

## Down-regulation of HER-2 Expression in Human Breast Cancer Cell HBC-4 and ZR75-1 by Nitrogen-mustard-N-oxide

SYUUITU SAI, YOSHIHIKO FURUYA, YASUHIRO FUJINO,  
AND YOSHIKAZU KURODA

*Department of Gastroenterological Surgery Kobe University Graduate School of Medicine  
7-5-2 Kusunoki-cho, Chuo-ku Kobe, Japan*

**Keywords:** Alkylating agents, Breast cancer, HER-2

**Background and Aims:** The human epidermal growth factor receptor type 2 (HER-2) seems to be sensitive to nitrogen mustard, because nitrogen mustard heavily alkylates on guanine. In this study, we examined the effects of nitrogen mustard on cell growth and expression of HER-2 proteins in cultured human breast cancer cells.

**Method:** The HER-2 protein levels on the cell surface of breast cancer cells were evaluated following treatment with various concentrations of Nitrogen-mustard-N-oxide using flow cytometry.

**Results:** The HER-2 proteins were detected as high and moderate in ZR75-1 and HBC-4, respectively. The addition of 0.5, 1 and 3 µg/ml of Nitrogen-mustard-N-oxide to the medium significantly decreased in the HER-2 protein with a sharp peak in both the ZR75-1 and HBC-4 cells. The mean value of the HER-2 protein expression in HBC-4 cells was 60%, 51% and 34% of the control, with the growth of 80%, 70% and 50% of the control at 72 hours, respectively. The mean value of HER-2 protein in ZR75-1 cells was 94%, 93% and 70% of the control, with the growth of 70%, 50% and 30% of the control at 72 hours, respectively. A lower dose of Nitrogen-mustard-N-oxide insufficient for growth inhibition did not show a decrease. Only the addition of Nitrogen-mustard-N-oxide in a sufficiently high dose to show growth inhibition down-regulated the HER-2 protein expression in human breast cancer cells.

**Conclusion:** We concluded that high concentrations of cyclophosphamide (CPA) might be able to inhibit cell growth through down-regulation of HER-2 protein level in breast cancer cell lines.

The human epidermal growth factor receptor type 2 (HER-2) protein belongs to the class of tyrosine kinase receptor proteins, and its ligand was established recently (5,22). HER-2 protein is known to form a heterodimer with other proteins of the epidermal growth factor (EGF) -family of HER-1, -3 and -4, and enhances the activity of receptors (1,9,15,16). HER-2 overexpression caused an escape from the immune system by tumor necrosis factor-induced apoptosis via the AKT/NF-Kappa B pathway (25). In breast cancer, HER-2 overexpression brings resistance to hormonal therapy (2,9), alkylating agents (6) and taxanes (24). Modifying HER-2 overexpression of tumor cells on chemosensitivity has been recently highlighted, but remains controversial. Modifying HER-2 expression by conventional therapeutic agents has not received sufficient attention. The HER-2 promoter gene core-binding site has been reported as G-rich 'GCCCCGGG' (12), and HER-2 seems to be sensitive to cyclophosphamide (CPA), because CPA heavily alkylates on guanine. CPA is broadly used in breast cancer treatment in combination with other chemotherapeutic agents

and is activated by cytochrome p 450 to the active form of CPA. In this study, we examined the effect of Nitrogen-mustard-N-oxide on HER-2 protein expression.

## MATERIALS AND METHODS

### Breast cancer cell lines

HBC-4 was supplied by Drs. T. Kawaguchi and H. Sugano (Cancer Institute, Tokyo, Japan) and ZR-75-1 was purchased from Dainippon Pharmaceutical Co. Ltd. (Osaka, Japan). The cells were maintained in T medium supplemented with 10% fetal calf serum (12). The cells were passage-cultured weekly prior to confluence.

