Expression and Gene Copy Number Analysis of *ERBB2* Oncogene in Prostate Cancer

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An anti-*ERBB2* antibody, trastuzumab, has been shown to be highly efficient in the treatment of metastatic breast cancers overexpressing the *ERBB2* gene. It has been suggested that overexpression and even amplification of *ERBB2* may play a role in the development of prostate cancer. Here, we have analyzed gene copy number and expression of the *ERBB2* gene in both androgen-dependent primary and metastatic tumors, as well as recurrent hormone-refractory tumors. The expression levels were compared to the expression of *ERBB2* in breast cancers with or without *ERBB2* gene amplification. Of 126 prostate tumors, chromogenic *in situ* hybridization (CISH) revealed only 1 case containing borderline (six to eight copies) amplifications of *ERBB2*. This hormone-refractory tumor, however, did not express *ERBB2* protein. Immunohistochemical staining of *ERBB2* protein was negative (0 or 1+ intensity) in all prostate samples ($n = 124$) analyzed. To quantitate the level of *ERBB2* mRNA expression in prostate tumors ($n = 34$) and cell lines ($n = 3$), as well as in breast tumors ($n = 30$) and cell lines ($n = 16$), real-time reverse transcriptase-polymerase chain reaction (LightCycler) methodology was used. The expression level was similar in all prostate tumor types and corresponded to the level of expression in breast carcinomas without *ERBB2* amplification. Breast tumors with *ERBB2* amplification expressed, on average, ~20 times ($P < 0.001$) higher mRNA levels than prostate tumors or breast carcinomas without the gene amplification. In conclusion, the expression of *ERBB2* in prostate cancer is relatively low, and is not altered during disease progression. Thus, it is unlikely that treatment modalities relying on the overexpression of *ERBB2* gene will be useful in treating prostate cancer. (*Am J Pathol* 2002, 160:339–345)

ERBB2 gene (*HER-2/neu*) encodes for a 185-kd transmembrane glycoprotein that contains tyrosine kinase ac-

tivity and belongs to the epidermal growth factor receptor family.¹ The gene is located at chromosomal region 17q11-q21.^{2,3} Amplification of the *ERBB2* gene, leading to its overexpression, has been found, for example, in 25 to 30% of human breast and ovarian cancers. The amplification and overexpression is associated with poor prognosis in these malignancies.^{4–6} The development of anti-*ERBB2* antibody, trastuzumab (Herceptin; Genentech, South San Francisco, CA), has almost revolutionized the treatment of metastatic breast cancer.^{7,8} The addition of trastuzumab to standard chemotherapy leads to a significantly higher rate and prolonged duration of response as well as to a lower rate of death. However, it seems that only tumors overexpressing the *ERBB2* gene respond to trastuzumab.⁹

Although *ERBB2* has been extensively studied in prostate cancer, its significance has remained unclear.^{10,11} Some authors have reported increased expression of the gene, as detected by immunohistochemistry, in association with the progression, or even in premalignant lesions.^{12–15} Others have not found such overexpression in any prostate carcinomas.^{16,17} At the molecular level, it has been shown that *ERBB2* enhances signaling activity of the androgen receptor in the presence of low levels of androgens, at least in two xenograft models, LAPC-4 and LNCaP.^{18,19} Thus, *ERBB2* could be involved in the progression of prostate cancer to androgen independence. Interestingly, trastuzumab treatment has been shown to have a growth inhibitory effect on the prostate cancer xenograft models CWR22 and LNCaP.²⁰ Also the gene copy number of *ERBB2* has been analyzed in several studies. Most investigations have not shown high-level amplification of the *ERBB2* gene in prostate cancer.^{12,21–25} Still, in a few studies amplification of *ERBB2* has been found in up to 40% of prostate carcinomas.^{13,26,27}

To clarify the role on *ERBB2* in prostate cancer, we analyzed the expression of the gene in prostate cancer cell lines and tumors using both immunohistochemistry and quantitative real-time reverse transcriptase-polymer-

Supported by the Academy of Finland, Cancer Society of Finland, Reino Lahtikari Foundation, Finnish Cancer Institute, Medical Research Fund of Tampere University Hospital, Sigrid Juselius Foundation, Emil Aaltonen Foundation, Pirkanmaa Cancer Society, City of Tampere, and by the contract with the Finnish Life and Pension Insurance companies.

