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The Mechanism of c-erbB-2 Gene Product Increase in Stomach Cancer Cell Lines[1]

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c-erbB-2 oncogene encodes a growth factor receptor whose amino acid sequence has exensive homology with human epidermal growth factor receptor. It is frequently overexpressed in human breast, ovary, lung, and stomach cancers, where its overexpression is related significantly to the prognosis. To investigate the possible role of c-erbB-2 oncogene in the oncogenesis of stomach cancer, we examined the genetic alterations of c-erbB-2 oncogene in 4 stomach cancer cell lines, SNU-1, SNU-5, SNU-16 and KATO III. There were no differences in c-erbB-2 mRNA level as well as c-erbB-2 gene copy number among them. But gp185^{erbB-2}, c-erbB-2 gene product, was increased from 2-to 4-fold in SNU-1 and SNU-5 cells, compared with that in SNU-16 or KATO III cells. Our results suggest that post-transcriptional regulation of gp185^{erbB-2} expression may underlie gp185^{erbB-2} overexpression in cancer cells.

Key Words: *c-erbB-2 oncogene-stomach cancer-post-transcriptional regulation*

INTRODUCTION

c-erbB-2 is a growth factor receptor oncogene which encodes a 185kd glycoprotein, gp185^{erbB-2}, with tyrosine kinase activity (King et al., 1985; Schechter et al., 1984; Bargmann et al., 1986, b; Yamamoto et al., 1986). gp 185^{erbB-2} has some functional relationships to as well as the extensive amino acid sequence homology with human EGF receptor (Akiyama et al., 1986; Wada et al., 1990; Quian et al., 1992). However, neither EGF nor TGF-α binds to gp185^{erbB-2} (Bargmann et al., 1986a), and the physiologic role of gp185^{erbB-2} is still obscure because the putative ligands of gp185^{erbB-2} have been discovered very recently (Holmes et al., 1992; Peles et al., 1992).

c-erbB-2 oncogene can transform NIH/3T3 cells when overexpressed but the level of expression is critical for transformation (Di Fiore et al.,. 1987). Transforming potential of c-erbB-2 oncogene is totally dependent upon tyrosine kinase activity as in other tyrosine kinase family oncogenes (Weiner et al., 1989). The increase of tyrosine kinase activity of c-erbB-2 can be mediated by three different mechanisms; N-terminal truncation, shown in v-erbB, viral counterpart of EGF receptor (Downward et al., 1984; Ullrich et al., 1984), point mutation in transmembrane domain, shown in neu oncogene, rat homologue of c-erbB2- gene (Bargmann et al., 1986) and mRNA overexpression. The first two mechanisms, however, have not yet demonstrated in primary human tumor (Hall et al., 1990; Lemoine et al., 1990). The only mechanism of c-erbB-2 tyrosine kinase activation observed in primary tumor is an increase in the total amount of tyrosine kinase activity, that is, increase in gp185^{erbB-2} amount.

Overexpression of gp185^{erbB-2} is observed in 10~40% of the carcinomas of the breast (Slamon et al., 1987; 1989; Ali et al., 1988; Bacus et al., 1990; Paterson et al., 1991), ovary (Berchuck et al., 1990; Slamon et al., 1989; Haldone et al., 1990), lung (Weiner et al., 1990; Kern et al., 1990; Tateishi et al., 1991) and in about 10% of gastric cancers (Slamon et al., 1987; Ali et al., 1988; Park et al 1989; Yokota et al., 1989;

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Jain et al., 1991; Yonemura et al., 1991a; 1991b; Lemoine et al., 1991). The mechanism of gp185^{erbB-2} increase in cancer tissues was known to be mRNA overexpression with or without gene amplification (Slamon et al., 1987; 1989). The nucleotide sequence of overexpressed c-erbB-2 mRNA has been thought to be unchanged. There were no sequence differences between cloned cDNA from primary tumor tissue and that of placenta (Slamon et al., 1989), and no mutations in transmembrane domain could be found in 100 breast cancer cases by PCR analysis (Lemoine et al., 1990).

The overexpression of c-erbB-2 oncogene acts as an independent prognosis factor in the carcinomas of the breast and the ovary (Slamon et al., 1989; Berchuck et al., 1990). In brest cancers, particularly in node-positive adenocarcinomas, gp185^{erbB-2} increase correlated with recurrence rate and poor prognosis (Slamon et al., 1987; 1989; Borg et al., 1990). There are some reports indicating that c-erbB-2 overexpression may predict bad prognosis in stomach cancer, too (Yonemura et al., 1991a; 1991b).