### Chemicals

Nitrogen-mustard-N-oxide (Nitromin, Mitsubishi Pharma Co. Ltd., Osaka, Japan), an anti-c-erbB-2 monoclonal antibody (Nichirei Co., Tokyo, Japan) that reacts with the extracellular domain of HER-2 product (gp-185) and Fluorescein- (FITC) conjugated AffiniPure Goat Anti-Mouse IgG (H+L, Jackson ImmunoResearch Lab Inc., West Grove, PA, USA) were purchased.

### HER-2 staining

The HER-2 protein levels on the cell surface of breast cancer cells were evaluated following treatment with various concentrations of Nitrogen-mustard-N-oxide. HBC-4 and ZR-75-1 cell were incubated on T medium added 10% fetal calf serum with 0.5, 1, 3  $\mu\text{g/ml}$  Nitrogen-mustard-N-oxide at 72 hours. The cells were detached from plates with 10 mM ethylene diamine tetraacetic acid (EDTA), rinsed in culture medium, washed twice with phosphate buffer saline, and concentrated using 500rpm centrifugal separator for 60 seconds. Extracted cells were incubated with T medium added 10% fetal calf serum with the 30  $\mu\text{l}$  of anti-c-erbB-2 monoclonal antibody. After washing with normal saline to remove unnecessary monoclonal antibody, the cells were stained with 0.2  $\mu\text{g}$  FITC conjugated AffiniPure Goat anti-Mouse IgG sequentially. The cells were washed again to remove the inaccuracy stain. The FITC-stained cells were evaluated FL-1on a FACScan (Becton Dickinson, Mountainview, CA, USA) and analyzed using CellQuest software (Becton Dickinson). Isotype-matched control samples were evaluated in all the experiments to determine the non-specific background fluorescence. The cell counts were obtained using a counting chamber, and viability was ascertained by the trypan blue dye exclusion test

## RESULTS

### Growth inhibition of HBC-4 and ZR75-1 cells by Nitrogen-mustard-N-oxide

The growth inhibitory effects of Nitrogen-mustard-N-oxide on ZR75-1 and HBC-4 cells are shown in Figure 1. The growth of HBC-4 cells was 80%, 70% and 50% of the control with 0.5, 1, 3  $\mu\text{g/ml}$  Nitrogen-mustard-N-oxide at 72 hours, respectively (Fig.1). The growth of ZR75-1 cells was 70%, 50% and 30% of the control with 0.5, 1, 3  $\mu\text{g/ml}$  Nitrogen-mustard-N-oxide at 72 hours. Cell growth was inhibited in a dose-dependent manner at above 1  $\mu\text{g/ml}$  Nitrogen-mustard-N-oxide.

### Modification of HER-2 protein expression by Nitrogen-mustard-N-oxide in HBC-4 and ZR75-1 cells

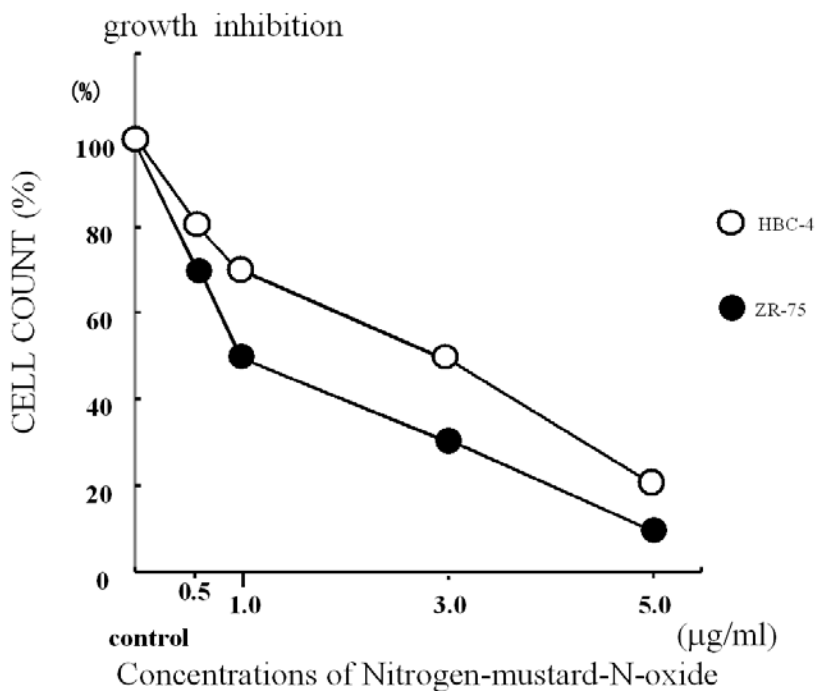
Modification of HER-2 protein expression by 1, 3, 10  $\mu\text{g/ml}$  Nitrogen-mustard-N-oxide in HBC-4 and ZR75-1 cells was detected by flow cytometry (Fig. 2). Nitrogen-mustard-N-oxide at 1, 3  $\mu\text{g/ml}$  down-regulated HBC-4 cells (Fig. 2A) and ZR75-1 cells (Fig. 2B), and at 10  $\mu\text{g/ml}$  these cells could not maintain a viable meaningful peak in

