

Characterization of a novel cell line established from a patient with Herceptin-resistant breast cancer

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Abstract

Clinical resistance to the *HER-2* oncogene-targeting drug trastuzumab (Herceptin) exists, but studies of the resistance mechanisms are hampered by the lack of suitable experimental model systems. We established a carcinoma cell line (designated JIMT-1) from a pleural metastasis of a 62-year old patient with breast cancer who was clinically resistant to trastuzumab. JIMT-1 cells grow as an adherent monolayer and form xenograft tumors in nude mice. JIMT-1 cells have an amplified *HER-2* oncogene, which showed no identifiable mutations in its coding sequence. JIMT-1 cells overexpress *HER-2* mRNA and protein, and the levels of *HER-1*, *HER-3*, and *HER-4* mRNA and protein were similar to the trastuzumab-sensitive cell line SKBR-3. The cell line lacks expression of hormone receptors (estrogen receptors and progesterone receptors) and is phenotypically of epithelial progenitor cell origin, as evidenced by immunohistochemical positivity for both cytokeratins 5/14 and 8/18. JIMT-1 cells were insensitive to trastuzumab and another *HER-2*-inhibiting drug, pertuzumab (2C4), *in vitro* and in xenograft tumors. Small molecule tyrosine kinase inhibitors Ci1033 and ZD1839 inhibited the JIMT-1 cell

growth but to a lesser degree than in trastuzumab-sensitive BT-474 cells. The lack of growth inhibition was rationalized by the unaltered Akt phosphorylation in JIMT-1 cells. Erk1/2 phosphorylation was slightly reduced but still evident in JIMT-1 cells. We conclude that the JIMT-1 cell line provides a valuable experimental model for studies of new trastuzumab-resistance mechanisms. [Mol Cancer Ther 2004;3(12):1585–92]

Introduction

The *HER-2/neu* oncogene encodes a 185-kDa transmembrane tyrosine kinase receptor that is amplified and overexpressed in ~20% of human breast cancers (1). *HER-2* positivity is associated with a high risk of relapse and death and, according to some studies, with an altered response to hormonal and cytotoxic therapies (1). A major breakthrough in anticancer therapy was the finding that monoclonal antibodies against *HER-2* protein can inhibit growth of cancer cells that overexpress this receptor on the cell surface (2). This led to the development of trastuzumab (Herceptin), a humanized monoclonal *HER-2* antibody, which shows remarkable activity against metastatic breast cancer (3, 4). Trastuzumab currently is widely used in metastatic breast cancer and is under extensive clinical study as an adjuvant postsurgical therapy for early disease (5).

When trastuzumab is given as a single agent for first-line treatment of *HER-2*-overexpressing metastatic breast cancer, objective clinical responses are achieved in 40% of patients (6). If used in combination with taxanes or vinorelbine, the response rate can be up to 60% to 80% (6). In the remaining patients, no tumor regression is observed, although the *HER-2* gene is amplified and the protein is overexpressed both in the primary tumor and in metastases (7). Thus, other currently unknown factors besides *HER-2* positivity must play a role in determining the degree of sensitivity to trastuzumab. Identification of potential resistance mechanisms is crucial when developing new molecular markers, which could be used to identify nonresponding patients among those currently considered eligible for Herceptin. Moreover, identification of resistance mechanisms may lead to development of new drugs, which could overcome the resistance or have additive or synergistic antitumor effect when given in combination with trastuzumab.

Studies of trastuzumab resistance are hampered by the lack of suitable experimental model systems. Whereas a number of *HER-2*-positive breast cancer cell lines have been used in experimental studies, none of them have been characterized as being intrinsically resistant or insensitive to trastuzumab. Here we describe and characterize the functional status of *HER-2* receptor in a new cell line and xenograft derived from a metastasis of a patient with breast cancer who was clinically resistant to trastuzumab.

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Materials and Methods

Drugs

Humanized monoclonal antibodies trastuzumab (Herceptin, Roche) and pertuzumab (2C4, Genentech, San Francisco, CA) were preserved at +4°C for short-term or at -20°C for long-term storage. Rituximab (Mabthera, a humanized anti-CD20 antibody, Roche, Basel, Switzerland) was used as a control. A pan-ErbB inhibitor Ci1033 (Pfizer, Plymouth, MI) and epidermal growth factor receptor (EGFR) inhibitor ZD1839 (Iressa, AstraZeneca Pharmaceuticals, Macclesfield, United Kingdom) were diluted in DMSO as 10 mmol/L stock solutions. These stock solutions were stored at -20°C and diluted with PBS at concentrations indicated in each experiment.