Accepted for publication October 3, 2001.

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ase chain reaction (RT-PCR) (LightCycler). *ERBB2* gene copy number was determined by chromogenic *in situ* hybridization (CISH). The expression levels of *ERBB2* in prostate cancer were compared to breast carcinomas with or without the gene amplification.

Materials and Methods

Cell Lines and Tumor Samples

Three prostate cancer cell lines (LNCaP, DU-145, PC-3) and 16 breast cancer cell lines (SK-BR-3, ZR-75-1, MDA-436, MDA-361, MDA-134, MDA-415, MDA-157, MCF-7, MCF-A10, BT-474, UACC-893, DU-4475, CAMA-1, HBL-100, HCC-1419) were obtained from the American Type Culture Collection (ATCC, Manassas, VA), and cultured according to the recommended conditions. For the analysis of *ERBB2* expression by RT-PCR freshly frozen specimens of 5 benign prostate hyperplasias, 21 primary prostate carcinomas (prostatectomy specimens), and 8 locally-recurrent hormone-refractory prostate carcinomas (TURP specimens from patients treated with hormonal therapy) were obtained from Tampere University Hospital. For the immunohistochemical and CISH analyses formalin-fixed paraffin-embedded tumor samples of 54 primary untreated (prostatectomy specimens) and 50 locally recurrent hormone-refractory prostate carcinomas (TURP specimens) from Tampere University Hospital, as well as 22 untreated lymph-node metastases of prostate cancer from Lund University Hospital, were obtained. Freshly frozen and corresponding formalin-fixed paraffin-embedded tumor blocks from 30 untreated primary breast carcinomas obtained from Tampere University Hospital were also analyzed by RT-PCR, immunohistochemistry, and CISH. For the immunohistochemical and CISH analyses, tissue microarray blocks were constructed according to published guidelines.²⁸

Chromogenic in Situ Hybridization

CISH was done on 5- μ m-thick archival formalin-fixed paraffin-embedded tissue sections with minor modifications to the previously described protocol.²⁹ In brief, the sections were deparaffinized and incubated in 0.1 mol/L of Tris-HCl (pH 7.3) in a temperature-controlled microwave oven (at 92°C for 10 minutes). The sections were then allowed to cool down for 20 minutes and washed with phosphate-buffered saline (PBS). Enzymatic digestion was done by applying 100 μ l of digestion enzyme on to slides for 10 to 15 minutes at room temperature (Digest-All III solution; Zymed Inc., South San Francisco, CA). The slides were then washed with PBS and dehydrated with graded ethanol. The ready-to-use digoxigenin-labeled *HER-2/neu* probe (consisting of two contig BAC clones; Zymed, Inc.) was applied onto the slides. The slides were denatured on a hot plate for 3 minutes, and the hybridization was performed overnight at 37°C. After hybridization, the slides were washed with 0.5 $\times$ standard saline citrate for 5 minutes at 75°C, followed by three washes in PBS/0.2% Tween20 (at room temperature). The *HER-2/*

neu probe was detected with sequential incubations with mouse anti-digoxigenin (diluted 1:300; Roche Biochemicals, Mannheim, Germany), anti-mouse-peroxidase polymer (Povervision, Immunovision Inc., Daly City, CA), and diaminobenzidine chromogen. Tissue sections were lightly counterstained with hematoxylin and embedded.

The CISH hybridizations were evaluated using $\times 40$ and $\times 60$ dry objectives and using 10 $\times$ 22 wide-field oculars. Amplification was defined as six or more signals per nucleus in more than 50% of cancer cells, or when a gene copy cluster was seen. Cases with other types of copy number alterations were considered as aneuploid.

