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Post-transcriptional control of c-*erb* B-2 overexpression in stomach cancer cells

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Abbreviations: UTR, untranslated region; Met, methionine; FBS, fetal bovine

Abstract

The growth factor receptor oncogene, c-*erb* B-2, is frequently overexpressed in the adenocarcinomas of breast, ovary, lung and stomach. Although the mechanism of *erb* B-2 overexpression is thought as the result of transcriptional upregulation in many primary human carcinomas, expression rate of c-*erb* B-2 at mRNA level is usually lower than the level of translated protein. We also found that the expression of *erb* B-2 in SNU-1 stomach cancer cells was greater at post-transcription level (Bae *et al*., 1993). To explore the underlying mechanism of *erb* B-2 protein overexpression, we have chosen two cells lines, SNU-1 and SNU-16 where transcription rate of *erb* B-2 was closely resemble to each other while expressed protein levels were quite different. The synthesis rate of *erb* B-2 protein in SNU-1 cells was faster than SNU-16 cells while levels of *erb* B-2 mRNA were found to be similar in both cell lines. The half-life of the expressed *erb* B-2 protein was not significantly different in both cell lines. Analysis of the 5' untranslated region (UTR) of *erb* B-2 mRNA (-1~-323) showed no sequence abnormality in both cell lines. However, ribonuclease protection assay using cloned 5 UTR sequence revealed that the size of 5' UTR of *erb* B-2 mRNA which associate with transcription initiation site(s) in SNU-1 cells was longer than that in SNU-16. These results suggest that the increased *erb* B-2 protein synthesis rate possibly due to the redundant selection of transcription initiation might be a mechanism of *erb* B-2 overexpression in SNU-1 cells.

Keywords: c-*erb* B-2, oncogene, overexpression, stomach cancer

Introduction

Overexpression of *erb* B-2 protein is observed in 10-40% of the carcinoma of breast, ovary, lung and stomach cancer (Slamon *et al*., 1987; Weiner *et al*., 1990; Park *et al*., 1989). The mechanism of *erb* B-2 protein overexpression is known to be gene amplification and/or mRNA overexpression (Slamon *et al*., 1987; 1989). In addition to overexpression of *erb* B-2 at mRNA level, several reports suggested that post-transcriptional upregulation of c-*erb* B-2 might exist (Burhing *et al*., 1995; Oshima *et al*., 1995; Child *et al*., 1999). We earlier reported post-transcriptional overexpression of *erb* B-2 (Bae *et al*., 1993). In that study, *erb* B-2 protein level was upregulated without mRNA increase in SNU-1 and SNU-5 stomach cancer cells compared to SNU-16 or KATO III cells. Moreover, indifference in the overall protein synthesis rate between SNU-1 and SNU-16 cells suggest that participation of some post-transcriptional events in *erb* B-2 overexpression in these cell lines. Expression rate of *erb* B-2 protein in breast cancer was heterogeneous and does not correlated with *erb* B-2 mRNA expression rate.

Many oncogenes including c-*erb* B-2 are known to have 5' UTR with high GC content. 5' untranslated region of mRNA is known as one of the major cis-acting elements affecting protein translation rate. The structure of 5' UTR and existence of ATG codon in 5' UTR may affect ribosome scanning along mRNA (Hershey, 1991). So there is an open possibility that altered 5' UTR structure might affect expression level of such oncogenes.

In this study, we compared the synthesis and degradation rates of *erb* B-2 protein in SNU-1 and SNU-16 cells and found that *erb* B-2 protein overexpression in SNU-1 cells resulted from increased translation rate, not from altered protein degradation rate. There was no sequence difference in 5' UTR of c-*erb* B-2 mRNA between SNU-1 and SNU-16 cells. Instead, the length of 5' UTR in c-*erb* B-2 mRNA was varied between in SNU-1 and SNU-16 cells. These results suggested that selection of transcriptional initiation might be another mechanism of c-*erb* B-2 overexpression.

Materials and Methods

Cell culture, genomic DNA, total cellular RNA prepa-

ration

All stomach cancer cells were cultivated in RPMI 1640 containing 10% FBS under 5% CO_2. Genomic DNA was extracted from cultured stomach cancer cells using proteinase K digestion followed by phenol extraction (Maniatis *et al*., 1989). Total cellular RNA was extracted using acid-guanidine-phenol method as described previously (Bae *et al*., 1996).