## HER-2 EXPRESSION WITH NITROGEN-MUSTARD-N-OXIDE

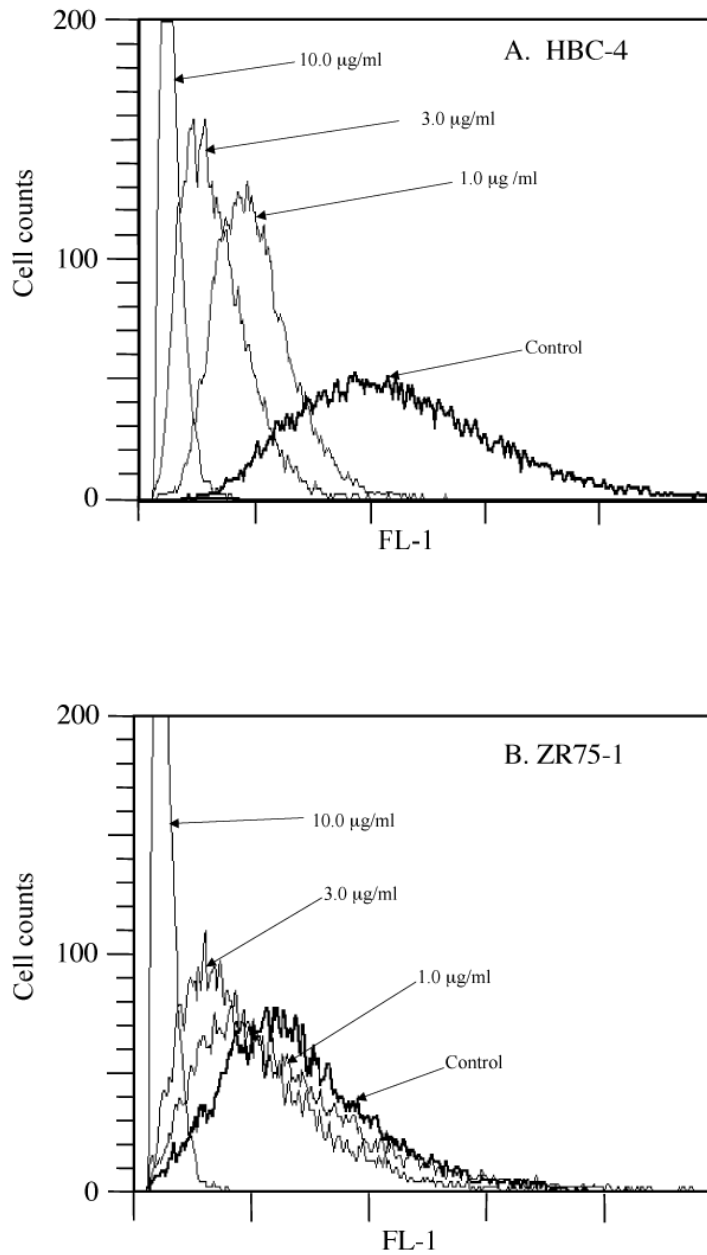
the HER-2 protein. Dead cells treated with 10  $\mu\text{g/ml}$  Nitrogen-mustard-N-oxide lost HER-2 protein expression within 24 hours.

