[CANCER RESEARCH 62, 5485–5488, October 1, 2002]

Inhibition of Ligand-mediated HER2 Activation in Androgen-independent Prostate Cancer

Nerissa Mendoza, Gail Lewis Phillips, Johnny Silva, Ralph Schwall, and Dineli Wickramasinghe[1]

Molecular Oncology, Genentech Inc., South San Francisco, California 94080

ABSTRACT

Hormone-independent tumor growth and metastasis are associated with increased mortality in human prostate cancer. In this study, we evaluate a potential role for ligand-mediated activation of HER2 receptor tyrosine kinase in androgen-independent prostate cancers. HER2, HER3, and epidermal growth factor receptor were detected in the androgen-independent cell line 22Rv1. Heregulin stimulation results in receptor phosphorylation and cell proliferation that is inhibited by increasing concentrations of anti-HER2 recombinant humanized monoclonal antibody (rhuMAb) 2C4. Furthermore, inhibition of tumor growth was observed in xenografts derived from 22Rv1 cells when treated with rhuMAb 2C4 in a dose-dependent manner. These studies provide a framework, both *in vitro* and *in vivo*, to examine the molecular mechanisms of ligand-driven HER2 activation in androgen-independent tumorigenesis.

INTRODUCTION

Prostate cancer progression to androgen independence may be driven by several mechanisms. One such mechanism implicates overexpression and activation of HER2 receptor tyrosine kinase (1). In breast cancer, genomic amplification and overexpression of HER2 has been observed and is predictive of poor prognosis (2). The anti-HER2 antibody, Herceptin, which recognizes the extracellular domain of HER2 is used effectively as a therapeutic agent in breast cancer. Reports of HER2 expression in prostate cancers have been contradictory, and a potential role for HER2 amplification and overexpression in prostate cancer needs further clarification (3, 4). An alternate mechanism of HER2 activation is driven by ligand-mediated receptor activation. HER2, in complex with other members of the receptor family, EGFR, HER3, and HER4 is activated by ligand binding of the heterodimer complex (5, 6). Ligand activation of these receptor complexes drives cell proliferation and has been implicated in a variety of cancers (7–10). Anti-HER2 rhuMAb 2C4 has been shown to inhibit heregulin-induced activation of HER2 phosphorylation (6, 7). In prostate cancer, the mechanism(s) that drives AI[2] tumorigenesis is not well understood. To address a potential role for HER2 in AI prostate cancer, we investigated ligand activation of HER2 heterodimers in AI 22Rv1 cells. Furthermore, we examined the effect of rhuMAb 2C4 inhibition of ligand-mediated HER2 activation both *in vitro* and *in vivo*. The AI 22Rv1 tumor cell line was particularly suitable for *in vitro* and extended *in vivo* analysis using the same cells. 22Rv1 is derived from a CWR22R tumor (11), which is a relapsed AI tumor derivative from a primary human prostate cancer (12, 13). The cells have been characterized extensively, particularly with respect to karyotype and prostate-specific antigen expression (11).

Our results indicate that HER2, HER3, and EGFR are expressed in this cell line. Heregulin stimulation of 22Rv1 cells results in increased receptor phosphorylation and cell proliferation that are inhibited by rhuMAb 2C4. In addition, we show that HER3 associates with HER2 only on heregulin stimulation. Furthermore, we demonstrate that rhuMAb 2C4 inhibited tumor growth in a dose-dependent manner in this AI xenograft model, which suggests a potential therapeutic role for anti-HER2 treatment in AI prostate cancer.

MATERIALS AND METHODS

22Rv1 cells, deposited by Sramkoski *et al.* (11), were obtained from American Type Culture Collection (Manassas, VA). 22Rv1 cells were maintained in Ham's F-12:high-glucose DMEM (50:50) supplemented with 10% heat-inactivated FBS and 2 mM L-glutamine. For FACS analysis, cells were detached with 5 mM EDTA in 150 mM NaCl, washed once with medium, and resuspended in 1% FBS/PBS. Cell suspensions were counted, adjusted to 1×10^6/ml and rotated, at 4°C for 1 h with 10 μg/ml 4D5 (anti-HER2), or 2F9 (anti-HER3), or 6C5 (anti-EGFR) monoclonal antibodies (14, Genentech, South San Francisco, CA) or assay buffer (1% FBS/PBS). Cells were washed, the cell pellets resuspended in assay buffer containing 15 μg/ml FITC-conjugated $F(ab')_2$ fragment of goat antimouse IgG (Cappel/ICN, Aurora, OH) and incubated for 1 h at 4°C. After this incubation period, the cell suspensions were washed and resuspended in assay buffer for analysis using a FACScan (Becton Dickinson, Bedford, MA). Cells expressing the specific receptors are represented as geometric mean fluorescence.