Overexpression of c-erbB-2 oncogene is about 10% in stomach cancers, not as high as in breast or ovarian tumors but is still the genetic alteration of oncogene found most frequently in stomach cancers. We have sought c-erbB-2 gene amplification or overexpression in the stomach cancers in Korea but barely detected c-erbB-2 gene overexpression. It is not obvious whether our previous results indicate the genuine low incidence of the genetic alterations of c-erbB-2 gene in Korea or result from dilution of cancer cells due to contamination of normal cells or stroma. So, we examined 4 stomach cancer cell lines, SNU-1, SNU-5, SNU-156 and KATO III (Park et al., 1987; 1990), for investigating what role c-erbB-2 oncogene may play in the carcinogenesis of stomach cancers.

MATERIALS AND METHODS

Cell lines

Stomach cancer cell lines, SNU-1, SNU-5 and SNU-16, established by Prof. J.G. Park (Park et al., 1987; 1990), were analyzed. SK-BR-3 (a human breast cancer cell line), KATO III (a human stomach cancer cell line) were obtained frm ATCC.

Southern hybridization

The genomic DNA was purified from each cell lysate by proteinase K digestion followed by phenol-chloroform extractions and ehtanol precipitation. The genomic DNA was fractionated through a 0.8% agarose gel after EcoRI digestion and transferred to Hybond N membrane as described by Maniatis (1989). The blot was hybridized with ^{32}P-labeled 1.6 Kbp internal EcoRI fragment of c-erbB-2 cDNA at 42°C for 16 hors under 10% dextran sulfate. After hybridization, the blot was washed and exposed to Kodak X-Omat AR film at-70°C for 1 to 3 days.

Northern hybridization

Total cellular RNA, extracted by guanidinium/CsCl method, was separated on a 1% denaturing agarose gel containing 20% formaldehyde, and transfered to Hybond N+ membrane. The blot was hybridized and washed as described in southern hybridization.

Anti-gp185^{erbB-2} sera preparation

The anti-gp185^{erbB-2} antibody was raised from rabbits using two oligopeptide antigens. The oligopeptide antigens were kindly provided by Prof. P. G. Suh of POSTECH. One composed of 15 amino acid residues from 865th to 879th (sequence: LARLLDIDETEYHAD) in the tyrosine kinase domain of gp185^{erbB-2} and the other composed of 15 residues from 1241st to 1255th (sequence: PTAENPEYLGLDVPV) at the carboxy terminal. The oligopeptides were coupled to keyhole limpet hemocyanin by methylmaleidosuccinic acid and injected into rabbits subcutaneously. After 4 boost injections at 1 month's interval, sera were collected and evaluated by immunoprecipitation.

Western blot

Cells were lysed with RIPA buffet (50mM HEPES, pH7.0, 150mM NaCl, 0.1% phenylmethylsulfonylfluoride (PMSF), 0.1μg/ml aprotinin), and the protein concentration of each cell lysate was determined by BCA method. An aliquot of each cell lysate containing the same amount of protein was separated on an 8% polyacrylamide gel containing SDS and electroblotted to nitrocellulose paper. The blot was blocked with non-fat dried milk and probed with anti-gp185^{erbB-2} rabbit serum. Detection of gp185^{erbB-2} was carried out using HRP-conjugated anti-rabbit IgG F (Ab)' fragment antibody and ECL-western blotting system (Amersham Co.).

RESULTS

Comparison of c-erbB-2 gene copy number

The genomic DNAs extracted from 4 stomach cancer cell lines, SNU-1, SNU-5, SNU-16, KATO III, were digested completely with EcoRI and blotted. Sub-

sequent hybridization with ^{32}P-labeled c-erbB-2 cDNA showed 2 fragments of similar densities. 4.4kb and 6.1kb, in all 4 cell lines, which were reported to be hybridized to internal EcoRI fragment of c-erbB-2 cDNA (Fig. 1-B). Copy number of c-erbB-2 cDNA in SNU-1 cells was not differnt from that of placenta (Fig. 1-A).

For quantiation of c-erbB-2 gene copy number, the genomic DNA from 4 cell lines was dotblotted and hybridized with β-actin cDNA for amount control and c-erbB-2 cDNA sequentially (Fig 2). The amount of blotted DNA was equal (Fig. 2-A) in all 4 lanes and no difference of c-erbB-2 gene copy number was detected (Fig. 2-B). This result indicated that c-erbB-2 gene copy numbers in these stomach cancer cells were not different.