### Modification of HER-2 protein expression in HBC-4 and ZR75-1 cells by Nitrogen-mustard-N-oxide

A lower dose of Nitrogen-mustard-N-oxide insufficient for growth inhibition did not show decrease in HER-2 protein expression in either cell type. The intensity of HER-2 protein was measured by FITC, which is bound to HER-2 proteins. The mean value of FITC in HBC-4 was 276.35, 236.11, and 157.84 with 0.5, 1, 3  $\mu\text{g/ml}$  Nitrogen-mustard-N-oxide at 72 hours. But mean value was 464.05 in control. So, the mean value of the HER-2 protein expression in HBC-4 cells was 60%, 51% and 34% of the control. And the mean value of FITC in ZR-75 was 268.38, 264.47, and 199.45 with 0.5, 1, 3  $\mu\text{g/ml}$  Nitrogen-mustard-N-oxide at 72 hours, respectively. And the mean value was 284.61 in control. The mean value of HER-2 protein expression in ZR75-1 cells was 94%, 93% and 70% of the control, respectively. (Fig.3).

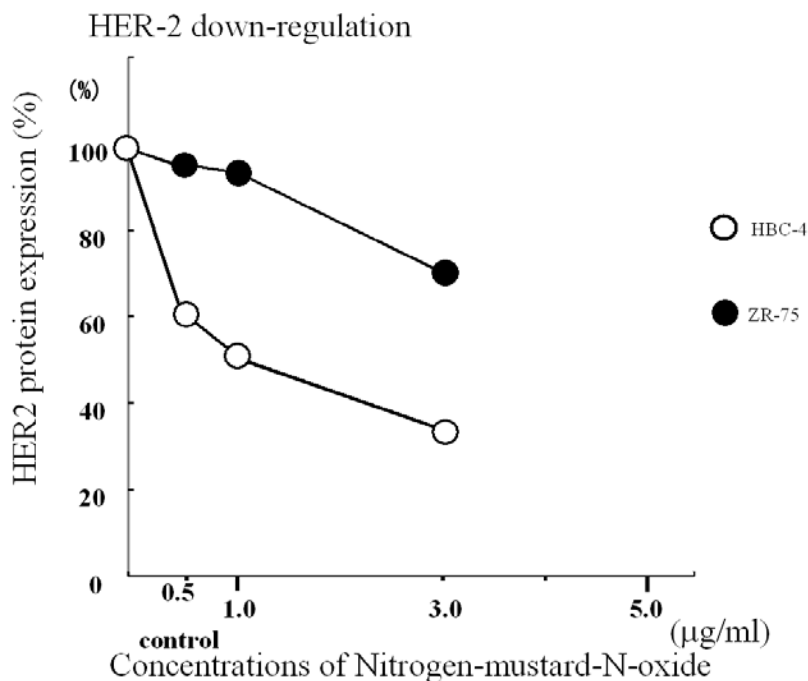


**Fig. 1.** Dose-responsive curves in growth inhibition of HBC-4 (○) and ZR75-1 (●) cells by Nitrogen-mustard-N-oxide at 72 hours. Cell counts obtained with a counting chamber are shown compared to the control (%).



**Fig. 2.** Dose-responsive modification of HER2 expression on HBC-4 (A) and ZR75-1 (B) cell surfaces detected by flow cytometry compared to the control (%).