Ribonuclease protection assay

The genomic DNA fragment containing c-*erb* B-2 exon 1 (-1 to -323 region) was amplified using Taq DNA polymerase [Perkin-Elmer] using 7-deaza dGTP instead of dGTP for resolving possible stable secondary structure during PCR. For preparation of riboprobe, amplified c-*erb* B-2 genomic DNA fragment was subcloned into pGEM3z and transcribed using T7 RNA polymerase in the presence of $\alpha[^{32}P]$-CTP *in vitro*. Total RNA (25 μg) was hybridized at 50 °C for 16 h with ^{32}P-labeled *erb* B-2 riboprobe and digested with RNase One [Promega] subsequently (Bae *et al*., 1996). The RNase One digestion products were separated through a 6% polyacrylamide gel containing 7 M urea. The resulting gel was dried and exposed to Kodak X-Omat AR film at -70°C for 18 h.

Metabolic labeling of ^{35}S-Methionine and immunoprecipitation

For short-term (< 4 h) labeling, cells at mid-log phase were resuspened in Met-free RPMI [Gibco/BRL] containing 5% dialyzed FBS for 20 min and then incubated in the same medium containing 0.2 mCi of ^{35}S-Met [Amersham]. For long-term labeling, Met-free RPMI containing 10% normal RPMI 1640, 10% FBS and 0.05 mCi of ^{35}S-Met was used instead of Met-free RPMI containig 5% dialysed FBS and 0.2 mCi of ^{35}S-Met. Cells labeled with ^{35}S-Met were resuspended in HNN buffer and lysed by passing through 23G needle. Each cell lysate was incubated for 4 h with anti-*erb* B-2 polysera at the dilution of 1 : 80 and then for 1 h with protein A-Sepharose [Amersham]. Antigen-antibody-protein A-sepharose complex was washed three times with HNN buffer and fractionated through 8% SDS-PAGE.

After polyacrylamide gel electrophoresis, Gels were fixed in 10% glacial acetic acid, 15% isopropyl alcohol and then soaked for 15-30 min in Amplify [Amersham], a fluorographic reagent. Gels were dried under vaccum and exposed to the preflashed films as described by Maniatis *et al*. (1989).

Results

Half life of *erb* B-2 protein in SNU-1 cells

Earlier report showed that expression level of *erb* B-2 protein in SNU-1 and SNU-5 cells 2 to 4 times higher than SNU-16 or KATO III cells without gene amplification or mRNA overexpression (Bae *et al*., 1993). Moreover, this overexpression of *erb* B-2 was gene-specific in that overall protein synthesis rate in SNU-1 or SNU-16 cells was similar. For exploring underlying mechanism, cellular metabolic state of *erb* B-2 protein were investigated by examining rate of degradation and synthesis in SNU-1 cells. The degradation rate of *erb* B-2 protein was measured by pusle-chase labeling followed by immunoprecipitation. The incorporation rate of ^{35}S-methionine was similar in both cell lines. The specific activity (radioactivity/μg protein) of the cell lysate at zero time was 3.9×10^4 cpm/mg in SNU-1 cells, 3.8×10^4 cpm/mg in SNU-16 cells, respectively, which indicated that *erb* B-2 protein overexpression in SNU-1 cells was gene-specific.

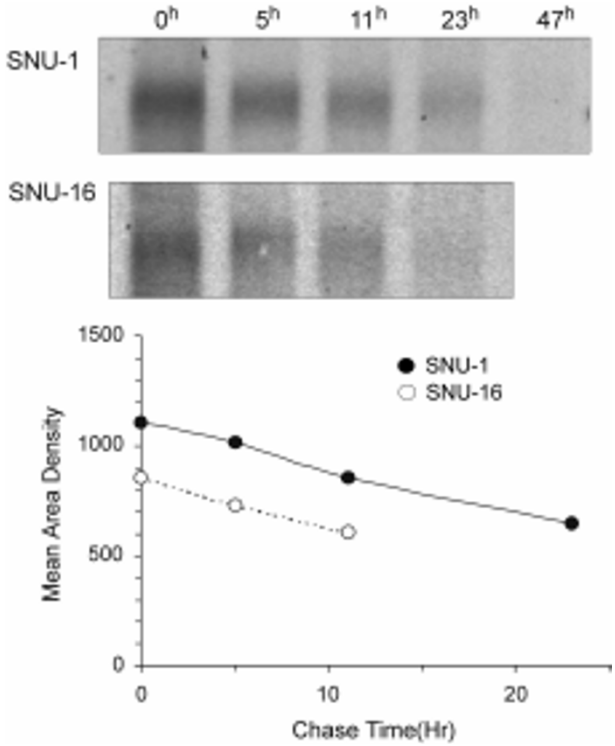

Figure 1. Degradation rate of *erb* B-2 gene product in SNU-1 cells and SNU-16 cells. SNU-1and SNU-16 cells at mid-log phase were pulse-labeled for 12 h and chased for 0, 5, 11, 23, 47 h. Cell lysate at each time point was immunoprecipiated using anti-*erb* B-2 polysera and exposed to preflashed X-Omat AR film for 6 days at -70°C as described in methods. *Erb* B-2 protein bands were much weaker in SNU-16 cells due to lower level of *erb* B-2 protein expression. The densities of *erb* B-2 protein band were expressed as area densities and plotted to chase time.