For immunoprecipitation experiments, 22Rv1 cells were serum starved for 4 h and incubated with 100 nM rhuMAb 2C4 (Ref. 14; Genentech, South San Francisco, CA) for 30 min at room temperature. Cells were stimulated with heregulin β1(Genentech, South San Francisco, CA) at 1 nM for 15 min at room temperature. SKBR3 (American Type Culture Collection) and 22Rv1. Cells were lysed with Tris-buffered saline and 1% Ipegal-630 and 1× Phosphatase Inhibitor Cocktail II (Sigma, St. Louis, MO) and Complete Protease Inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN). Supernatant was immunoprecipitated with anti-HER2 Ab-8, MS-325 (NeoMarkers, Fremont, CA) overnight at 4°C followed by binding to protein G-Sepharose and then was washed four times. For Western blotting, proteins were separated by SDS-PAGE and transferred to 0.45 μm of nitrocellulose membrane. HER2, EGFR, HER3, HER4, and phosphotyrosine were detected by immunoblotting with the following antibodies, MS-730 (NeoMarkers), SC-03 (Santa Cruz Biotechnologies, Santa Cruz, CA), and MS-310 (NeoMarkers), SC-8050 (Santa Cruz Biotechnologies) and 4G10 05-321 (Upstate, Waltham, MA), respectively. The antibodies were diluted in Tris-buffered Saline/0.1% Tween 20 with 5% nonfat dry milk. Blots were detected by chemiluminescence, ECL Plus (Amersham, Piscataway, NJ).

Proliferation assays were performed with cells that were seeded into 96-well black microtiter plates at a density of 8000 cells/well in medium containing 10% FBS and were allowed to adhere overnight. The following day, the medium was removed and replaced with medium containing 0.1% FBS. After a 24-h period of serum starvation, the cells were treated with different concentrations of rhuMAb 2C4 for 1 h at room temperature, followed by the addition of either 1 nM heregulin or 1 nM TGF-α (Sigma). Cells were incubated for 4 days at 37°C. At the end of the incubation period, cell proliferation was measured using CellTiter-Glo Luminescent Viability assay (Promega, Madison, WI), and viable cells were reflected in relative luminescence units.

For xenograft studies, 1,000,000 22Rv1 cells were injected into 8-week-old female BALB/c nude mice (Charles River Labs, Wilmington, DE) in a 1:1 ratio of Matrigel (Collaborative Research, Bedford, MA) as described previously (11). When tumors reached ~100 mm^3, mice were randomly assigned to groups ($n = 10$) and were given i.p. injections of 100, 30, 10 mg/kg of rhuMAb 2C4, 100 mg/kg of isotype-matched control antibody, or vehicle control in a 100-μl volume. Injections and tumor measurements were carried out twice

Received 6/10/02; accepted 8/2/02.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] To whom requests for reprints should be addressed, at Molecular Oncology, One DNA Way, South San Francisco, CA 94080.

[2] The abbreviations used are: AI, androgen-independent; rhuMAb, recombinant humanized monoclonal antibody; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; TGF, tumor growth factor; EGFR, epidermal growth factor receptor.

weekly for up to 25 days. Tumors were isolated and analyzed by H&E and immunohistochemistry for HER2 status (HercepTest; DAKO, Carpinteria, CA).

RESULTS

Because HER2 forms heterodimers with other EGFR/HER receptor family members, the AI cell line 22Rv1 was evaluated for expression of EGFR, HER2, HER3, and HER4. Lysates from 22Rv1 cells were run by SDS-PAGE and were Western blotted onto nitrocellulose. The blots were probed with specific EGFR/HER receptor antibody, and EGFR, HER2, and HER3 were detected (Fig. 1*A*). In contrast, HER4 was not detected (data not shown), as anticipated, based on previous reports (15). HER2 levels were low in 22Rv1 cells compared with the level of control cell line SKBR3 (Fig. 1*A*). A characteristic doublet of highly expressed HER2 was detected in SKBR3 cells as shown previously (16). The expression of EGFR/HER receptors was confirmed using FACS analysis, which corroborated the Western blot data (Fig. 1*B*).