Immunohistochemistry

The sections were first deparaffinized, followed by antigen retrieval (autoclave cooking at 121°C for 2 minutes in 10 mmol/L of sodium citrate buffer, pH 6.0). p185^{HER2} protein was detected with the monoclonal antibody CB11 (Novocastra Laboratories, Newcastle, UK). The antibody was diluted 1:200 in Povervision blocking solution (Immunovision Inc.) and incubated overnight at +4°C using Shandon Sequenza immunostaining cover plates (Shandon, Pittsburgh, PA). A conjugate of anti-mouse antibody and horseradish peroxidase was used for visualization (30 minutes at room temperature) with diaminobenzidine as a chromogen (PowerVision, Immunovision Inc.). Sections were counterstained with hematoxylin. Evaluation of the staining was done as described previously.²⁹ Briefly, intense membranous staining in >50% of cancer cells was considered as 3+. Staining present in a smaller proportion of the cells or lower intensity was considered as 2+ staining. No staining or faint staining in a few cells were scored as 0 and 1+, respectively.

RT-PCR

The freshly frozen specimens were first histologically examined for the presence of carcinoma cells using hematoxylin and eosin-stained slides. Only samples containing more than 60% of cancerous or hyperplastic tissue were selected for the gene expression analyses. One to three 20- μ m frozen sections were cut using a cryotome. Total RNAs were isolated from the sections using Qiagen RNeasy MiniKit (Qiagen Inc., Valencia, CA), and used for the first-strand cDNA synthesis with Superscript II reverse transcriptase and oligo d(T)₁₂₋₁₈ primer according to the manufacturer's protocol (Life Technologies, Inc., Gaithersburg, MD). Total RNA from prostate and breast cancer cell lines was isolated using Trizol reagent (Life Technologies, Inc.) according to the manufacturer's instructions, and reverse-transcribed as described above. For preparing the standard curve, 5 μ g of totRNA from prostate cancer cell line LNCaP (ATCC), was reverse-transcribed. After the first strand cDNA synthesis serial dilutions (one to five) were made to correspond cDNA transcribed from 500, 100, 20, 4, 0.8, and 0.16 ng of totRNA.

Primers for the *ERBB2* and TATA box-binding protein (*TBP*) genes were designed with the assistance of the

Table 1. The Primer and Probe Sequences Used in the Real-Time RT-PCR

Gene	Primer sequences (5'-3')	Hybridization probe sequences (5'-3')
<i>ERBB2</i>	AGC CGC GAG CAC CCA AGT TTG GTG GGC AGG TAG GTG AGT T	CCTGCCAGTCCCGAGACCCACCT-Fluorescein Red640-CATGCTCCGCCACCTCTACCAGGG
<i>TBP</i>	GAATATAATCCCAAGCGGTTTG ACTTCACATCACAGTCCCC	TTTCCCAGAACTGAAAATCAGTGCC-Fluorescein Red640-TGGTTCGTGGCTCTCTTATCCTCATG

Primer3 program (available at <http://www-genome.wi.mit.edu/cgi-bin/primer3.www.cgi>). To avoid amplification of any genomic DNA, the forward and reverse primers for each gene were chosen from different exons. The sizes of PCR products were designed to be under 400 bp to optimize the RT-PCR measurements. Primer and probe sequences for the genes are given in Table 1.