Clinical History of the Donor Patient

The sample used to initiate the JIMT-1 cell line was derived from a patient diagnosed with breast cancer at the age of 62 years. The tumor was a grade 3 invasive ductal breast cancer (T2N1M0), for which the patient underwent an operation with radical mastectomy and axillary lymph node evacuation. Metastases were found in 1 of 12 lymph nodes examined. The patient was recruited to a randomized, adjuvant trastuzumab therapy trial (the FinHER study). Adjuvant therapy was started 7 weeks after operation. According to the trial schedule, the patient was randomized to receive nine courses of weekly trastuzumab (4 mg/kg initial dose, continued with 2 mg/kg) and vinorelbine (25 mg/m²) followed by three courses of standard dose CEF: cyclophosphamide (600 mg/m²), epirubicin (60 mg/m²), and fluorouracil (600 mg/m²) i.v. thrice weekly. Postoperative radiation therapy (50 Gy) was applied to the ipsilateral regional lymph nodes and thoracic wall after chemotherapy.

Two weeks after completion of radiation therapy, ipsilateral pleural effusion was diagnosed. Cytologic examination of the aspirated pleural fluid revealed carcinoma cells. Therapy for distant metastatic disease was initiated with thrice-weekly single-agent trastuzumab (8 mg/kg initial dose). The disease progressed during the first three weeks (extensive accumulation of pleural fluid bilaterally), and a palliative pleural puncture was clinically necessary. Material for cell culture came from the second aspirate. Therapy was continued with a weekly trastuzumab (2 mg/kg) and paclitaxel (80 mg/m²) combination. After 3 weeks, accumulation of pleural fluid still continued aggressively. Subsequent palliative procedures (pleurodesis and Denver shunt) did not improve the patient's condition and she died 12 weeks after the first diagnosis of distant metastasis.

The JIMT-1 Cell Line

Approval to use the cells for culture was obtained from the patient and the local ethical committee prior to the study. Aspirated pleural fluid was centrifuged and placed in culture dishes. The cells were grown in various culture media for 6 months, during which a medium containing Ham's F-12/DMEM (50%/50%), penicillin/streptomycin (100 units/100 mg), L-glutamine (2 mmol/L), fetal bovine

serum (10%, HyClone, lot CMA 0114, Logan, UT), and insulin (0.3 units/mL, Protaphan 100 units/mL, Novo Nordisk, Bagsvaerd, Denmark) was found to be optimal. The resulting cell line, designated JIMT-1, grows continuously when the culture medium was changed thrice per week. Confluent cultures were trypsinized for 5 minutes at +37°C, and split into new cultures with ratios of 1:2 or 1:3. The cells have by April 2004 undergone 40 passages. Cryopreservation was done in 90% fetal bovine serum/10% DMSO. To test xenograft tumor formation, ~ (3-5) × 10⁶ trypsinized JIMT-1 cells were injected s.c. into nude mice in PBS.

For comparative purposes, HER-2-positive and trastuzumab-sensitive cell lines SKBR-3, BT-474, and N-87 were grown under conditions recommended for each line. For a positive control in the HER-2 phosphorylation experiment, MCF-7 cells were starved overnight in medium containing 1% serum and half of them stimulated with 50 ng/mL neuregulin (NRG-1-β1, R&D Systems, Minneapolis, MN) for 10 minutes at 37°C. For drug-sensitivity experiments, cell cultures were serum starved overnight in serum-free media.