Comparison of c-erbB-2 mRNA expression

We examined c-erbB-2 expression level because c-erbB-2 oncogene could be overexpressed without amplification of the gene (Slamon et al., 1989). The total cellular RNA from 4 stomach cancer cell lines was analyzed by northern blot. The sizes of c-erbB-2 mRNs were about 4.6kb as reported (Yamamoto et al., 1986) in all cell lines. In comparison with autoradiogram obtained by hybridization with ^{32}P-labeled β-actin probe (Fig. 3-B), the amount of the c-erbB-2 message was similar among cell lines (Fig. 3-A). So we concluded that the amounts of c-erbB-2 mRNA were almost the

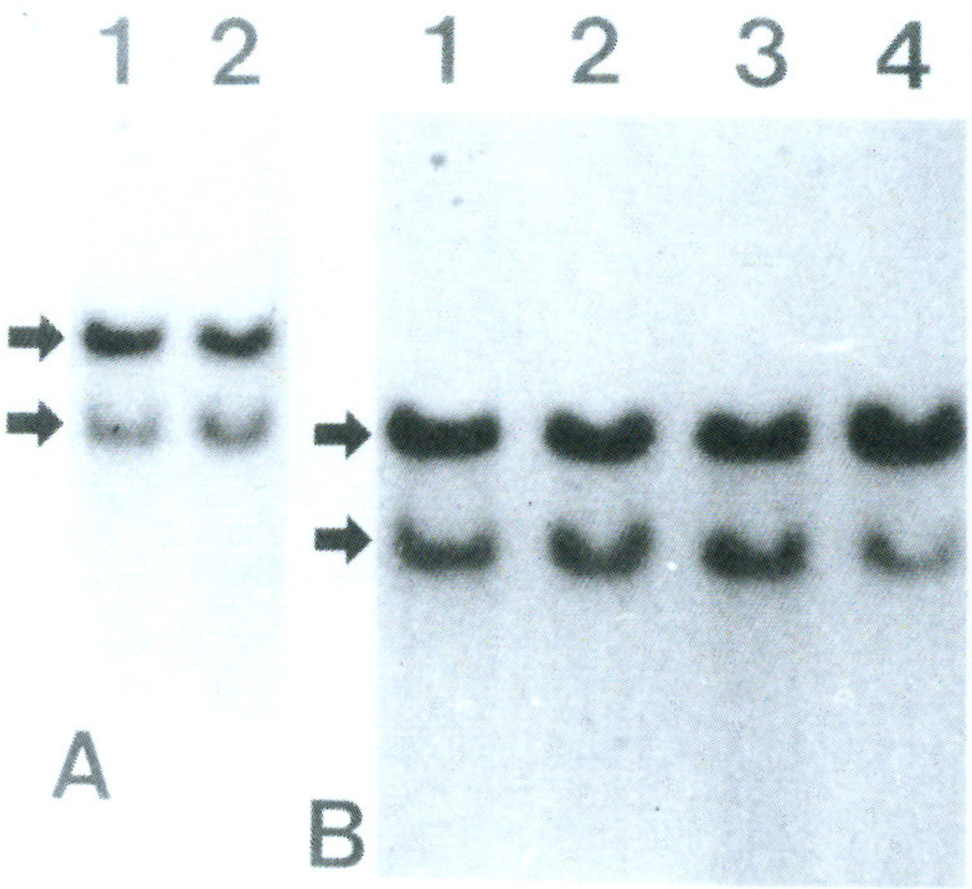
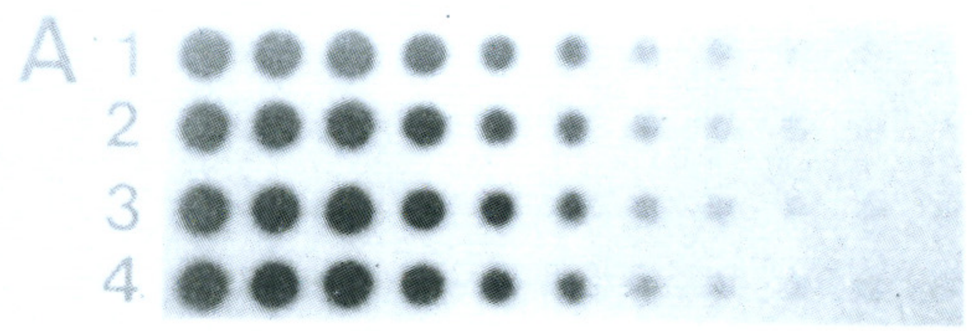


Fig. 1. Southern blot analysis of genomic DNA. Twenty-five micrograms of high molecular weight DNA, extracted from each cell line, were digested with EcoRI and analyzed by southern hybridization as described in Mechods, A: lane 1; placenta, lane 2; SNU-1, B: lane 1; SNU-1, lane 2; SNU-5, lane 3; SNU-16, lane 4; KATO III.