The PCR reactions were performed in the LightCycler apparatus³⁰ using the LC-FastStart DNA Hybridization Probes Kit (Roche Diagnostics, Mannheim, Germany). Thermocycling for each reaction was done in a final volume of 20 μ l containing 2 μ l of cDNA sample (or standard), 4 mmol/L of MgCl₂, 0.5 μ mol/L of each primer, 0.2 μ mol/L of fluorescein, and 0.4 μ mol/L of LC Red640-labeled probes as well as 1 $\times$ ready-to-use reaction mix including *Taq* DNA polymerase, reaction buffer, and dNTP mix. After 10 minutes of initial denaturation at 95°C, the cycling conditions of 50 cycles consisted of denaturation at 95°C for 3 seconds, annealing at 65°C (for *ERBB2*) or 58°C (for *TBP*), and elongation at 72°C for 7 seconds. The LightCycler apparatus measured the fluorescence of each sample in every cycle at the end of the annealing step. After proportional background adjustment, the fit point method was used to determine the cycle in which the log-linear signal was distinguished from the background, and that cycle number was used as the crossing-point value. The software produced the standard curve by measuring the crossing point of each standard and plotting them against the logarithmic values of concentrations. The expression level of *ERBB2* was normalized by the housekeeping gene *TBP*. *TBP* was chosen for the reference gene, because there are no known retropseudogenes for it and the expression of *TBP* is lower than in many commonly used abundantly expressed reference genes.³¹ After the PCR, every sample was also run in an agarose gel electrophoresis to ensure that a right size product was amplified in the reaction.

Statistical Analyses

The association of the gene copy number and tumor type with the expression level was calculated with nonparametric Kruskal-Wallis test with Dunn's multiple comparisons test as well as with the Mann-Whitney *U* test.

Results

ERBB2 Gene Amplification by CISH

Figure 1 shows an example of high-level amplification in a breast carcinoma as well as a prostate tumor without copy number alteration. None of the 54 untreated primary

prostate cancers showed high-level amplification of *ERBB2* oncogene (Table 2). Fourteen (26%) cases showed an aneuploid gene copy number (three to five copies per cell). Eight (36%) of the untreated lymph node metastases were aneuploid, and rest of the cases (*n* = 14) showed two copies of the gene. Of the 50 hormone-refractory tumors 17 (34%) were aneuploid. One hormone-refractory tumor (2%) showed six to eight copies of *ERBB2* gene (per cell), which is considered as a borderline amplification. The remaining 32 samples (64%) showed only two copies of the gene. Eight of the 30 (27%) breast cancer control specimens showed high-level amplification of *ERBB2*.

Expression of ERBB2 Protein by Immunohistochemistry

None of the androgen-dependent primary (*n* = 54) prostate tumors, lymph-node metastases (*n* = 20), or locally recurrent hormone-refractory tumors (*n* = 50) showed clearly positive immunostaining (with 2+ or 3+ intensity) (Table 2). In contrast, all eight breast carcinomas containing *ERBB2* amplification showed typical strong membranous staining indicating overexpression of the gene (Figure 1).

Expression of ERBB2 Gene by RT-PCR

Figure 2 shows a standard curve of the real-time RT-PCR measurement of *ERBB2* mRNA. The dynamic range was wide, corresponding to cDNA transcribed from 0.16 to 500 ng of total RNA and linear relationship between fractional cycle and fluorescent threshold was strong ($r^2 \sim 1$). In addition to *TBP*, the expression of β -actin was measured and used alternatively for normalization in most of the samples (data not shown). The results were similar with both control genes. Because of the potential problems with the β -actin retropseudogenes, *TBP* was chosen for normalization in the whole material.

The breast cancer cell lines with *ERBB2* amplification (BT-474, UACC-893, HCC-1419, SK-Br-3, MDA-361),³²⁻³⁴ showed almost 20 $\times$ higher expression of the gene (*P* = 0.0009) than cell lines without amplification (Figure 3). ZR75-1 with only a gain of *ERBB2* expressed approximately five times more mRNA of the gene than the other cell lines without high-level amplification, whereas MDA-361 with the gene amplification expressed only 2.5 times more of the gene than cell lines without the amplification. Of the cell lines with *ERBB2* amplification, the level of amplification is lowest in the MDA-361.³² Of the three prostate cancer cell

Figure 1. CISH analysis of *ERBB2* copy number in breast (A) and prostate (B) carcinoma. Multiple signals indicating high-level amplification of the gene is seen in breast cancer. Only two copies of *ERBB2* are found in prostate cancer. Immunohistochemical analysis of *ERBB2* protein expression in breast (C) and prostate (D) carcinoma. Strong membranous staining indicating overexpression of the gene is seen in breast cancer, whereas no immunostaining is found in prostate cancer. Black and white photos.

lines (of which none contains *ERBB2* amplification), only LNCaP expressed the gene in the range of ZR75-1 and MDA-361.