Genetic and Phenotypic Characterization

DNA fingerprinting, karyotyping by multicolor fluorescence *in situ* hybridization (mFISH), comparative genome hybridization, and gene expression profiling of JIMT-1 cells have been described elsewhere.⁶ The primary tumor specimen from the patient and a formalin-fixed, paraffin-embedded xenograft were studied immunohistochemically for estrogen receptor (clone 6F11, Novocastra Laboratories, Newcastle, United Kingdom), progesterone receptor (clone 312, Novocastra), HER-2 (clone CB-11, Novocastra), Ki-67 (clone MM1, Novocastra), PTEN (clone 6H2.1, Cascade Biosciences, Winchester, MA), TP53 (clone DO-7, Novocastra), cytokeratin 8/18 (clone 5D3, Novocastra), cytokeratin 5/14 (clones XM26 and LL002, LabVision, Fremont, CA), vimentin (clone V9, LabVision), smooth muscle actin (clone 1A4, LabVision), and p63 (Ab-4, LabVision). FISH and chromogenic *in situ* hybridization (CISH) were done to study the amplification of *HER-2* (7).

DNA Sequencing

The genomic DNA of JIMT-1 cell line was amplified using a standard PCR protocol. The forward and reverse oligonucleotide primers used to amplify *HER-2* exons 1 to 27 are available on request. The PCR products were purified using a Montage DNA purification column (Millipore Co., Bedford, MA). Direct sequencing of PCR products was done using BigDye3 termination chemistry (Applied Biosystems, Foster City, CA) and an ABI 310 genetic analyzer (Applied Biosystems) according to the manufacturer's instructions.

Expression of HER-1 to HER-4 Proteins

HER-1 to HER-4 protein expression and the degree of constitutive HER-2 protein phosphorylation were analyzed by Western blotting, as previously described (8). Primary antibodies against EGFR (1005), NEU (C-18), HER-3 (C-17),

⁶ K. Rennstam, et al. Cytogenetic characterization and gene expression profiling of the Herceptin-resistant breast cancer cell line JIMT-1, submitted for publication.

and HER-4 (C-18, Santa Cruz Biotechnology, Santa Cruz, CA) were used to detect HER-1, HER-2, HER-3, and HER-4 proteins, respectively. All primary antibodies were used at a dilution of 1:1,000 and visualized by means of peroxidase-conjugated goat anti-rabbit immunoglobulin G (1:10,000 dilution, Jackson ImmunoResearch Laboratories, West Grove, PA), enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ), and autoradiography.

Real-time Quantitative Reverse Transcription – PCR

To quantitate HER gene family expression at the mRNA level, real-time reverse transcription–PCR (TaqMan) was done as described elsewhere (9). Total RNA from cultured cells were isolated using a GenElute mammalian total RNA kit (Sigma-Aldrich Chemie GmBn, Steinheim, Germany). One microgram of DNase I (Gibco Life Technologies, Inc., Gaithersburg, MD)–treated total RNA was reverse transcribed to cDNA using M-MLV RNase H minus reverse transcriptase (Promega, Madison, WI) and random hexamer primers (Promega). Samples were analyzed in triplicate; in each measurement, SD of the threshold cycle (C_T) values was <2% of the mean. Expression of each HER transcript was presented as the percentage of HER mRNA expression relative to the expression of the internal control gene (for β -actin).

Trastuzumab Binding

To study binding of trastuzumab to HER-2 protein, 1 mg of total cell lysate from JIMT-1 xenograft tumor was precleared for 3 hours and subjected to overnight immunoprecipitation with 10 μ g of trastuzumab as previously described (8). The presence of HER-2 in the precipitates was detected by Western blotting using Neu C-18 antibody as described above. An irrelevant humanized monoclonal antibody rituximab (recognizing lymphocyte surface antigen CD20) was used as negative control. To study heterodimerization of HER-2 and HER-3, 500 μ g of total cell lysates were subjected to immunoprecipitation with polyclonal antibody C-18 that recognizes HER-2 (sc-284, Santa Cruz Biotechnology), as previously described (8). The presence of HER-3 in the precipitates was detected by Western blotting using polyclonal HER-3 antibody C-17 (sc-285, Santa Cruz Biotechnology) as described above.

Detection of HER-2 Ectodomain Shedding

The presence of HER-2 ectodomain was measured with human sp185^{HER-2} instant ELISA kit (Bender Medsystems GmbH, Vienna, Austria). Serum samples of mice bearing JIMT-1 and N-87 xenografts and cell culture media from 3-day cultures of JIMT-1 and BT-474 were diluted 1:20 for the ELISA measurements.