22Rv1 cells were stimulated with heregulin or TGF-α, and cell proliferation was measured in response to ligand-mediated activation. Stimulation of serum-starved 22Rv1 cells with 1 nM heregulin showed increased cell proliferation compared with control (Fig. 2), whereas TGF-α did not mediate a similar proliferative response (data not shown). rhuMAb 2C4 inhibited cell proliferation, driven by 1 nM heregulin, in a dose-dependent manner (Fig. 2). In contrast, increasing concentrations of rhuMAb 2C4 alone did not affect cell proliferation. These observations suggest that rhuMAb 2C4 inhibits 22Rv1 cell proliferation driven by ligand-mediated HER2/HER3 activation. To directly examine the effects of rhuMAb 2C4 on HER receptor phosphorylation, 22Rv1 cells were treated with or without heregulin in the presence or absence of rhuMAb 2C4. Heregulin-treated cells, immunoprecipitated with anti-HER2 antibody and blotted with phosphotyrosine antibody, demonstrated receptor phosphorylation, whereas 2C4 treatment inhibited this phosphorylation (Fig. 3). HER3 was detected in coimmunoprecipitates of HER2 only on heregulin stimulation, and EGFR was not coimmunoprecipitated, as anticipated (data not shown). However, treatment with rhuMAb 2C4 attenuates heregulin-driven association of HER3 with HER2 (Fig. 3). Because our *in vitro* observations suggest that rhuMAb 2C4 inhibits heregulin-mediated HER2 activation and resultant proliferation, we extended the analysis *in vivo* to examine the effect of rhuMAb 2C4 on the inhibition of tumor growth.

One million 22Rv1 cells, in a 1:1 ratio with Matrigel, were inoculated s.c. into BALB/c nude mice, and tumor growth was monitored. The histopathology of tumors revealed the growth of poorly differentiated prostatic adenocarcinoma. Immunohistochemical analysis

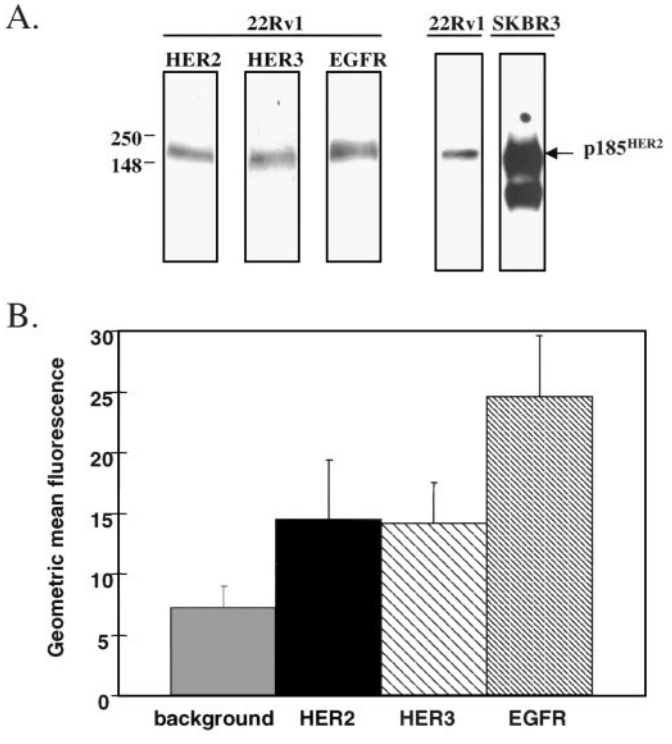

Fig. 1. *A*, EGFR/HER receptor expression in 22Rv1 cells observed by Western blot analysis. Lysate from 22Rv1 cells was subjected to SDS-PAGE and transferred to nitrocellulose. Blots were probed with antibodies that recognized the specified EGFR/HER receptors. Comparative Western blot of HER2 expression in 22Rv1 and SKBR3 cells. *B*, FACS analysis of EGFR/HER receptor expression in 22Rv1 cells. Specific antibodies that recognized EGFR, HER2, and HER3 were used to label 22Rv1 cells as described in "Materials and Methods," and were counted by FACS.

