, Brunt and Swanson (1992) detected c-erb-B-2 oncoprotein in only 2/8 HCC in their series by the use of a monoclonal antibody on paraffin sections. Our results, along with those of the above authors, suggest that the significance of c-erb-B-2 expression in HCCs as a true oncogenic event must be considered with trepidation.

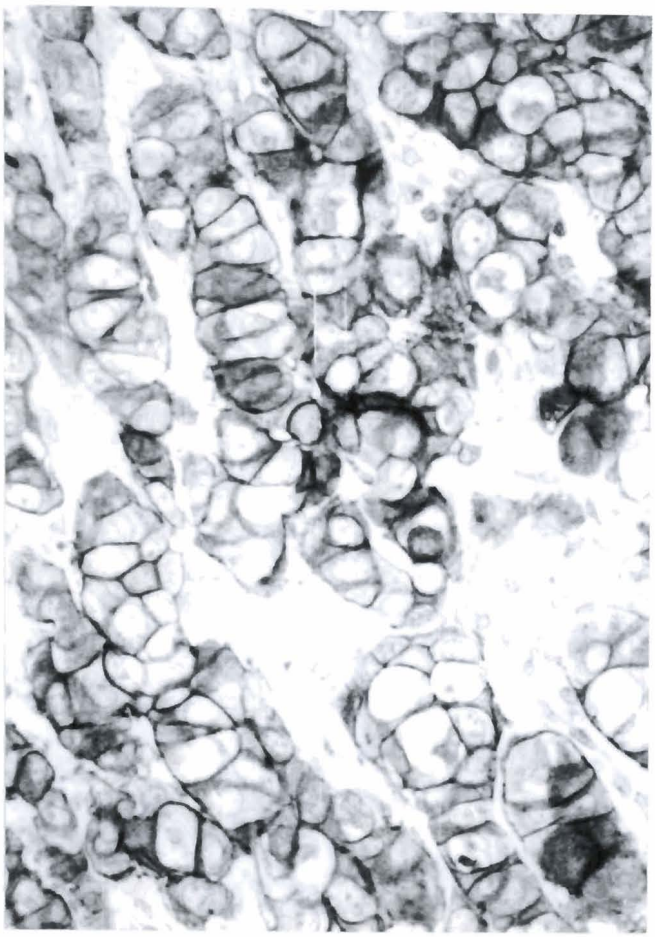

Fig. 3. Membranous expression of EGFR in tumour cells of HCC. ABC. x 400

It is well known that there is a strong association between HCC and chronic infection with hepatitis-B-virus (Beasley, 1987). Several studies have shown that HBV DNA is integrated in the genome of many HCCs developing in patients with chronic HBV infection (Matsubara, 1991). Despite early expectation of a common target sequence near an oncogene or a suppressor gene, no consistent site of integration has yet been found (Matsubara, 1991). Since HBV does not carry its own oncogenes, it may activate the expression of a neighbouring cellular oncogene or might disrupt a cellular regulator gene.

A number of studies have shown activated oncogenes dominantly expressed, such as hst, lca, and ras family genes (Yang et al., 1988) while scattered observations suggest rearrangement or myc and H-ras (Chandar et al., 1989). Unfortunately, up to date, no predominant oncogene has been found.

The presence of the HBV was detected in 23.8% of HCC cases. It is likely that this percentage understimates the true frequency of HBV in these tumours for a number of reasons such as: 1) insufficient tissue availability for immunohistochemical evaluation; 2) due to the heterogeneity of HBsAg in liver tissue it is possible that areas with positive expression were not available for interpretation; 3) as a method for HBV detection, immunohistochemistry is less sensitive than others such as polymerase chain reaction (PCR), which can detect a single copy of HBV (Shih et al., 1990).

According to our results, no significant correlation between c-erb-B-2 expression and HBsAg detection was observed. Our results are further supported by the data of Motoo et al. (1986) and Lee et al. (1988) which suggest that there is no evidence for HBV DNA integration adjacent to other known cellular oncogenes. In addition, Lee et al. (1988) suggest that continued expression of HBsAg or the presence of HBV DNA RNA in human HCC cells is not necessary for enhanced expression of ras or c-myc proteins.

Recent immunohistochemical data indicate that over production of c-erb-B-2 oncoprotein in few cases of

HBV- or HCV-related cirrhosis or submassive necrosis may be an epiphenomenon of hepatitis B or C virus infection, since it was observed only in bile ducts and not in hepatocytes (Brunt and Swanson, 1992).

It is interesting that in our series EGFR was rarely expressed in the HCCs and the adjacent hepatic tissue. To our knowledge, Fukusato et al. (1990) have been the only investigators to report a high frequency of EGFR immunohistochemical expression in a series of 11 HCCs. The discrepancy between our results and theirs may be due to the different reagents and methods used as well as to the small number of HCCs studied by the above authors. On the other hand the structural and functional similarities of c-erb-B-2 oncoprotein and EGFR which are members of the type 1 growth factor receptor family, may account for their analogous expression in various malignancies.

The low frequency of c-erb-B-2 expression in HCCs of this series in combination with the data reported by other authors (Mori et al., 1987; Zhou et al., 1987; Brunt and Swanson, 1992) support the above notion.

In conclusion, the absence of a significant correlation between c-erb-B-2 expression and the degree of HCC differentiation suggests that c-erb-B-2 cannot be used as an indicator of biological aggressiveness in HCCs.

Moreover, the limited expression of c-erb-B-2 oncoprotein and EGFR in HCCs indicates that these two oncogene products may not be predominantly involved in the transformation of hepatocytes to the malignant phenotype.

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