Immunoblotting of Akt and Erk1/2

Total cell lysates were separated using SDS-PAGE followed by Western blotting with the sc-8321 antibody (Santa Cruz Biotechnology) recognizing both the phosphorylated and nonphosphorylated forms of Akt or with the P-4112 antibody (Sigma, Schnellendorf, Germany) against Akt phosphorylated on Ser⁴⁷³. For studies of Erk1/2 activation we used antibody recognizing Erk [p44/42 mitogen-activated protein kinase (MAPK) 9102, Cell Signaling

Technologies, Beverly, MA] and its phosphorylated form, phospho-Erk T202/Y204 antibody 9101S (New England Biolabs, Beverly, MA). As a blotting control, actin antibody sc1616 (Santa Cruz Biotechnology) was used. A peroxidase-conjugated secondary antibody was used in conjunction with enhanced chemiluminescence (Amersham, Freiburg, Germany) to visualize the bands that were quantitated using Gel-Pro analyzer (Media Cybernetics, Silver Spring, MD).

In vitro Assay of Drug Sensitivity

The effect of trastuzumab on the growth of JIMT-1 and BT-474 cells was examined by using the alamarBlue method (TREK Diagnostic Systems, Inc. Cleveland, OH). The cells were cultured in serum-free conditions overnight, trypsinized, and plated at a density of 5,000 cells per well in 96-well, flat-bottomed, tissue culture plates. The effect of trastuzumab and pertuzumab were tested at concentrations of 0, 1, 10, and 100 μ g/mL of culture medium containing 0.1% FCS. Concentrations tested for C11033 and Iressa were 0.1, 1, and 10 μ mol/L. Cell viability was tested by 18 hours incubation of the alamarBlue before fluorescence measurement according to the manufacturer's instructions. Fluorescence was measured with excitation at 544 nm and emission at 590 nm using a Wallac Victor2 plate reader (Perkin-Elmer, Turku, Finland). Fluorescence values of samples were normalized with values of culture media without cells.

In vitro Assay of Drug Sensitivity

The JIMT-1 cells were also tested for trastuzumab sensitivity when grown as xenograft tumors in 5- to 8-week-old female *nu/nu* NMRI nude mice ($n = 29$, Harlan Netherlands, Horst, Netherlands). The tests were done with the approval of the ethical committee of the University of Tampere. Treatments were started after tumors reached 100 mm³ in volume. Trastuzumab ($n = 7$) and pertuzumab ($n = 7$) were given i.p. at a dose of 5 mg/kg once a week and compared with the same dose of a similar humanized antibody rituximab ($n = 7$), which is directed against CD20 antigen not expressed in JIMT-1 cells. N-87 xenografts were used as a known trastuzumab-sensitive control ($n = 8$) under similar experimental conditions. The N-87 xenografts were mock treated ($n = 4$) with isotonic sodium chloride solution once a week. Tumor growth was measured with a caliper and tumor volume was calculated using the formula $T_{vol} = \pi/6 \times \text{larger diameter} \times (\text{smaller diameter})^2$.

Results

The JIMT-1 Cell Line

A new breast cancer cell line, designated JIMT-1, was established from a freshly centrifuged sample of pleural metastasis fluid. Cell growth was stabilized in 6 months, after which the cells grew as an adherent monolayer and reached confluence in 7 to 10 days. The cells had the appearance of medium-sized epithelioid cells with variable nuclear size. The cells have undergone 40 passages; they show continuous growth and can recover from cryopreservation. Injection of JIMT-1 cells s.c. into nude mice led to formation of xenograft tumors in ~90% of the animals tested. Histopathologically, the xenograft tumors represent a high-grade adenocarcinoma (not shown).

Table 1. Immunohistochemical characterization of a novel breast cancer cell line JIMT-1

Protein expression	Result
Estrogen receptor	Negative
Progesterone receptor	Negative
HER-2 (mab CB-11)	Overexpression (3+)
p53	High expression
p63	Positive
PTEN	High expression
Ki-67	High expression
Cytokeratin 5/14 (basal)	Positive
Cytokeratin 8/18 (luminal)	Positive
Vimentin	Positive
Smooth muscle actin	Negative

Immunohistochemical Characterization

Immunohistochemistry of the patient's primary breast tumor and a JIMT-1 xenograft showed no staining for estrogen and progesterone receptors but strong immunopositivity for p53. High cell-proliferation rate was evidenced by abundant Ki-67 labeling (Table 1). The epithelial origin of the JIMT-1 cells was confirmed by a strongly positive pan-cytokeratin immunostaining (Table 1).