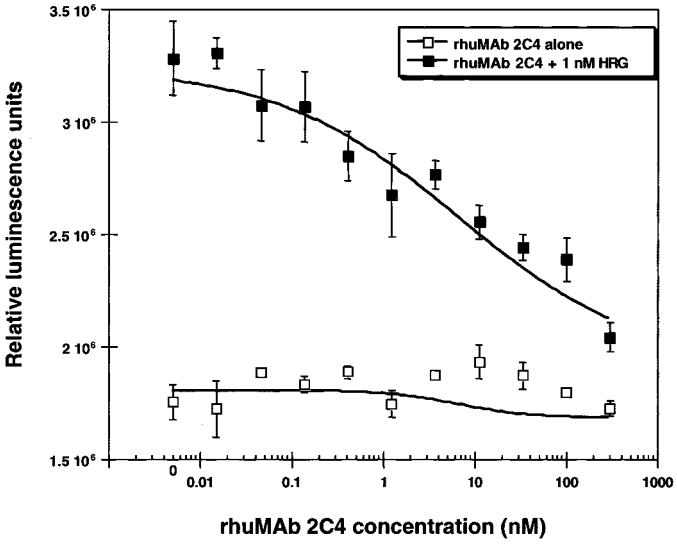

Fig. 2. rhuMAb 2C4 inhibition of 22Rv1 cell proliferation stimulated by heregulin. Serum-starved 22Rv1 Cells were treated with/without 1 nM heregulin (*HRG*) in the presence of increasing concentrations of rhuMAb 2C4, as described before. Dose-dependent inhibition by rhuMAb 2C4 of heregulin-stimulated cell proliferation is reflected as a decrease in relative luminescence.

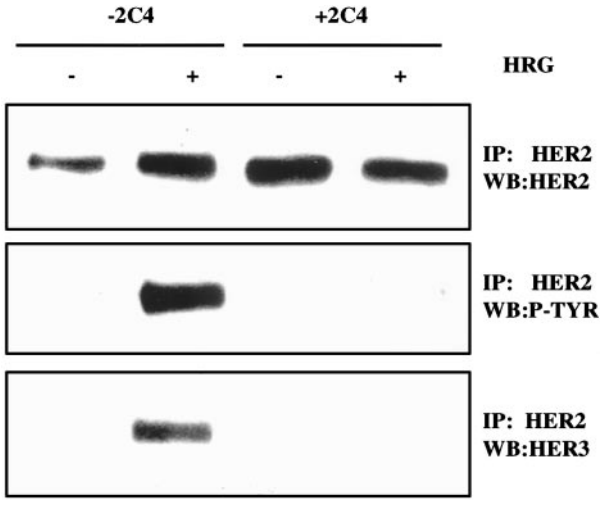

Fig. 3. Inhibition of heregulin-mediated activation of HER2. 22Rv1 cells were treated with or without heregulin (*HRG*) in the presence or absence of rhuMAb 2C4. Lysates were harvested as described in "Materials and Methods." 22Rv1 cell lysate was immunoprecipitated with anti-HER2 antibody. Immunoprecipitates (IP) were run by SDS-PAGE, transferred and probed with antibodies to phosphotyrosine (*P-Tyr*), HER2, or HER3.