As shown in Figure 1, the difference between the half life of *erb* B-2 protein in SNU-1 cells and that in SNU-16 cells was not significantly different and not enough to explain the *erb* B-2 protein increase in SNU-1 cells although overall signal intensity was much less in SNU-16 cells due to the low level of *erb* B-2 protein expression,

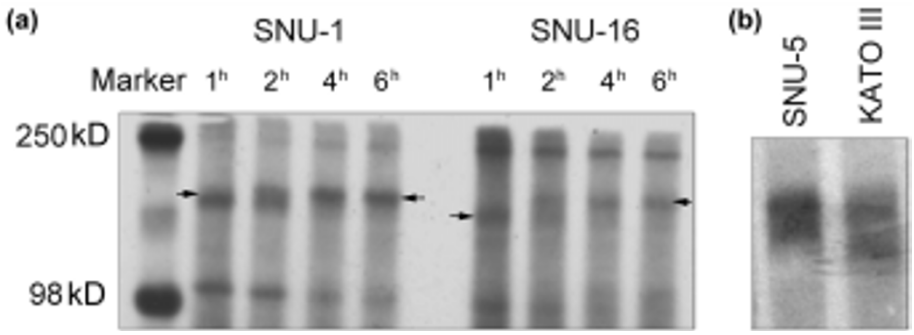

Figure 2. Synthesis of *erb* B-2 protein in SNU-1 and SNU-16 cells. (a) SNU-1 and SNU-16 cells were labeled for 1, 2, 4, and 6 h with 0.2 mCi/ml or 0.5 mCi/ml of ^{35}S-Met, respectively. Cell lysate at each time point was immunoprecipitated using anti-*erb* B-2 polysera as described in methods. All immunoprecipitates were fractionated through the same gel and treated under same conditions for comparison between SNU-1 and SNU-16 cells. The fluorogram showed that main product of *erb* B-2 protein synthesized for the first 1 h period was immature form (170 kD, lower arrow). Fully processed *erb* B-2 protein (185 kD, upper arrow) was seen after 2 h time point. (b) SNU-5 and KATO III cells were labeled for a fixed time period, 2 h with ^{35}S-Met (0.2 mCi/ml). All fluorograms were obtained after 2 days exposure to Kodak X-Omat AR film.

which left some difficulty in assessing the half life of *erb* B-2 protein in SNU-16 cells. This result suggested that *erb* B-2 protein half lives between these two cells was not significantly different and overexpression of *erb* B-2 protein in SNU-1 cells might be due to the other causes, *i.e.* protein synthesis rate.

Synthesis rate of *erb* B-2 protein in SNU-1 cells

Since there was very little difference in the rate of *erb* B-2 protein degradation, the synthesis rate by measuring ^{35}S incorporation rate into *erb* B-2 protein was carried out. A higher concentration of ^{35}S-Met (0.5 mCi/ml) was used to facilitate the visualization of *erb* B-2 protein in SNU-16 cells than in SNU-1 cells (0.2 mCi/ml). As seen in Figure 2, *erb* B-2 protein labeled for 1 h, representing newly synthesized protein, appeared to be slightly smaller molecule size than *erb* B-2 protein labeled for 2 or more h. It is an interesting observation that a newly synthesized *erb* B-2 protein in both SNU-1 and SNU-16 cells appeared as a slightly smaller molecular size than matured protein where one can visualize the post-translational processing (*i.e.* glycosylation) post one h. Diffuse band pattern in *erb* B-2 protein labeled for 2 h also supported this idea. The amount of ^{35}S-labeled *erb* B-2 protein for 6 h was not increased compared to that for 4 h possibly by depletion of Met in the labeling medium. Since we used Met-free medium and dialyzed fetal bovine serum to maximize labeling efficiency, the concentration of Met in the labeling medium might not be sufficient for more than 2 h.