Subtyping with antibodies specific to luminal cytokeratins (8/18) and basal cytokeratins (5/14) indicated a "progenitor cell" phenotype being positive for both classes of cytokeratins. The progenitor cell type was further confirmed with positive p63 and vimentin stainings (Table 1). Immunostaining with smooth muscle actin was negative, excluding the possibility that JIMT-1 is a myoepithelial tumor. Positive immunostaining with anti-PTEN antibody indicated that its activity is retained and is unlikely to explain the constitutively active Akt-1 pathway (see below). Moreover, the gene copy number ratio of PTEN was unaltered according to the array comparative genome hybridization.³

Status of the *HER-2* Gene

The primary tumor of the patient was initially screened for eligibility to trastuzumab therapy with immunohistochemistry and CISH. Both 3+ overexpression by immunohistochemistry (Fig. 1A) and amplification by CISH were found (not shown). Gene amplification (~20–30 copies per cell) was found also by *FISH* in the pleural metastatic cells (data not shown) and in the JIMT-1 cells (Fig. 1B) after 10 passages. The gene amplification was found also in the xenograft tumor by CISH (Fig. 1C) as well as 3+-type immunohistochemical overexpression (Fig. 1D). Sequence analysis of exons 1 to 27 revealed no identifiable

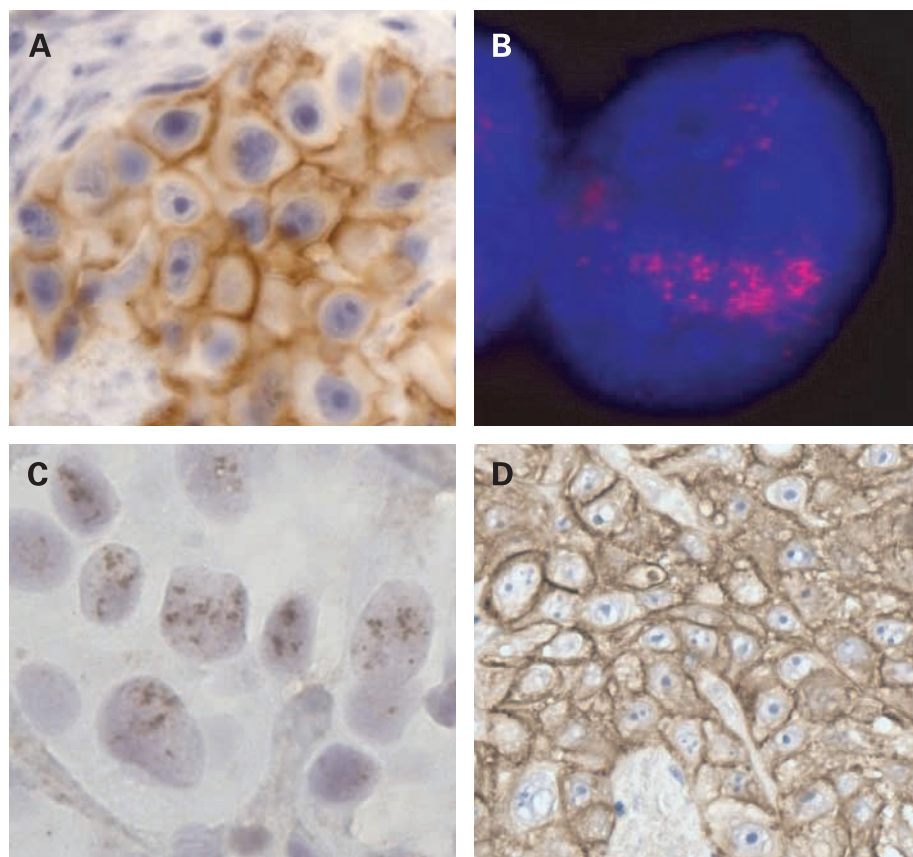


Figure 1. **A**, HER-2 overexpression (3+) shown by immunohistochemistry in the patient's primary tumor. **B**, HER-2 amplification by single-color FISH (red signals). **C**, CISH shows HER-2 amplification (cluster of brown peroxidase reaction deposits) in the nuclei of JIMT-1 xenograft cells. **D**, HER-2 protein overexpression in the xenograft tumor tissue.