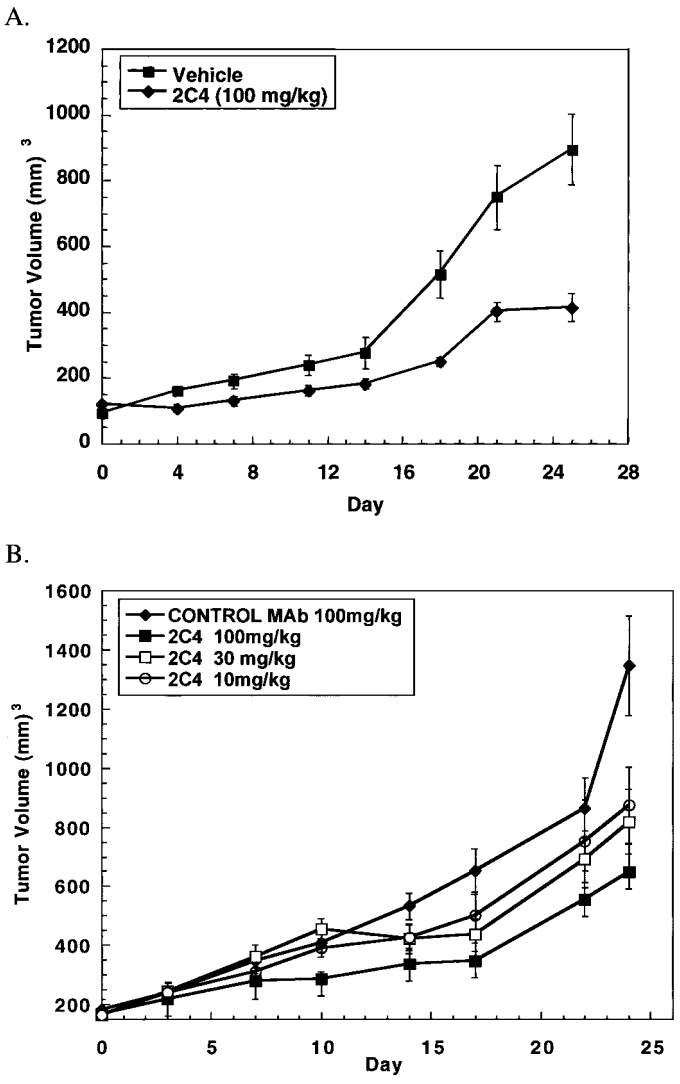

Fig. 4. Inhibition of tumor growth by rhuMAb 2C4 in the 22Rv1 xenograft model. One million 22Rv1 cells were injected with Matrigel into BALB/c nude mice. *A*, 10 mice/group were randomly selected after tumor establishment and were treated with vehicle alone or 100 mg/kg rhuMAb 2C4. *B*, 10 mice/group were randomly selected after tumor establishment and were treated with 100 mg/kg isotype-matched control antibody (*CONTROL MAb*) or with 100, 30, and 10 mg/kg rhuMAb 2C4 twice weekly for the duration of study. Tumor measurements taken twice weekly are shown.

showed low levels of HER2 expression (2+ HER2 by DAKO staining) in 22Rv1 tumor sections (data not shown). Once tumors were established, 100 mg/kg rhuMAb 2C4 or vehicle was administered to 10 mice/group, i.p., twice weekly for the duration of study. Inhibition of tumor growth was observed in rhuMAb 2C4-treated mice compared with the vehicle-treated control group (Fig. 4*A*).

To examine the dose response of rhuMAb 2C4 in these AI prostate tumors, 10, 30, and 100 mg/kg rhuMAb 2C4 were injected i.p., twice weekly into 10 mice/group, as described in the previous study. Isotype-matched control antibody was also injected into 10 mice as a control group. As before, the highest dose group of rhuMAb 2C4 (100 mg/kg) demonstrated inhibition of tumor growth compared with the control group, and a dose response was observed in comparing the different treatments with 30 and 10 mg/kg rhuMAb 2C4 (Fig. 4*B*). These data show that rhuMAb 2C4 inhibited tumor growth in a dose-dependent manner. Collectively, our studies demonstrated that rhuMAb 2C4 inhibits ligand-driven receptor phosphorylation and cell proliferation of 22Rv1 tumor cells. These *in vitro* observations are extended in our *in vivo* analyses by rhuMAb 2C4 inhibition of tumor growth in this AI prostate tumor model.

DISCUSSION

The role of HER2 in AI prostate tumors has been controversial because conflicting reports of HER2 receptor overexpression have been reported. Unlike human breast cancers in which gene amplification and up-regulation of HER2 receptor is well documented, there is no clear evidence to suggest a similar correlation in AI prostate cancer. Craft *et al.* (1) demonstrate *in vitro* that overexpression of HER2 is correlated with AI prostate tumorigenesis, and they elucidate a possible mechanism for AI prostate cancer.

Our analysis explores a uniquely different mechanism through ligand-mediated HER2 activation, cellular proliferation, and tumorigenesis. The detection of low HER2 levels suggests that overexpression of HER2 does not drive 22Rv1 cell proliferation. Other investigators have reported similar expression patterns of HER2, HER3, HER4, and EGFR in the CWR22 and CWR22R parental tumors (15, 17), and our data for the derivative 22Rv1 are consistent with these observations. Our results show that heregulin mediates the activation of the HER2/HER3 receptor complex in these AI 22Rv1 cells. Moreover, we show that rhuMAb 2C4 blocks receptor phosphorylation, most likely through inhibition of heregulin-mediated HER2/HER3 receptor dimerization. We also demonstrate *in vitro* that heregulin-driven cell proliferation of 22Rv1 is inhibited by rhuMAb 2C4. HER3 harbors an inactive kinase domain, and ligand-mediated kinase activation is driven by HER2 in these heterodimer complexes. Tyrosine phosphorylation of HER2 immunoprecipitates, detected by the 4G10 antibody, recognizes phosphorylation of HER2 and HER3 in this complex. Our results show that rhuMAb 2C4 effectively blocks phosphorylation of the heterodimer complex.