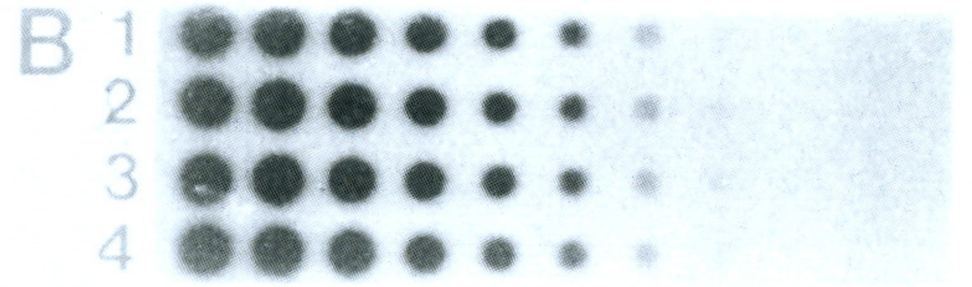


Fig. 2. Dot blot analysis of c-erbB-2 gene. Fifty micrograms of high molecular weight DNA from each cell line were fragmented by sonification and analyzed by dot-blot. The blot was hybridized random primed β-actin (2-A) or c-erbB-2 (2-B) probe. lane 1: SNU-1, lane 2: SNU-5:, lane 3: SNU-16, lane 4: KATO III.