There were no significant differences in the expression levels of *ERBB2* between the benign prostate hyperplasia and prostate cancers, either androgen-dependent or refractory, or the breast tumors without *ERBB2* gene amplification (Figure 4). The level of expression was below the

lowest standard (0.16) in 33% of the benign prostate hyperplasia, 19% of the untreated and 25% of the hormone-refractory prostate carcinomas as well as in 32% of the untreated breast carcinomas. The median value of *ERBB2* expression in the breast cancer tumors with the *ERBB2* gene amplification was $\sim 20\times$ higher than in the breast cancers without the amplification or prostate tumors ($P < 0.0001$).

Table 2. Gene Copy Number and Protein Expression of *ERBB2* Gene in Prostate Cancer

Tumor type	Gene copy number of <i>ERBB2</i> by CISH			Expression of <i>ERBB2</i> by IHC	
	Diploid	Aneuploid	High-level amplification	0 to 1+	2+ to 3+
Primary	40	14	0	54	0
Lymph node metastases	14	8	0	20	0
Hormone-refractory tumors	32	17	1	50	0

Figure 2. Standard curve of the *ERBB2* expression measurement by real-time RT-PCR. **A:** Serially diluted standard containing cDNA transcribed from 500, 100, 20, 4, 0.8, and 0.16 of total RNA. Cycle number is plotted against fluorescent signal obtained in every cycle at the end of the annealing step. **B:** Standard curve—plotting cycle number (crossing point), in which fluorescence levels of each standard are the same at the lower part of the log-linear phase of the amplification curve, against logarithmic value of the each standard.

Discussion

Using CISH analysis, we have shown here that *ERBB2* oncogene is not highly amplified in either androgen-dependent or -independent primary or metastatic prostate carcinomas. Only one case with six to eight copies of *ERBB2* was found. Based on the copy number, this tumor could be classified as borderline amplification. However, the tumor showed no *ERBB2* protein overexpression by immunohistochemistry. Therefore, we conclude that *ERBB2* is not the true target gene of the amplification in this particular case. These findings are in a good agree-

Figure 3. Relative expression of *ERBB2* in prostate and breast cancer cell lines by real-time RT-PCR. The highest expression is found in the breast cancer cell lines containing the *ERBB2* gene amplification.

Figure 4. Relative expression of *ERBB2* in prostate and breast tumor samples analyzed by real-time RT-PCR. Median value of expression is shown by a horizontal line. The amplification of the *ERBB2* gene in breast tumors (*ERBB2*+) was detected by CISH. The expression of *ERBB2* was significantly higher in the breast carcinomas with the gene amplification than in the other tumor types ($P < 0.001$, Kruskal-Wallis test). There were no differences in the expression levels of *ERBB2* in the other tumor groups.

ment with most of the previously published studies, which have analyzed *ERBB2* amplification in prostate cancer either by Southern blotting or fluorescence *in situ* hybridization.^{12,21–24} However, there are a few published studies reporting *ERBB2* amplification in prostate cancer as detected by fluorescence *in situ* hybridization.^{13,26,27} One possible explanation for these discrepancies is that the definition of gene amplification varies from one study to another. Here, we used a definition, which has been validated in breast cancer studies in which significant *ERBB2* amplification is considered to contain more than five copies of the gene.^{9,29} Only such amplification is associated with response to trastuzumab and poor prognosis in breast cancer. The fact that we found one borderline case, which did not overexpress the gene, indicates the difficulty in the definition of significant amplification.