Table 2. Genotypic and phenotypic characterization of the members of the HER-receptor family in the breast cancer cell line JIMT-1

Gene	Amplification status	mRNA* (%)
<i>HER-2</i>	Yes (20–40 copies/cell)	65
<i>HER-1 (EGFR)</i>	No	2.9
<i>HER-3</i>	N.D.	0.9
<i>HER-4</i>	No	0.01

*% β -Actin by quantitative RT-PCR (TaqMan).

mutations but one known allelic variation (according to <http://www.ensembl.org>) in exon 27. This nonsynonymous single nucleotide polymorphism (CCC→GCC) in codon 1170 causes an amino acid substitution (proline→alanine).

Expression Levels of HER-1 to HER-4 mRNA and Protein

The expression levels of each of the HER receptor mRNAs in JIMT-1 cells were compared with those of the breast cancer cell line SKBR-3 using the TaqMan quantitative real-time RT-PCR method (Table 2). The results showed that *HER-2* mRNA was highly overexpressed in JIMT-1 and SKBR-3 (being 65% and 179% of the expression of β -actin mRNA, respectively). Expression levels of *HER-1*, *HER-3*, and *HER-4* mRNAs were very similar in JIMT-1 and SKBR-3 and were much lower than that of *HER-2* (*HER-1*, 2.9% and 10% of β -actin; *HER-3*, 0.9% and 0.5%; *HER-4*, 0.01% and 0.004%, respectively, in JIMT-1 and SKBR-3 cells; Table 2). Expression levels of HER-1 to HER-4 proteins were also analyzed by Western blotting. HER-2 was highly overexpressed in JIMT-1 and SKBR-3 (data not shown).

Functional Characterization of HER-2 in JIMT-1 Cells

To study whether trastuzumab is able to bind to the HER-2 protein on the JIMT-1 cells, we immunoprecipitated lysate from JIMT-1 cells with trastuzumab and analyzed the results by Western blotting. The results showed that trastuzumab immunoprecipitated p185^{HER-2} similarly in JIMT-1, whereas no band was detected with negative control antibody rituximab (Fig. 2). By immunoprecipitation with HER-2 and immunoblotting with HER-3 antibody, we found that in JIMT-1 cells HER-2 dimerizes with HER-3 even in the presence of trastuzumab. In comparison, in SKBR-3 cells trastuzumab totally prevents the dimerization (Fig. 3). The concentration of p105^{HER-2} ectodomain measured from cell

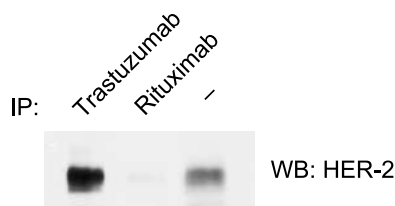


Figure 2. Western blot of HER-2 after immunoprecipitation with trastuzumab, demonstrating the ability of trastuzumab to bind and precipitate HER-2 protein extracted from JIMT-1 cells. Rituximab was used as a negative control antibody.

IP: HER2 SC-284
Western: HER3 SC-285

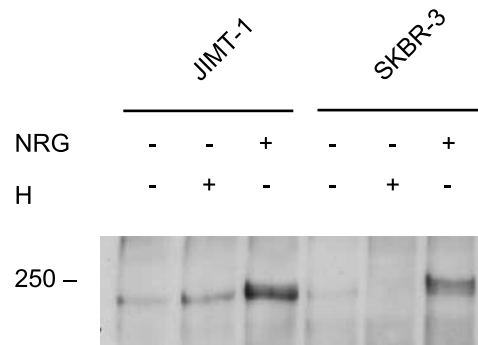


Figure 3. Western blot of HER-3 after immunoprecipitation with anti-HER-2, demonstrating HER-2/HER-3 heterodimer formation after neuregulin (*NRG*) stimulation. Heterodimers are detectable in JIMT-1 also constitutively and after trastuzumab (*H*) treatment.

culture media and serum samples from xenograft-bearing mice showed clearly detectable but six times lower concentrations in JIMT-1 than in BT-474 (cell cultures) or two to four times lower concentrations in sera compared with mice with N-87 xenografts (Fig. 4).