The time course for post-translational modification was similar in both cells. However, when ^{35}S-labeled *erb* B-2 protein for 1 or 2 h time period was compared, the amount of newly synthesized *erb* B-2 protein for the labeling period in SNU-1 cells was greater than that in SNU-16 cells even though higher amount of ^{35}S-Met was used for labeling SNU-16 cells (Figure 2a, see Figure legend). Such results indicate that *erb* B-2 overexpression in SNU-1 cells was likely due to increased protein synthesis rate.

To confirm this hypothesis, the synthesis rate of *erb* B-2 protein in SNU-5 cells, closely related to SNU-1 cells, was carried out under the same experimental conditions. Figure 2b showed the amount of *erb* B-2 protein synthesized for 2 h in SNU-5 cells was greater than that in KATO III cells, another stomach cancer cells showing simliar level of *erb* B-2 mRNA expression. Taken together, we concluded that *erb* B-2 overexpression in SNU-1 and SNU-5 cells resulted from increased rate of *erb* B-2 protein synthesis.

Analysis of 5' untranslated region (UTR) of c-*erb* B-2 mRNA

Since 5' UTR might affect the synthesis rate of protein and untranslated region of c-*erb* B-2 mRNA is known to contain high levels of GC, 5' UTR of c-*erb* B-2 mRNA in SNU-1 and SNU-16 cells were analyzed. We cloned and sequenced the genomic fragment (-1 to -323) containing 5' UTR of c-*erb* B-2 mRNA using primers (upstream primer: GAGAAAGTGAAGCTGGGAGTTG, downstream primer: CCATGGTGCTCACTGCGGCT). We could amplify this region only when we used 7-deaza-dGTP, which resulted from extremely high GC content of this region. Five indifferent clones obtained from SNU-1 cells and 3 indifferent clones from SNU-16 (Figure 3) were sequenced. However, any mutations leading to either change in number of upstream ATG or significant change in GC content of 5' UTR were not detected. So we excluded the possible role of upstream ATG sequences in overexpression of *erb* B-2 protein in SNU-1 cells. We found 2 sequence differences (underlined sequence in Figure 3) compared to the published sequence (Gene bank accession number M16789). Since all the published *erb* B-2 promotor sequences are

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                              GAG AAAGTGAAGC TGGGAGTTGC CACTCCCAGA -301
CTTGTTGGAA TGCAGTTGGA GGGGGCGAGC TGGGAGCGCA CTTGCTCCCA -251
CTTCG(Published sequence)
ATCACAGGAG AAGGAGGAGG TGGAGGAGGA GGGCTGCTTG AGGAAGTATA -201
                                  → Primary Transcript
AGAATGAAGT TGTGAAGCTG AGATTCCCCT CCATTGGGAC CGGAGAAACC -151
AGGGGAGCCC CCCGGGCAGC CGCGCGCCCC TTCCCACGGG GCCCTTTACT -101
GCGCCGCGCG CCCGGCCCCC ACCCCTCGCA GCACCCCCGG CCCCGCGCCC  -51
                        (Published Sequence)GC
TCCCAGCCGG GTCCAGCCGG AGCCATGGGG CCGGAGCCGC AGTGAGCACC   -1
→ Translation Initiation
ATGGAGCTGG
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Figure 3. Sequence analysis of 5' UTR of c-*erb* B-2 mRNA in SNU-1 cells. The genomic DNA fragment containing 5' UTR was amplified using Taq DNA polymerase and sequenced (see text). Upstream ATG sequences were marked as clear box and the sequences different from previous publication were underlined. The sequence was the representative of 5 indifferent clones to rule out PCR error.

showing same sequences, these sequence differences might be related with usage of 7-deaza-dGTP in PCR. 7-deaza-dGTP can weaken hydrogen bond between G and C but might increase misincorporation rate in PCR.

The transcription initiation site was next examined by ribonuclease protection assay. As seen in Figure 4, three distinct fragments were protected from RNase digestion in all 3 stomach cancer cells (SNU-1, SNU-16, and KATO III), which suggest that there were three distinct transcription initiation sites. In both SNU-16 and KATO III cells, smaller two fragments were mainly protected while in SNU-1 cells, those two fragments were barely visible. Instead, a larger fragment was the mainly protected in SNU-1 cells.