Although we did not find *ERBB2* amplification in prostate cancer, we still wanted to evaluate the level of expression of the gene. First, we used immunohistochemical staining, which is the most commonly used technique to detect overexpression of the *ERBB2* gene. We did not find 2+ or 3+ intensity of immunostaining of *ERBB2* in any of the prostate tumors by immunohistochemistry. As positive and negative controls, we used breast carcinomas that showed clear overexpression consistent with gene amplification. Although our findings are in an agreement with some published reports,^{16,17} several immunohistochemical studies have reported overexpression of *ERBB2* in prostate cancer.^{12–14} For example, Signoretti and colleagues¹² recently reported immunostaining of *ERBB2* in 25% of the untreated and 78% of the androgen-independent prostate tumors analyzed. Thus, the authors concluded that *ERBB2* overexpression is associated with progression of the disease to androgen independence. The discrepancies in the immunohistochemical findings are probably because of the different antibodies and staining technique that were used, as well as because of

inconsistent interpretation of the staining results. For example, Signoretti and colleagues¹² used AO485 antibody from DAKO (Carpinteria, CA), whereas CB11 antibody (Novocastra Laboratories) was used in this study. The immunostaining with the antibody CB11 and high-temperature antigen retrieval as performed here has been shown to correlate well with gene amplification by fluorescence *in situ* hybridization and CISH, as well as with poor prognosis in breast cancer.^{29,35,36} Thus, we believe the staining method used has high sensitivity and specificity in detection of overexpression of *ERBB2*, which associates with gene amplification.

Still, because of the above-mentioned shortcomings of immunohistochemistry, we decided also to use the real-time RT-PCR approach to measure the expression of *ERBB2*. In real-time PCR, the quantification of the template is based on detection of the cycle in which the reaction enters the exponential phase, instead of measuring the amount of end product. Thus, none of the reagents are rate limiting in the reaction at the time of measurement of the fluorescence. For the detection of the amplified fragment, we used hybridization probes consisting of two different oligonucleotides that hybridized to the internal sequence of the fragment during the annealing phase of PCR cycles. Thus, to produce signal four different oligonucleotides (two primers and two probes) have to be annealed to their targets confirming that only the PCR product of interest and nothing else is measured. Several studies have already shown that such real-time RT-PCR is a highly quantitative and reliable method.^{31,37–41}

A majority of the prostate tumors showed detectable mRNA levels (higher than the lowest standard) of *ERBB2* by real-time RT-PCR. The level of expression was low both in cell lines and tumors, and there were now significant differences in the expression between the androgen-dependent or -independent tumors. The levels of expression corresponded to the expression found in breast carcinomas that do not contain the *ERBB2* gene amplification. The breast tumors containing *ERBB2* gene amplification expressed ~20× higher levels of the gene than the prostate tumors. Three of the eight breast carcinomas and one cell line containing the amplification showed mRNA expression in approximately the same levels as the highest expressors of the breast or prostate tumors and cell lines without the amplification. These three breast tumors with relatively low expression of *ERBB2* by RT-PCR, despite the gene amplification, showed, however, overexpression by immunohistochemistry suggesting that either the PCR reaction or the quality of RNA has not been optimal in these samples. This conclusion is also supported by the fact that three breast tumors without *ERBB2* gene amplification, which according to RT-PCR expressed *ERBB2* at comparable levels as the three above-mentioned cases containing the gene amplification, did not show overexpression by immunohistochemistry. The RT-PCR runs for these cases were repeated twice. Whether the low-level expression of the gene, which can be detected by very sensitive RT-PCR technique, in the prostate tumors has any functional significance remains to be studied.

In conclusion, the results indicate that amplification and overexpression of the *ERBB2* (*Her-2/neu*) oncogene is absent or at least very rare in prostate cancer. The level of expression of *ERBB2* was similar in all prostate tumor types (benign prostate hyperplasia, hormone-naïve or hormone-refractory carcinomas) and it was in the same levels as in breast carcinomas without the gene amplification. Thus, it is unlikely that treatment strategies based on *ERBB2* overexpression, such as trastuzumab, will be effective in the treatment of prostate cancer.

Acknowledgments

We thank Mrs. Sari Toivola and Mrs. Mariitta Vakkuri for technical assistance.

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