Rarity of EGFR and c-ErbB-2 Overexpressions in Hepatocellular Carcinoma: An Immunohistochemical Study

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Background: The overexpression of epidermal growth factor receptor (EGFR) and c-erbB-2 oncoproteins has been implicated in the development of many types of cancer. However, the role of EGFR and c-erbB-2 overexpression in hepatocellular carcinoma (HCC) has not been fully elucidated.

Methods: The aim of this study was to evaluate the immunohistochemical expression of EGFR and c-erbB-2 oncoprotein in a series of 52 HCCs.

Results: All but one of the HCC tumor tissues were negative for EGFR monoclonal antibody, clone H11. All of the HCC tumor tissue samples were negative for EGFR monoclonal antibody, clone 29.1.1. However, strong EGFR immunoreactivity was detected in sinusoidal endothelial cells of HCC in 25 tumors (48%) using EGFR 29.1.1 antibody. The expression of c-erbB-2 was observed in 6% (3/52) of the HCCs. No significant correlation was found between p53 mutation and the expression of c-erbB-2.

Conclusion: Our results suggest that both EGFR and c-erbB-2 oncoprotein overexpressions in tumor cells are rare and do not seem to predominantly contribute to the malignant phenotype in HCC.

Key Words: Carcinoma, Hepatocellular, Receptor, Epidermal Growth Factor, erbB-2 Immunohistochemistry

The epidermal growth factor receptor (EGFR) family includes four homologous transmembrane receptor protein kinases: EGFR, c-erbB-2, c-erbB-3, and c-erbB-4. This receptor family plays a crucial role for regulating cell proliferation, differentiation and transformation, and particularly for the transmission of growth signal transduction in cells. Ligand-induced activation of these receptors results in formation of homo- and heterodimers that undergo transphosphorylation and transactivation.1-3 The overexpression of EGFR and c-erbB-2 has been found in a variety of human malignancies and their role in the development and progression of cancer has been described.2,4-6 Accumulating evidences have demonstrated that anti-EGFR or anti-c-erbB-2 therapies are effective in several types of cancer.7-10 However, the role of EGFR and c-erbB-2 overexpression in hepatocellular carcinoma (HCC) is still controversial.11-15 Several studies have also demonstrated an increased expression of EGFR and c-erbB-2 in human HCC.11,14,15 On the other hand, other studies have demonstrated a lack of correlation between HCC development and EGFR or c-erbB-2 overexpression.12,13

Wild-type p53 is a tumor suppressor gene product that blocks progression of cells through the cell cycle.16 It has been shown that p53 can either activate or suppress the activity of a number of target genes. Wild-type p53 contributes to growth inhibition by regulating, directly or indirectly, the expression of genes that are required for ongoing proliferation.17 A mutation in the p53 gene often results in a prolonged half-life of the protein compared to wild type, and the loss of normal functions.18 Therefore, positive nuclear staining suggests mutated or overexpressed p53. These findings stimulated our study to investigate the expression and cellular localization of EGFR and c-erbB-2 by using immunohistochemistry in resected human HCC specimens. In addition, we examined the relationship between overexpression of p53 by using immunohistochemistry, and the expression of EGFR and c-erbB-2 in HCC.
MATERIALS AND METHODS

Patients and specimens

The Human Ethics Committee of Chonbuk National University Medical School approved this study. We retrospectively studied the HCC specimens obtained from 52 patients who were treated between 1990 and 1999 at the Chonbuk National University Hospital. All patients underwent surgical resection of their tumors. Of the 52 patients with HCC, 40 were men and 12 were women, and the mean age of the patients was 54 years (range, 29-76 years). HBV and HCV serologies were positive in 31 and 5 patients, respectively. The non-tumorous liver tissue was composed of 25 cirrhotic specimens and 9 chronic hepatitis specimens.

