Amplification and overexpression of the c-erbB-2 protooncogene in human gastric cancer

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Summary: The c-erbB-2 protooncogene encodes a possible growth factor receptor. This gene has been studied as to whether it can be regarded as a prognostic indicator in human breast carcinoma. As amplification and overexpression of the gene have been reported in several adenocarcinomas, 24 specimens of human gastric cancers were examined by immunohistochemical staining (24 cases), by Southern blotting (23/24) and by Northern blotting (16/24). Amplification of the gene was detected in two moderately differentiated tubular adenocarcinomas (8.7%), and overexpression of c-erbB-2 mRNA was detected in three moderately differentiated tubular adenocarcinomas (18.8%). By immunohistochemical staining of paraffin-embedded tissues using a polyclonal antibody to c-erbB-2 gene products, the cell membrane was stained positively in three cases of gastric cancers which overexpressed c-erbB-2 mRNA. Peritoneal metastases were found in six gastric cancers, including two moderately differentiated tubular adenocarcinomas in which amplification of c-erbB-2 occurred. These results suggest that amplification and overexpression of c-erbB-2 may be correlated with metastases in differentiated adenocarcinoma of the stomach. Gastroenterol Jpn 1992;27:172-178.

Key words: c-erbB-2; gastric cancer; metastasis; protooncogene

Introduction

Recently, various factors have been examined to determine their influence on the prognosis of gastric cancer, however, accurate determinants of prognosis have not been established. In recent years, more than 50 protooncogenes have been identified, which can be classified into two groups. One group contains self-activated protooncogenes involved in tumorigenesis, while the other group contains oncogenes which belong to the growth factor-receptor system and are involved in the proliferation of cancer cells. The former includes N-myc in neuroblastoma¹ and the latter includes c-erbB-2 in breast carcinoma². Both protooncogenes have been reported as possible prognostic indicators of cancer. The autocrine theory which proposes that cancer cells produce growth factors, which interact with their own receptors has been reported³. Furthermore, overexpression of growth factor receptors in various human carcinomas has also been reported⁴,⁵.

We analyzed the amplification and overexpression of the c-erbB-2 gene, which is one of the growth factor receptors, in 24 human gastric cancers, in order to determine its effect on the prognosis of human gastric cancer.

Materials and Methods

Tissue specimens

Tissues from 24 primary gastric cancers were collected during surgery at Dokkyo University Hospital, School of Medicine. Specimens from
cancer tissues and adjacent normal gastric mucosa were obtained simultaneously. Part of the tissue was formalin-fixed and paraffin-embedded, and the remainder was frozen immediately and stored in liquid nitrogen.

**DNA probes**

The 3.4 kbp DNA fragment of pCER2046, containing cDNA of the c-erbB-2 gene, obtained from the Japanese Cancer Research Resources Bank (JCRB) was labelled with 32P-deoxycytidine 5'-triphosphate and random primer. As internal markers, UDh DNA, which was cloned from human placenta and β-actin DNA, was used for Southern and Northern blot analysis.

**DNA preparation and Southern blotting**

Frozen tissues were ground in liquid nitrogen and digested with 200 μg/ml proteinase K in lysis buffer containing 2% sodium dodecyl sulfate. Then DNA was purified by phenol-chloroform extraction and precipitated with ethanol. DNA samples (8 μg) were digested with EcoRI and electrophoresed through 0.8% agarose gels. The DNA fragments were transferred to nitrocellulose filters as described. The blotted filters were baked at 80°C for 2 hours. Then hybridization and autoradiography were carried out as described previously.

**RNA preparation and Northern blotting**

Frozen tissues were ground in liquid nitrogen and lysed quickly in 4 M guanidinium thiocyanate/25 mM sodium citrate/0.5% sarcosyl/0.1 M 2-mercapto ethanol. The lysate was homogenized and DNA was cut into small fragments using a syringe (21 G). Then 1/10 volume of 2 M sodium acetate (pH 4.0), one volume of phenol and 1/5 volume of chloroform-isooamyl alcohol (49:1) were added to the lysate and mixed by inversion. After incubation on ice for 15 minutes, debris was removed by centrifugation at 10,000 rpm and RNA in the supernatant was precipitated with ethanol. RNA (8 μg) was electrophoresed through 1.0% agarose gels in 3-(N-morpholino)propanesulfonic acid buffer containing 5% formaldehyde and transferred to nitrocellulose filters.

**Hybridization**

For Southern and Northern blot analyses, high stringency hybridization was conducted in the hybridization buffer containing 50% formamide and 5X SSC (1X SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 42°C for 24 hours. For rehybridization of the filter, 32P-labeled c-erbB-2 probe on a filter was stripped off by submerging the filter in boiling water for 5 minutes. Then the filter was rehybridized with the probe for internal markers, UDh and β-actin.

**Immunohistochemical staining**

The avidin-biotin complex (ABC) immunoperoxidase assay was performed on 4 μm sections from a formalin-fixed, paraffin-embedded tissue using an ABC kit (Vector Laboratories, Burlingame, USA) following the manufacturer's instructions. The rabbit polyclonal antibody to c-erbB-2 gene products (pAb1, Triton Biosciences Inc. Alameda, USA) was used as a primary antibody. A range of dilutions of the primary antibody was tested using 10 tumor specimens and a 1:15 dilution of the antibody was chosen in this study. For negative controls, serial sections were treated with normal rabbit serum instead of the primary antibody. In order to block endogenous avidin-binding activity (EABA), we used EABA-blocking kit (Vector Laboratories, Burlingame, USA). The nuclei were counterstained in hematoxyline to examine the nuclear grading.

**Results**

**Amplification of the c-erbB-2 gene in primary gastric cancers**

DNA isolated from 23 gastric cancers was digested with EcoRI and analyzed by Southern blotting using the c-erbB-2 probe (Table 1). As shown in Figure 1, DNA fragments of 16, 7.5, 6.4 and 4.4 kbp were detected in all samples. In two gastric cancers, nos. 15 and 20, amplification of the c-erbB-2 gene, ranging from 3- to 10-fold, was detected. In these amplified samples, the 1.6 kbp small DNA fragment could be detected in addition to the four DNA fragments. There five DNA fragments observed in our analysis corresponded