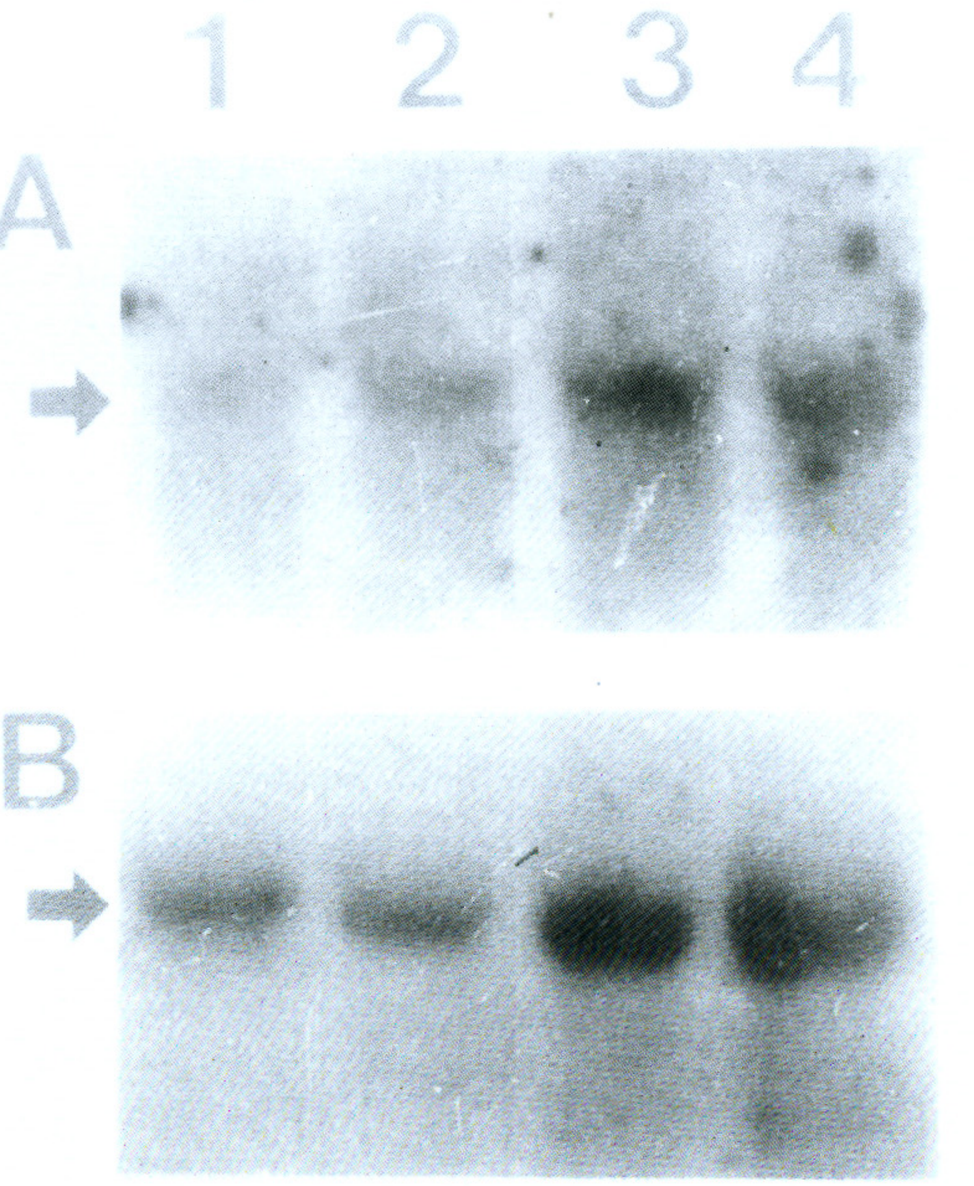


Fig. 3. Comparison of c-erbB-2 mRNA amount. Total cellular RNA was isolated by guanidinium/CsCl method and blotted on Hybond N+following separation on formaldehyde agarose gel electrophoresis. The blot hybridized ^{32}P-labeled c-erbB-2 probe (2-A) and then β-actin probe (2-B) after stripped. Lane 1: SNU-1, lane 2: SNU-5, lane 3: SNU-16, lane 4: KATO III.

same in all 4 stomach cancer cell lines.

Characterization of anti-gp185^{erbB-2} polyclonal sera

To investigate c-erbB-2 protein level, we decided to make anti-gp185^{erbB-2} polyclonal antibody. Two oligopeptide antigens, prepared as described in methods, were injected into rabbits. We could obtain six anti-gp185^{erbB-2} sera; two (PAb-TK-1, 2) of anti-tyrosine-kinase-domain of gp185^{erbB-2}, two (PAb-CT-1,2) of anti-C-terminal-end and the other (PAb-TC-1, 2) of antisera for both. For the titration of anti-gp185B^{erbB-2} antibody, immunoprecipitation was done (Fig. 4). PAb-TC-1 serum (Fig. 4-E) could detect gp185^{erbB-2} even dilution by the factor of 80 without the loss of sensitivity. So, we decided to use PAb-TC-1 serum for the following experiments.