Immunohistochemistry

For the p53 immunohistochemical staining, the immunoperoxidase method was used with the Streptavidin-biotinylated horseradish peroxidase complex (DAKO, Carpinteria, CA, USA). Sections 4 \( \mu m \) thick were cut from the formalin-fixed paraffin-embedded tissue blocks. After deparaffinization, they were incubated in methanol containing 0.3% hydrogen peroxide at room temperature for 20 min to block endogenous peroxidase, and then they were treated with a microwave antigen retrieval procedure in 0.01 M sodium citrate buffer for 10 min. After blocking the endogenous biotin, the sections were incubated with Protein Block Serum-Free (DAKO, Carpinteria, CA, USA) at room temperature for 10 min to block nonspecific staining and then incubated for 2 h at room temperature with anti-p53 antibody (DAKO, Carpinteria, CA, USA). After washing, the sections were incubated with a biotin-conjugated secondary antibody at room temperature for 30 min, and they were finally incubated with peroxidase conjugated streptavidin at room temperature for 30 min. Peroxidase activity was detected with the enzyme substrate 3-amino-9-ethyl carbazole. For EGFR and c-erbB-2 staining, the DAKO Envision system, employing dextran polymers conjugated with alkaline phosphatase, was used to avoid endogenous biotin contamination. After deparaffinization, the slides were treated with the microwave antigen retrieval procedure or proteinase K treatment for EGFR, Clone H11 (a monoclonal antibody obtained by a combined immunization with the micorsomal membrane of HC2 20 d2 xenograft cells and a 14 amino acid EGFRvIII-specific synthetic peptide, DAKO, Carpinteria, CA), EGFR, Clone 29.1.1 (a monoclonal antibody raised on human carcinoma cell line A431, Sigma, St. Louis, MO, USA) and c-erbB-2, (C-18, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 10 min was performed. After blocking the endogenous alkaline phosphatase, the sections were incubated for 2 h at room temperature with the above antibodies. Fast red solution was used for the localization of the antibodies. For the negative controls, the sections were treated the same way except they were incubated with Tris buffered saline instead of the primary antibody. Sections taken from known EGFR and c-erbB-2 positive cases of colon carcinoma were used as positive controls. Immunostaining was scored as follows: score 0, undetectable staining or membrane staining in <10% of the tumor cells; score 1+, weak membrane staining detected in >10% of the tumor cells; score 2+, weak to moderate complete membrane staining observed in >10% of the tumor cells; score 3+, strong complete membrane staining observed in >10% of the tumor cells. Samples with staining of at least 10% of the tumor cells were defined as a positive case. Stains for EGFR and c-erbB-2 protein were interpreted as "positive" only if membrane accentuation was obtained.

Statistical analysis

An association between the expression of c-erbB-2 and p53 protein overexpression was evaluated for by chi-square testing. A p value \( \leq 0.05 \) was considered significant.

RESULTS

The results are summarized in Table 1. The EGFR was detected in 1 out of 52 cases of HCC using the monoclonal antibody

<table>
<thead>
<tr>
<th>Name of antibodies</th>
<th>Number of positive cases/ Number of total cases</th>
<th>Tumor cell reactivity</th>
<th>Non-tumorous elements stained</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFR, Clone H11</td>
<td>1/52</td>
<td>Diffuse, 2+, cytoplasmic with membrane accentuation</td>
<td>Sinusoidal endothelial cells of HCC (48%) and cirrhotic liver (16%)</td>
</tr>
<tr>
<td>EGFR, Clone 29.1.1</td>
<td>0/52</td>
<td></td>
<td></td>
</tr>
<tr>
<td>c-erbB-2</td>
<td>3/52</td>
<td>Diffuse, 2+, membranous</td>
<td></td>
</tr>
</tbody>
</table>
H11 (Fig. 1A). All of the HCC tumor tissue samples were negative for EGFR, clone 29.1.1 expression. However, strong EGFR immunoreactivity was detected in the sinusoidal endothelial cells of HCC in 25 (48%) tumors and in 4 cirrhotic liver specimens (16%) using monoclonal antibody 29.1.1 (Fig. 1B). Only 3 cases of 52 (6%) HCC specimens showed positive staining for c-erbB-2. The c-erbB-2 was diffusely expressed in the cytoplasmic membranes of positive tumor cells and less frequently, in their cytoplasm (Fig. 1C). Non-neoplastic hepatocytes and bile ducts adjacent to the tumor also were negative. All the positive and negative controls stained appropriately (Fig. 1D). P53 overexpression was observed in 17 of 52 tumors (33%), and the staining was nuclear in all positive cases. We could not find a significant correlation between c-erbB-2 expression and p53 overexpression (p=0.98).
DISCUSSION