Because decreased Akt phosphorylation has been suggested as a requirement for the antiproliferative effect of trastuzumab, we next determined the levels of total Akt and phosphorylated Akt before and after trastuzumab treatment. Akt phosphorylation decreased on trastuzumab treatment in the sensitive breast cancer cell line SKBR-3, whereas it increased in JIMT-1 cells (Fig. 5). We also noticed

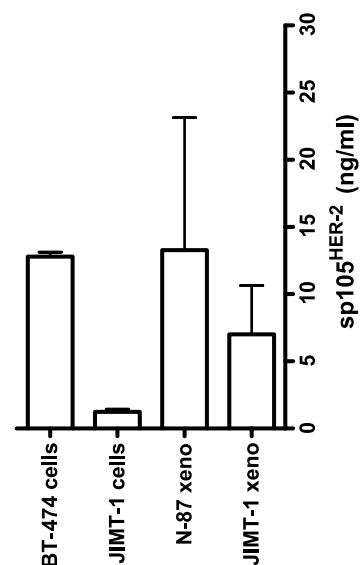


Figure 4. Concentration of sp105^{HER-2} by an ELISA assay, demonstrating the presence of ectodomain of HER-2 in JIMT-1 cells both in xenograft tumors and in cell culture.

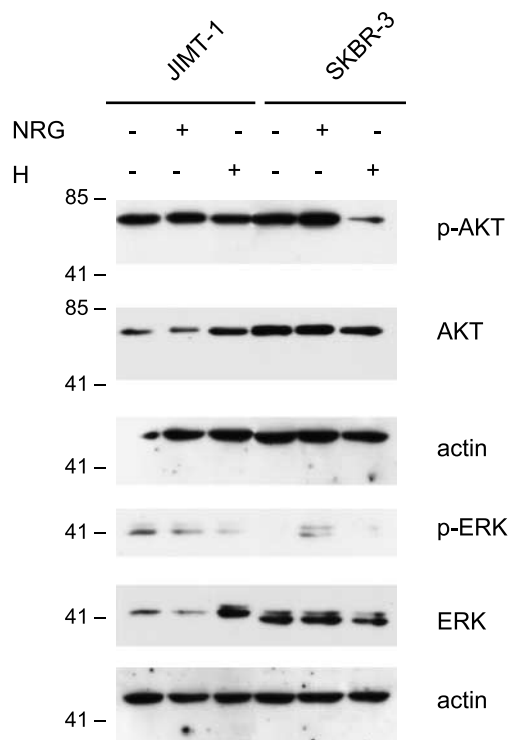


Figure 5. Western blots showing the activity of the main signaling pathways Akt and MAPK/Erk in JIMT-1 and SKBR-3 either stimulated with neuregulin 50 ng/mL (NRG) or treated with trastuzumab (H).

that phospho-extracellular signal-regulated kinase levels were high in untreated JIMT-1 cells. The ratio of phospho-Erk and total Erk in JIMT-1 decreased with trastuzumab treatment. However, phosphorylated Erk was still detectable in JIMT-1, unlike in SKBR-3.

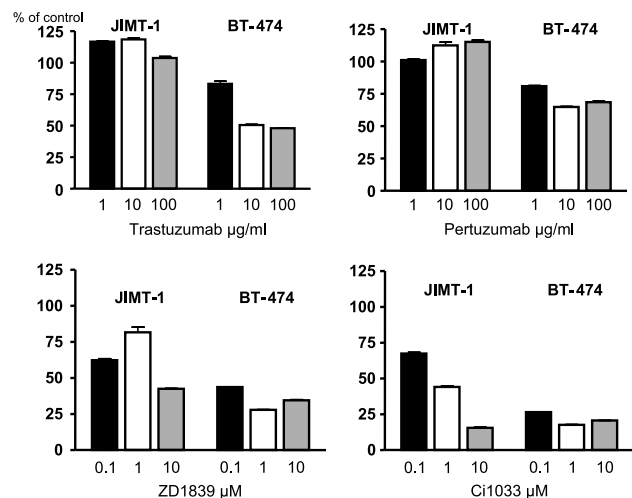


Figure 6. Growth of JIMT-1 and BT-474 cells in culture (day 3) by the concentrations of drugs added to the culture media. The values on the y-axis represent readouts of the alamarBlue assay relative to each control (= 100%).