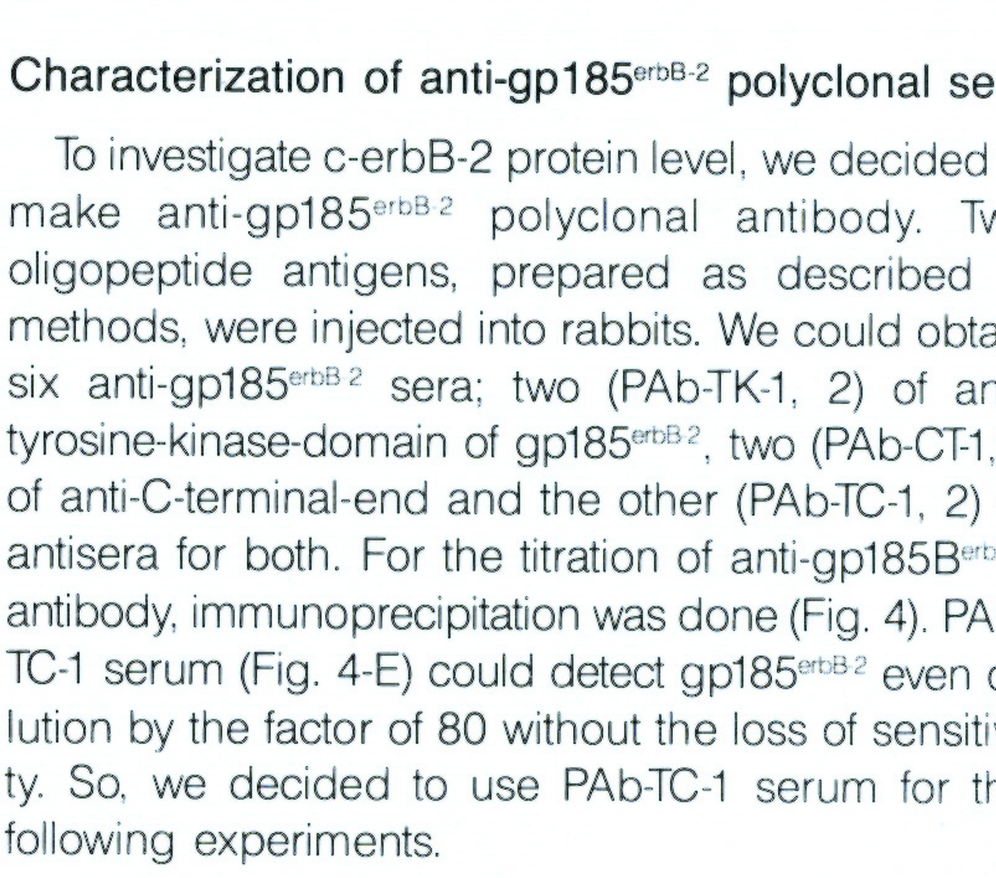


Fig. 4. Titration of anti-gp185^{erbB-2} Ab in polyclonal sera. Protein extracted from SNU-1 cells is immunoprecipitated with 6 polyclonal rabbit sera. Each immunoprecipitation reaction contained 50μg of protein and the dilution fold of sera was as indicated. Immune complexes were precipitated with heat-killed Staphylococcus aureus and washed 3 times with RIPA buffer (50mM HEPES, pH 7.4, 150mM NaCl, 1% NP-40). A: PAb-TK-1, B: PAb-TK-2, C: PAb-CT-1, D: PAb-CT-2, E: PAb-TC-1, F: PAb-TC-2 sera.

Comparison of gp185^{erbB-2} level

To compare the gp185^{erbB-2} level in 4 stomach cancer cell lines, each cell lysate was analyzed by western blot. Fig. 5. showed that PAb-TC-1 polyclonal serum could detect 185kd c-erbB-2 protein as a single band. To our surprise, gp185^{erbB-2} was increased in SNU-1 and SNU-5 cells (Fig. 5-A), in which c-erbB-2 mRNA overexpression was not observed. So we duplicated the same experiment with newly prepared protein samples and similar result was obtained (Fig. 6-A). It was obvious that c-erbB-2 protein was increased without gene amplification or mRNA overexpression in SNU-1 and SNU-5 cells. When we performed western blot analysis after serial dilution of cell lysate for more precise quantitation of gp185^{erbB-2}, SNU-1 and SNU-5 cells expressed gp185^{erbB-2} 2-to 4-fold, compared t SNU-16 or KATO III cells (Fig. 6-B, C).

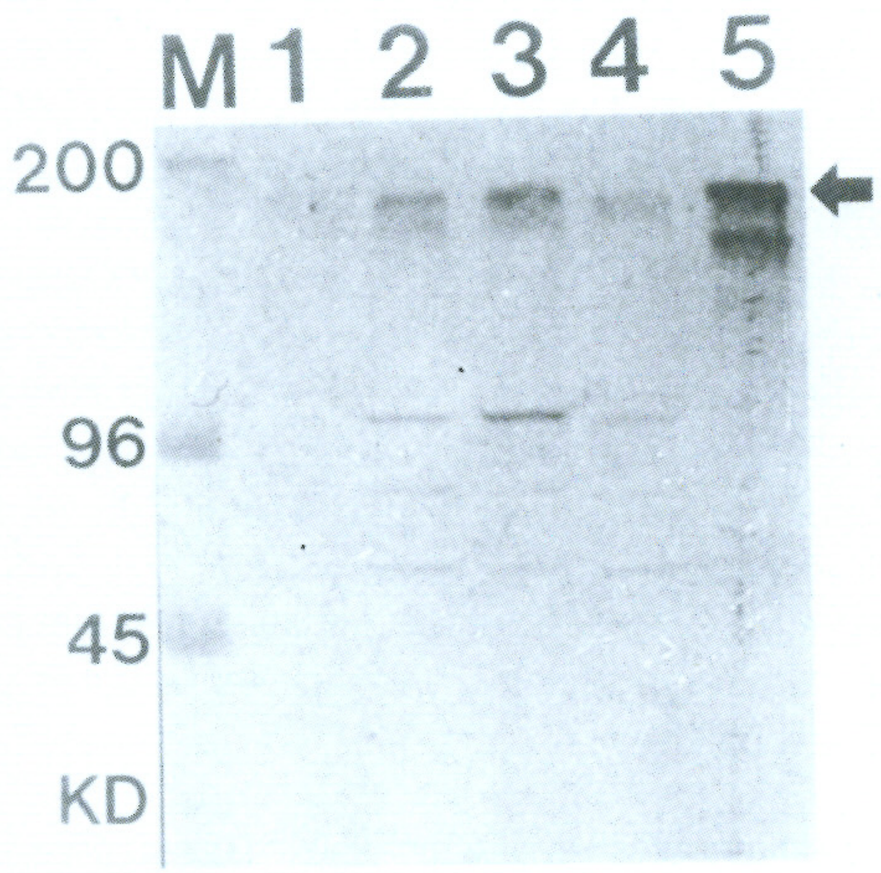


Fig. 5. Western blot analysis of gp185^{erbB-2} expression. A: Fifty micrograms of each cell lysate were fractionated on 8% SDS-polyacrylamide gel and electroblotted to nitrocellulose paper. The blot was treated with PAb-TC-1 (1:4,000 dilution), anti-rabbit IgG-HRP (1:3,000 dilution) sequentially, and c-erbB-2 protein was detected by ECL method. lane 1: KATO III, lane 2 SNU-1, lane 3: SNU-5, lane 4: SNU-16, lane 5: SK-BR-3 as positive control.