This inhibition extends to our *in vitro* cell proliferation and *in vivo* analyses. We show inhibition of tumor growth with rhuMAb 2C4 treatment of 22Rv1 mouse xenografts compared with the control group. It is formally possible, although unlikely, that 2C4 rhuMAb inhibition of tumor growth may be attributable to general immune mechanism(s) still intact in the nude mouse. Investigation of anti-HER2 treatment has been carried out in the parental CWR22 and CWR22R xenograft tumor models (18–20). However, *in vitro* analysis of CWR tumors has been hampered by an inability to maintain and characterize these transplant model cells in culture. Extensive characterization by cytogenetic analysis shows the 22Rv1 cell line represents one hyperdiploid stem line from CWR22 tumor. Injection of 22Rv1 cells into a xenograft model shows formation of a poorly differentiated adenocarcinoma with irregular glandular appearance and elevated prostate-specific antigen (11). Our histopathology analyses of 22Rv1 tumor xenografts are in agreement with previous observations. The tumors express low levels of HER2 (data not shown), consistent with our *in vitro* observations of low HER2 expression by Western blot and FACS analysis. In conclusion, the 22Rv1 cell line offers the distinct advantage of studying ligand-receptor activation in AI tumors *in vitro* and also correlatively *in vivo*. This model lends itself to the exploration of the role of ligand-mediated HER2 activation and the prospect of anti-HER2 for therapeutic use in AI prostate tumors.

ACKNOWLEDGMENTS

We acknowledge Franklin Okumu (Genentech Inc.) for providing rhuMAb 2C4. We thank Hartmut Koeppen for histopathology and the immunohistochemical analysis of the tumor samples.