## HER-2 EXPRESSION WITH NITROGEN-MUSTARD-N-OXIDE



**Fig. 3.** Modification of HER2 expression by 0.5, 1, 3, 10 µg/ml Nitrogen-mustard-N-oxide in HBC-4 (○) and ZR75-1 (●) cells detected by flow cytometry at 72 hours.

### DISCUSSION

CPA, a masked compound of nitrogen mustard, is a key drug in combination chemotherapies for breast cancer. CAF (CPA, Adriamycin and 5-fluorouracil) and CMF (CPA, methotrexate and 5-fluorouracil) are the standard therapies for breast cancer, and they provide benefits especially for premenopausal breast cancer patients (14). CPA is thought to be the most active component of CMF, while CAF additionally contains adriamycin and is reported to be effective against HER-2 over-expressing breast cancer in high-dose regimens (19).

One mechanism of the cytotoxic effect of CPA is the ablating effect; CPA is known to damage the ovaries and finally to cause menopause with cumulative doses above 5.2 g in women in their forties and above 9.3 g in women in their thirties (17). Another mechanism of the cytotoxic effect of CPA just exists in combination chemotherapies, yielding great benefits in estrogen receptor negative breast cancer (4,18). The molecular mechanism underlying the effectiveness in combination chemotherapy has been reported as bcl-2 (8), p53 (2), and HER-2. HER-2 is the most attractive, but involves a major difference between the clinical observation and known underlying mechanisms. In this study, Nitrogen-mustard-N-oxide caused down-regulation of the HER-2 protein only in high concentrations, accompanied by growth inhibition in breast cancer cells.

In a clinical setting, anthracycline is known to be effective in adjuvant chemotherapy for HER-2 positive breast cancer, while known underlying mechanisms are controversial (16). Orr's study has demonstrated that overexpression of exogenous HER-2 sometimes alters

drug sensitivities, but sometimes has no effect, and overexpression of HER-2 by itself is not sufficient to induce changes in chemosensitivity (21). However, Harris's study claims that up-regulation of topoisomerase II $\alpha$  in vitro and in clinical specimens is associated with increased response to doxorubicin (presumptively by an increase in drug substrate) (13).

Alkylation of GC rich consensus by sulfur mustards (other alkylating agents) has been reported to inhibit binding of the AP2 transcription factor, which is GC rich in vitro, by alkylating the AP2 consensus domain to interfere with the AP2 mediated process (11), thus enhancing growth inhibition. On the other hand, Rodenhuis's study reported that HER-2-positive breast cancer is relatively resistant to alkylating agents (23). However, our study showed that Nitrogen-mustard-N-oxide caused down-regulation of the HER-2 protein only in high concentrations. This suggests that CPA acts via HER-2 modulation when used in chemotherapy. Therefore, chemosensitivity without overexpression might be expected for cases of HER-2 overexpression, which commonly resists chemotherapy, combining with high concentrations of CPA. We cultured the residual cell lines to confirm the cells lived, and each cell survived.

In conclusion, high concentrations of CPA might be able to inhibit cell growth through down-regulation of HER-2 in breast cancer cell lines, and the effect of HER-2 modulation in combination therapy is under investigation.