DISCUSSION

Our results suggest the possibility that c-erbB-2 expression may be regulated by post-transcriptional events. We examined the protein synthesis in SNU-1 and KATO III cells by ^{35}S-incoporation rate to rule out the difference on overall protein synthesis rate. At least in these two cell lines, we couldn't observe any difference in protein synthesis rate (data not shown), which supported that c-erbB-2 expression should regulated by post-transcriptional events in SNU-1 cells.

There is much evidence of wide participation of post-transcriptional events in the regulation of gene expression in prokaryotes (Ilan, 1987). In eukaryotes, however,

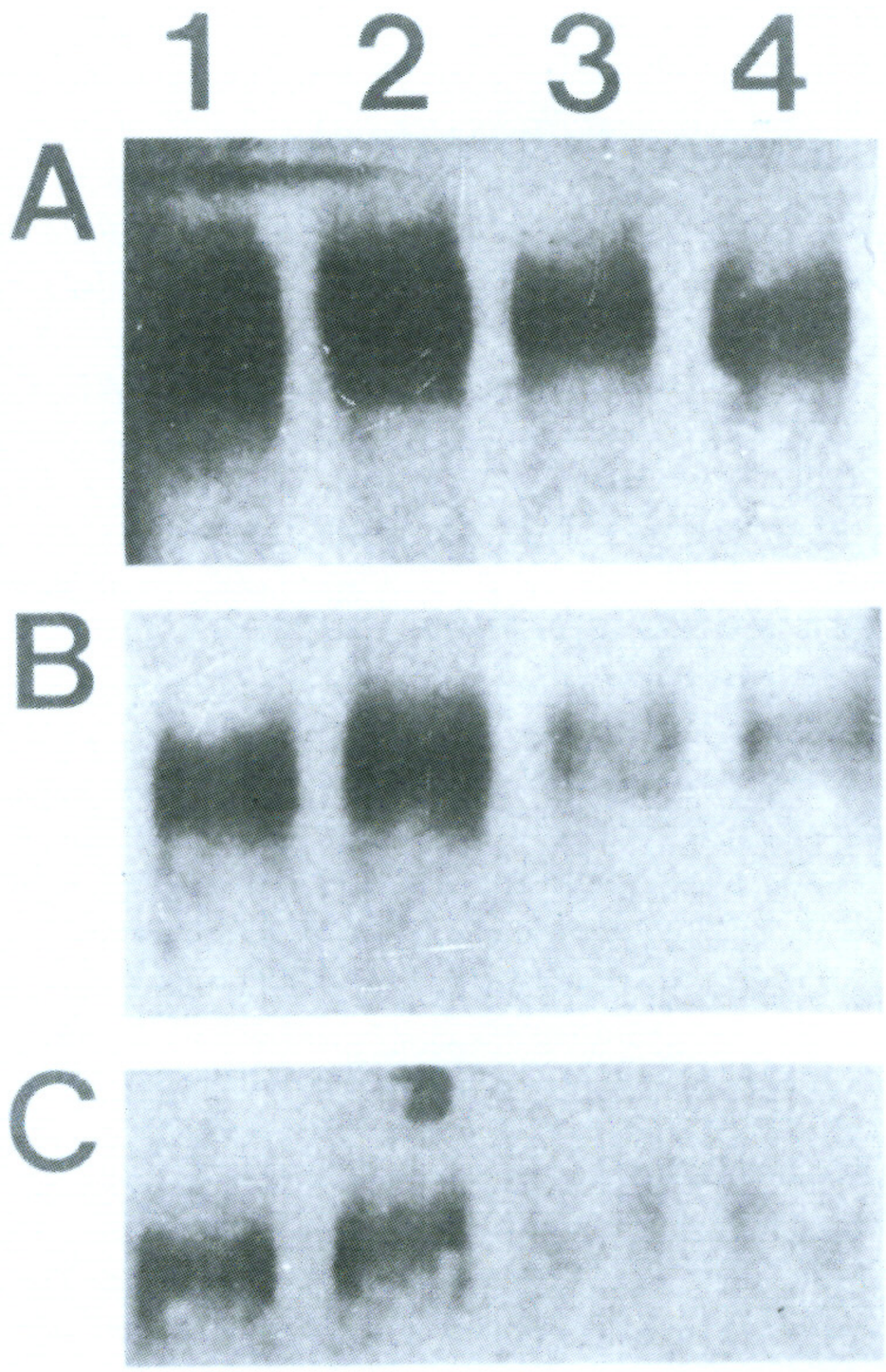


Fig. 6. Quantitation of gp185$^{erbB\text{-}2}$ amount Fifty micrograms of each cell lysate were diluted serially and analyzed by western blot. lane 1: SNU-1, lane 2: SNU-5, lane 3: SNU-16, lane 4: KATO III. A contained 50 μg of protein, B contained 25μg while C contained 12.5μg of protein from each cell line.

post-transcriptional events including mRNA transport, mRNA storage and translation are still not clear. But there are many observations supporting the importance of post-transcriptional events in gene expression of eukaryotes, such as translation of maternal mRNA in oocyte, heat-shock mRNA, ribosomal protein (Hershey, 1991: Perry and Meyhaus, 1990), insulin (Cordell et al., 1982), ornithine decarboxylase (Manzella et al., 1991), cyclin A, ribonucleotide reductase (Standart and Hung, 1990), interferon-β, tumor necrosis factor (Kruys et al., 1991), fem-3 protein (Ahringer and Kimble, 1991), creatine kinase (Ch'ng et al., 1991) and many other proteins.

Some oncoproteins have been also known to be regulated post-transcriptionally. Fos, a transcriptional activator, has been reported to be regulated by a translational inhibitor bound 3'untranslated region of c-fos mRNA (Kruys et al., 1991). Increase of gene product by prolongation of half life has been reported in EGF receptor (Gamou and shimizu, 1987) and N-myc (Cohn et al. 1990). We have observed that the detection rate of c-erbB-2 protein increase in immunohistochemical staining of frozen tissue sections is somewhat greater than that of mRNA increase in northern blot (data not shown). The discrepancy of the detection rates may be explained by the regulation of c-erbB-2 expression in the post-transcriptional processes as shown in this paper.