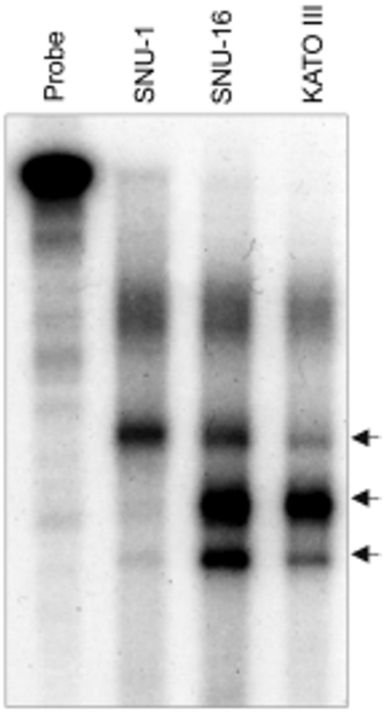

Figure 4. Anaysis of the length of 5' UTR of c-*erb* B-2 mRNA in SNU-1 and SNU-16 cells. The genomic DNA fragment was in vitro transcribed and subsequent RNA was used for ribonulcease protection assay. Autoradiogram was obtained using phosphoimage storage screen [Molecular Dynamics]. Three different fragments (arrows) were protected from RNase digestion.

These results suggest that the transcription initiation site of c-*erb* B-2 mRNA in SNU-1 cells was different from those in SNU-16 and KATO III cells and the upregulation mechanism of *erb* B-2 protein in SNU-1 cells might be due to longer 5' UTR. At the present, other genetic alterations such as translation initiation factors couldn't be excluded but redundant selection of transcription initiation sites might consider as a possible mechanism of *erb* B-2 overexpression in SNU-1 cells.

Discussion

c-*erb* B-2 oncogene is frequently overexpressed in primary human adenocarcinomas where overexpression of *erb* B-2 protein is related with poor prognosis. The molecular mechanism of *erb* B-2 overexpression in primary human cancer is known due to mRNA overexpression with/without gene amplification. However, the detailed mechanism of mRNA overexpression or gene amplification is still obscure. Moreover, in many cases, *erb* B-2 overexpression was detected by immunohistochemistry even though no c-*erb* B-2 mRNA overexpression was detected by northern blot anaylsis. In this study, we reported post-transcriptional overexpression mechanism of *erb* B-2 oncogene. As shown in Figure 2, synthesis rate of *erb* B-2 protein in SNU-1 cells was increased compared to SNU-16 cells while the half-life of *erb* B-2 protein was not significantly different (Figure 1). This enhanced protein synthesis rate was also observed in SNU-5 cells, showing *erb* B-2 protein overexpression without mRNA increase (Bae *et al.*, 1993; 1996).

In an effort to find out underlying molecular mechanism of enhanced *erb* B-2 synthesis, we have targeted the initiation step as the rate controlling step in translation.

The overall rate of protein elongation step in cells is reported to be near-maximum (5 amino acid/ribosome/sec) and has little effect on protein synthesis rate (Hershey, 1991). Among the factors affecting initiation step of protein synthesis, initiation factors and structure of mRNA are important (Hershey, 1991; Thach, 1992). Both the fact that *erb* B-2 mRNA has highly GC-rich 5' UTR (Figure 3) and the complexity of eukaryotic translation factors lead us to start our further study from exploring the possibility of genetic alterations in 5' UTR.

A possible presence of extra ATG upstream to translation initiation site which may make ribosome stall, or the altered secondary structure of 5' UTR which may block scanning ribosome along this region have also been considered (Child *et al*., 1999). We firstly, thought the latter possibility might be the case since the overall GC content of the genomic fragment (300 bp) including 5' UTR of c-*erb* B-2 mRNA was very high (67%) which raised the possibilities of secondary structure. The fact that this region has 3 extra ATG sequences but 2 of those upstream ATG are located upstream to the known transcription initation site (Tal *et al*., 1987; Grooteclaes *et al*., 1994) suggests that ribosome stalling might not occur. In fact, we could exclude the role of upstream ATG since there was no mutation resulting in any change of upstream ATG in c-*erb* B-2 mRNA in SNU-1 cells. Moreover, we couldn't detect any sequence differences in the promoter region including exon1 between SNU-1 and SNU-16 cells. Instead, we found that 5' UTR length of c-*erb* B-2 mRNA in SNU-1 cells was longer than that in SNU-16 cells. This results might be confusing since longer 5' UTR with high GC content was likely to have more stable secondary structure. However, 5' UTR of c-*erb* B-2 mRNA was relatively short, less than 200 bp, so shortening of 5' UTR didn't look like affecting the secondary structure greatly. Moreover, overall GC content was still high even though some shortening of 5' UTR occurred. Possible explanation of the effect of long 5' UTR was that ribosome entry to c-*erb* B-2 mRNA might be more facilitated. Other possible explanation would be the presence of other genetic alterations such as trans-acting initiation factors. At the present, we do not exclude the role of initiating factors, which is worth to be explored.

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