#### REFERENCES

1. **R.R. Beerli, D. Graus-Porta, K. Woods-Cook, X. Chen, Y. Yarden, and N.E. Hynes.** 1995. Neu differentiation factor activation of ErbB-3 and ErbB-4 is cell specific and displays a differential requirement for ErbB-2. *Mol. Cell. Biol.* **15**: 6496-6505.
2. **E.M. Berns, J.A. Foekens, R. Vossen, M.P. Look, P. Devilee, S.C. Henzen-Logmans, I.L. van Staveren, W.L. van Putten, M. Inganas, M.E. Meijer-van Gelder, C. Cornelisse, C.J. Claassen, H. Portengen, B. Bakker, and J.G. Klijn.** 2000. Complete sequencing of TP53 predicts poor response to systemic therapy of advanced breast cancer. *Cancer Res.* **60**: 2155-2162.
3. **A. Borg, B. Baldetorp, M. Ferno, D. Killander, H. Olsson, S. Ryden, and H. Sigurdsson.** 1994. ERBB2 amplification is associated with tamoxifen resistance in steroid-receptor positive breast cancer. *Cancer Lett.* **81**: 137-144.
4. **M. Colleoni, M. Bonetti, A.S. Coates, M. Castiglione-Gertsch, R.D. Gelber, K. Price, C.M. Rudenstam, J. Lindtner, J. Collins, B. Thurlimann, S. Holmberg, A. Veronesi, G. Marini, and A. Goldhirsch.** 2000. Early start of adjuvant chemotherapy may improve treatment outcome for premenopausal breast cancer patients with tumors not expressing estrogen receptors. The International Breast Cancer Study Group. *J. Clin. Oncol.* **18**: 584-590.
5. **L. Coussens, T.L. Yang-Feng, Y.C. Liao, E. Chen, A. Gray, J. McGrath, P.H. Seeburg, T.A. Libermann, J. Schlessinger, U. Francke, et al.** 1985. Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with neu oncogene. *Science* **230**: 1132-1139.
6. **S. Eppenberger-Castori, W. Kueng, C. Benz, R. Caduff, Z. Varga, F. Bannwart, D. Fink, H. Dieterich, M. Hohl, H. Muller, K. Paris, F. Schoumacher, and U. Eppenberger.** 2001. Prognostic and predictive significance of ErbB-2 breast tumor levels measured by enzyme immunoassay. *J. Clin. Oncol.* **19**: 645-656.
7. **Y. Furuya, N. Kohno, Y. Fujiwara, and Y. Saitoh.** 1989. Mechanisms of estrogen action on the proliferation of MCF-7 human breast cancer cells in an improved culture medium *Cancer Res.* **49**: 6670-6674.