It is also noticeable that two out of 4 cell lines showed gp185$^{erbB\text{-}2}$ increase without overexpression of c-erbB-2 mRNA. This favors the view that such a phenomenon is not an acquired process during cell line establishment. There is no evidence of gp185$^{erbB\text{-}2}$B-2 increase without mRNA overexpression in vivo. But this cannot rule out such a process in vivo because the present data have been obtained mainly from southern, northern blot and immunohistochemical staining. Although all three techniques are reported to be correlated with each other, the problem is that northern blot analysis cannot detect post-transcriptional regulation and immunohistochemical staining may not detect a small increase of c-erbB-2 protein. Immunohistochemical staining method, despite low sensitivity and relative inaccuracy, is used more prevalently in screening c-erbB-2 protein amounts in tissues because western blot, another method to assess protein amounts on the immunological base, is more influenced by contaminating normal cells (Slamon et al., 1989a). So, it remains an open question that post-transcriptional regulation of c-erbB-2 oncogene is a genuine mechanism of regulation of c-erbB-2 expression.

Another important question can be raised; what influence can the increase of c-erbB-2 protein by a factor of 2 to 4 may exert on the cellular oncogenesis? It is obvious that such a small increase of gp185$^{erbB\text{-}2}$ cannot transform cells because it is experimentally proven that transformation of cells by c-erbB-2 oncogene requires a 50-to 100-fold increase of its gene product (Di Fiore et al., 1987). But we want to point out the fact that c-erbB-2 protein is a growth factory receptor and generally speaking, oncogenesis requires genetic alterations of two or more oncogenes (Weinberg, 1989). The report that the breast cancers with a 2-to 5-fold increase of c-erbB-2 gene copy number also exhibit worse prognosis (Slamon et al., 1989a) represents the influence of a small increase of c-erbB-2 gene on the carcinogenesis of breast cancers. In stomach cancers, c-erbB-2 overexpression has been reported to be found in more advanced cases, im-

plicating the c-erbB-2 may participate in maintenance of gastric cancer (Yonemura et al., 1991a). These indicate that a small increase of c-erbB-2 protein may play a role in carcinogenesis of stomach cancer cells examined in this study as well as in the carcinogenesis of breast cancer.

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