It has been reported that 47-68% and 30-86% of human HCC tissue samples overexpress the EGFR and c-erbB-2 protein as demonstrated by immunohistochemistry, respectively. Voravud et al. have reported that the expression of c-erbB-2 was observed in 86% (12/14) of HCC using rabbit polyclonal anti-c-erbB-2 antibody (ICRF, Molecular Oncology Laboratory, Hammersmith Hospital). Nakopoulou et al. have reported that the expression of c-erbB-2 was observed in 30% (21/71) of HCC using monoclonal antibody 3 B5 raised against a synthesis peptide at the C-terminal end of the c-erbB-2 gene product (Oncogene Science). The above studies suggested the possible role of EGFR and c-erbB-2 for the development or progression of human HCC. In contrast to the above results, the present study showed that all but one of the HCCs was negative for EGFR when using two different EGFR monoclonal antibodies. Only three cases out of 52 specimens of HCC overexpressed c-erbB-2. Our findings indicate that EGFR and c-erbB-2 overexpression is rare in HCC. Our results are in agreement with those results recently reported by others. Prange and Schirmacher have demonstrated the lack of c-erbB-2 overexpression in HCC. The other studies have reported only 2.8% (1/36) and 7.7% (2/26) of HCC overexpressed c-erbB-2 as evidenced by membrane staining. Moreover, c-erbB-2 mRNA was not detected in the seven HCCs examined by Northern blot analysis. Only one of the 12 HCC cell lines revealed a significant level of c-erbB-2 expression by Western blotting. Similarly, Nakopoulou et al. have reported that only 3 cases out of 71 cases of HCC showed heterogenous membrane expression of EGFR by using mouse monoclonal anti-EGFR antibody (Biomaker). In our study, the presence of cell membrane staining was considered an essential criterion for the identification of EGFR or c-erbB-2 overexpression, and the dextran polymer method was used to avoid endogenous biotin contamination. The previous studies did not distinguish between cytoplasmic and membrane staining, or details of the scoring criteria used in their study were not provided. The significance of cytoplasmic staining without membrane immunoreactivity for EGFR or c-erbB-2 remains unclear. However, previous studies have suggested that a membrane staining using immunohistochemistry more accurately reflected the expression of HER-2/neu oncoprotein on cell membrane in a breast biopsy specimen. The membrane staining was never found in breast tumor specimens with a normal c-erbB-2 oncogene copy number. The discrepancy of the incidence of overexpression of EGFR and c-erbB-2 may be due to the different criteria for positivity, and the different antibodies and detection methods used. Our results, along with those of the above authors, suggest that the significance of EGFR and c-erbB-2 expression in HCCs as a true oncogenic event must be considered with trepidation. This negative result occurring with the use of specific monoclonal antibodies in an established immunohistochemical method for the analysis of EGFR and c-erbB-2 does not exclude the presence of these receptors in HCC, but it does suggest that if present, they occur in much smaller amounts than in colon or gastric cancer tissue.

Our study showed strong EGFR immunoreactivity to monoclonal antibody 29.1.1 in sinusoidal endothelial cells of HCC and those of regenerating nodules, but there was no immunoreactivity to monoclonal antibody H11. The reasons for the different expression patterns of EGFR when using different EGFR antibodies are unclear. The different antibodies used in this study, which detect different epitopes, may explain the apparent discrepancy in different localization of EGFR. Ross et al. have shown that increased angiogenic growth factor receptors in endothelial cells such as VEGF receptor, angiopoietin receptors, platelet-derived growth factor receptor beta and epidermal growth factor receptor during the revasculization of regenerating rat liver. Strong immunoreactivity in vascular endothelial cells of meningioma has been reported using same monoclonal antibody for EGFR, and the researchers suggested that EGFR might participate in angiogenesis of meningioma. When taken together, our results suggest that the EGFR expression in tumor endothelial cells may play a role in tumor angiogenesis in HCC. Additional investigations are necessary to determine the relationship between EGFR expression for tumor endothelium and angiogenesis. Finally, our current study did not demonstrate a statistically significant association between p53 overexpression and expression of c-erb-B2 in HCC tissue specimen.

In conclusion, our results suggest that both EGFR and c-erbB-2 overexpression do not seem to predominantly contribute to the malignant phenotype in HCC.

REFERENCES