## HER-2 EXPRESSION WITH NITROGEN-MUSTARD-N-OXIDE

8. **L.F. Gibson, J. Fortney, G. Magro, S.G. Ericson, J.P. Lynch, and K.S. Landreth.** 1999. Regulation of BAX and BCL-2 expression in breast cancer cells by chemotherapy. *Breast Cancer Res. Treat.* **55**: 107-117.
9. **D. Graus-Porta, R.R. Beerli, and N.E. Hynes.** 1995. Single-chain antibody-mediated intracellular retention of ErbB-2 impairs Neu differentiation factor and epidermal growth factor signaling. *Mol. Cell. Biol.* **15**: 1182-1191.
10. **D. Graus-Porta, R.R. Beerli, J.M. Daly, and N.E. Hynes.** 1997. ErbB-2, the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling. *EMBO J.* **16**: 1647-1655. [4] **D. Karunakaran, E. Tzahar, R.R. Beerli, X. Chen, D. Graus-Porta, B.J. Ratzkin, R. Seger, N.E. Hynes, Y. Yarden.** 1996. ErbB-2 is a common auxiliary subunit of NDF and EGF receptors: implications for breast cancer. *EMBO J.* **15**: 254-264.
11. **P.J. Gray.** 1995. Sulphur mustards inhibit binding of transcription factor AP2 in vitro. *Nucleic Acids Res.* **23**: 4378-4382.
12. **M. Grootclaes, D. Vernimmen, S. Plaza, F. Pasleau, D. Hodzic, and R. Winkler-Gol.** 1999. A new cis element is involved in the HER2 gene overexpression in human breast cancer cells. *Cancer Res.* **59**: 2527-2531.
13. **L.N. Harris, L. Yang, C. Tang, D. Yang, and R. Lupu.** 1998. Induction of sensitivity to doxorubicin and etoposide by transfection of MCF-7 breast cancer cells with heregulin beta-2. *Clin. Cancer Res.* **4**: 1005-1012.
14. **A. Howell, H. Bush, W.D. George, J.M. Howat, D. Crowther, R.A. Sellwood, R.D. Rubens, J.L. Hayward, R.D. Bulbrook, I.S. Fentiman, et al.** 1984. Controlled trial of adjuvant chemotherapy with cyclophosphamide, methotrexate, and fluorouracil for breast cancer. *Lancet* **2**: 307-311.
15. **D. Karunakaran, E. Tzahar, R.R. Beerli, X. Chen, D. Graus-Porta, B.J. Ratzkin, R. Seger, N.E. Hynes, and Y. Yarden.** 1996. ErbB-2 is a common auxiliary subunit of NDF and EGF receptors: implications for breast cancer. *EMBO J.* **15**: 254-264.
16. **Pritchard KI, Shepherd LE, O'Malley FP, Andrulis IL, Tu D, Bramwell VH, and Levine MN; National Cancer Institute of Canada Clinical Trials Group.** 2006. HER2 and responsiveness of breast cancer to adjuvant chemotherapy. *N Engl J Med.* May 18; **354**(20): 2103-2011.
17. **H. Koyama, T. Wada, Y. Nishizawa, T. Iwanaga, and Y. Aoki.** 1977. Cyclophosphamide-induced ovarian failure and its therapeutic significance in patients with breast cancer. *Cancer* **39**: 1403-1409.
18. **D.W. Miles, W.H. Harris, C.E. Gillett, P. Smith, and D.M. Barnes.** 1999. Effect of c-erbB2 and estrogen receptor status on survival of women with primary breast cancer treated with adjuvant cyclophosphamide/methotrexate/fluorouracil. *Int. J. Cancer.* **84**: 354-359.
19. **H.B. Muss, A.D. Thor, D.A. Berry, T. Kute, E.T. Liu, F. Koerner, C.T. Cirrincione, D.R. Budman, W.C. Wood, M. Barcos, et al.** 1994. c-erbB-2 expression and response to adjuvant therapy in women with node-positive early breast cancer. *N. Engl. J. Med.* **330**: 1260-1266.
20. **J.C. Newby, S.R. Johnston, I.E. Smith, and M. Dowsett.** 1997. Expression of epidermal growth factor receptor and c-erbB2 during the development of tamoxifen resistance in human breast cancer. *Clin. Cancer Res.* **3**: 1643-1651.
21. **M. S. Orr, P.M. O'Connor, and K.W. Kohn.** 2000. Effects of c-erbB2 overexpression on the drug sensitivities of normal human mammary epithelial cells *J. Natl. Cancer Inst.* **92**: 987-994. [25] **B.P. Zhou, M.C. Hu, S.A. Miller, Z. Yu, W. Xia, S.Y. Lin, and M.C.**

- Hung.** 2000. HER-2/neu blocks tumor necrosis factor-induced apoptosis via the Akt/NF-kappaB pathway. *J. Biol. Chem.* **275**: 8027-8031.
22. **Stein R. A., and Staros J. V.** 2006. Insights into the evolution of the ErbB receptor family and their ligands from sequence analysis. *BMC Evol Biol.* 2006 Oct 6, **6**:79.
  23. **Rodenhuis S., et al.** 2006. Efficacy of high-dose alkylating chemotherapy in HER2/neu-negative breast cancer. *Ann Oncol.* 2006 Apr. **17(4)**: 588-96. Epub 2006 Jan 30.
  24. **D. Yu, B. Liu, M. Tan, J. Li, S.S. Wang, and M.C. Hung.** 1996. Overexpression of c-erbB-2/neu in breast cancer cells confers increased resistance to Taxol via mdr-1-independent mechanisms. *Oncogene* **13**: 1359-1365.
  25. **B.P. Zhou, M.C. Hu, S.A. Miller, Z. Yu, W. Xia, S.Y. Lin, and M.C. Hung.** 2000. HER-2/neu blocks tumor necrosis factor-induced apoptosis via the Akt/NF-kappaB pathway. *J. Biol. Chem.* **275**: 8027-8031.