REFERENCES

1. Craft, N., Shostak, Y., Carey, M., and Sawyers, C. L. A mechanism for hormone-independent prostate cancer through modulation of androgen receptor signaling by the HER-2/neu tyrosine kinase. Nat. Med., *5:* 280–285, 1999.
2. Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ulrich, A., and McGuire W. L. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science (Wash. DC), *235:* 177–182, 1987.
3. Scher, H. I. HER2 in prostate cancer—a viable target or innocent bystander? J. Natl. Cancer Inst. (Bethesda), *92:* 1866–1867, 2000.
4. Signoretti, S., Monitroni, R., Manola, J., Altimari, A., Tam, C., Bubley, G., Balk, S., Thomas, G., Kaplan, I., Hlatky, L., *et al*. Her-2-neu expression and progression toward androgen independence in human prostate cancer. J. Natl. Cancer Inst. (Bethesda), *92:* 1918–1925, 2000.
5. Carraway, K. L., Sliwkowski, M. X., Akita, R. W., Platko, J. V., Guy, P. M., Nuijens, A., Diamonti, A. J., Vandlen, R. L., Cantley, L. C., and Cerione, R. A. The *erbB3* gene product is a receptor for heregulin. J. Biol. Chem., *269:* 14303–14306, 1994.
6. Sliwkowski, M. X., Schaefer, G., Akita, R. W., Lofgren, J. A., Fitzpatrick, V. D., Nuijens, A., Fendly, B. M., Cerione, R. A., Vandlen, R. L., and Carraway, K. L. Coexpression of erbB2 and erbB3 proteins reconstitutes a high affinity receptor for heregulin. J. Biol. Chem., *26:* 14661–14665, 1994.
7. Lewis, G. D., Lofgren, J. A., McMurtrey, A. E., Nuijens, A., Fendly, B. M., Bauer, K. D., and Sliwkowski, M. X. Growth regulation of human breast and ovarian tumor cells by heregulin: evidence for the requirement of ErbB2 as a critical component in mediating heregulin responsiveness. Cancer Res., *56:* 1457–1465, 1996.
8. Westphal, M., Meima, L., Szonyi, E., Lofgren, J., Meissner, H., Hamel, W., Nikolics, K., and Sliwkowski, M. X. Heregulins and the ErbB-2/3/4 receptors in gliomas. J. Neurooncol., *35:* 335–346, 1997.
9. Xu, F., Yu, Y., Le, X. F., Boyer, C., Mills, G. B., and Bast, R. C. The outcome of heregulin-induced activation of ovarian cancer cells depends on the relative levels of HER-2 and HER-3 expression. Clin. Cancer Res., *5:* 3653–3660, 1999.
10. Venkateswarlu, S., Dawson, D. M., St Clair, P., Gupta, A., Willson, J. K., and Brattain, M. G. Autocrine heregulin generates growth factor independence and blocks apoptosis in colon cancer cells. Oncogene, *21:* 78–86, 2002.
11. Sramkoski, R. M., Pretlow, T. G., Giaconia, J. M., Pretlow, T. P., Schwartz, S., Sy, M. S., Marengo, S. R., Rhim, J. S., Zhang, D., and Jacobberger, J. W. A new human prostate carcinoma cell line, 22Rv1. In Vitro Cell. Dev. Biol. Anim., *35:* 403–409, 1999.
12. Pretlow, T. G., Wolman, S. R., Micale, M. A., Pelley, R. J., Kursh, E. D., Resnick, M. I., Bodner, D. T., Jacobberger, J. W., Delmoro, C. M., Giaconia, J. M., *et al*. Xenografts of primary human prostatic carcinoma. J. Natl. Cancer Inst. (Bethesda), *85:* 394–398, 1993.
13. Nagabhushan, M., Miller, C. M., Pretlow, T. P., Giaconia, J. M., Edgehouse, N. L., Schwartz, S., Kung, H. J., deVere White, R. W., Gumerlock, P. H., Resnick, M. I., Amini, S. B., and Pretlow, T. G. CWR22: the first human prostate cancer xenograft with strongly androgen-dependent and relapsed strains both *in vivo* and in soft agar. Cancer Res., *56:* 3042–3046, 1996.
14. Fendly, B. M., Winget, M., Hudziak, R. M., Lipari, M. T., Napier, M. A., and Ullrich, A. Characterization of murine monoclonal antibodies reactive to either the human epidermal growth factor receptor or *HER2/neu* gene product. Cancer Res., *50:* 1550–1558, 1990.
15. Wainstein, M. A., He, F., Robinson, D., Kung, H. J., Schwartz, S., Giaconia, J. M., Edgehouse, N. L., Pretlow, T. P., Bodner, D. R., Kursh, E. D., *et al*. CWR22: androgen-dependent xenograft model derived from a primary human prostatic carcinoma. Cancer Res., *54:* 6049–6052, 1994.
16. Harwerth, I. M., Wels, W., Marte, B. M., and Hynes, N. E. Monoclonal antibodies directed to the erbB-2 receptor inhibit *in vivo* tumor cell growth. J. Biol. Chem., *267:* 15160–15167, 1992.
17. Robinson, D., He, F., Pretlow, T. G., and Kung, H. J. A tyrosine kinase profile of prostate carcinoma. Proc. Natl. Acad. Sci. USA, *93:* 5958–5962, 1996.
18. Agus, D. B, Scher, H. I., Higgins, B., Fox, W. D., Heller, G, Fazzari, M, Cordon-Cardo, C., and Golde, D. W. Response of prostate cancer to anti-Her-2/neu antibody in androgen-dependent and -independent human xenograft models. Cancer Res., *59:* 4761–4764, 1999.
19. Agus, D. B., Akita, R. W., Fox, W. D., Lofgren, J. A., Higgins, B., Maiese, K., Scher, H. I., and Sliwkowski M. X. A potential role for activated HER-2 in prostate cancer. Semin. Oncol., *27:* 76–83, 2000.
20. Agus, D. B., Akita, R. W., Fox, W. D., Lewis, G. D., Higgins, B., Pisacane, P. I., Lofgren, J. A., Tindall, C., Evans, D. P., Maiese, K., Scher, H. I., and Sliwkowski, M. X. Targeting ligand-activated ErbB2 signaling inhibits breast and prostate tumor growth, Cancer Cell. In press, 2002.

Inhibition of Ligand-mediated HER2 Activation in Androgen-independent Prostate Cancer

Nerissa Mendoza, Gail Lewis Phillips, Johnny Silva, et al.

Cancer Res 2002;62:5485-5488.