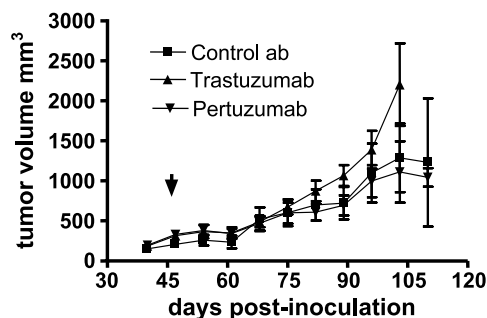


Figure 7. Growth curves of JIMT-1 xenograft tumors in trastuzumab-treated ($n = 7$), pertuzumab (2C4)-treated ($n = 7$), and control (rituximab-treated; $n = 7$) mice. Arrowhead, beginning of the antibody treatment i.p.

Sensitivity to HER Receptor – Inhibiting Drugs

The growth-inhibitory effect of trastuzumab was assessed using the alamarBlue assay, which reflects the number of viable cells at each time point studied. BT-474 cell line was used a trastuzumab-sensitive control cell line. On day 3, the inhibitory effect of trastuzumab on BT-474 cells was clearly visible (Fig. 6). Therefore, this time point was chosen for alamarBlue assays. These experiments indicated that at concentrations ranging from 1 to 100 µg/mL, trastuzumab failed to inhibit the growth of JIMT-1 cells in low-serum (0.1% FCS) conditions as it did in the trastuzumab-sensitive cell line BT-474 (Fig. 6). The other HER-2-directed monoclonal antibody pertuzumab had an inhibitory effect in BT-474 but not in JIMT-1 cells. Likewise, there was no effect on JIMT-1 cell growth when trastuzumab and pertuzumab were given in combination (100 µg/mL + 100 µg/mL, data not shown). JIMT-1 cells were also more resistant to low concentrations of the small tyrosine kinase inhibitors Ci1033 and ZD1839. On BT-474 the effect of these inhibitors was 2 to 2.2 times stronger (Ci1033) and 1.5 to 2.6 times stronger (ZD1839) than on JIMT-1. The highest concentration used (10 µmol/L) was probably nonspecifically toxic and equally effective on both cell lines (Fig. 6).

Trastuzumab and Pertuzumab in JIMT-1 Xenografts

As shown in Fig. 7, JIMT-1 tumors grew almost equally well in mice with or without treatment with anti-HER-2 antibodies. The growth-inhibitory effect of trastuzumab was evident in N-87 xenografts, which was used as a control xenograft model (data not shown).

Discussion

We report here the characterization of a novel cell line derived from a patient with trastuzumab-resistant breast cancer. The primary tumor of the patient showed amplification and overexpression of HER-2, thereby identifying the patient as eligible for Herceptin therapy. She received Herceptin as adjuvant therapy in a randomized clinical trial (in combination with vinorelbine) and again for distant metastatic disease. Despite the therapy, distant metastatic

disease manifested rapidly (in 6 months) and could not be controlled by subsequent Herceptin treatments. Thus, in a clinical context, the resistance was complete and of primary (intrinsic and noninduced) type. However, due to the nature of adjuvant Herceptin treatment, initial therapeutic effect on nondetectable micrometastatic cells followed by acquired resistance cannot be ruled out. In line with the clinical history, trastuzumab was unable to inhibit the growth of JIMT-1 cells *in vitro* when used at concentrations inhibitory to the growth of the HER-2-sensitive cell line BT-474. JIMT-1 cells were also insensitive to trastuzumab when grown as xenograft tumors in nude mice. To the best of our knowledge, the JIMT-1 cell line is the first experimental model for primary trastuzumab resistance derived from a patient with Herceptin-resistant HER-2-positive breast cancer.

In general, the mechanisms of resistance to trastuzumab are not known. At the clinical level, it has been speculated that cellular heterogeneity in HER-2 expression might lead to overgrowth of HER-2-negative and thus trastuzumab-nonresponsive cells in the metastases (6). This was ruled out in our patient because a high-level gene amplification was present in the pleural metastatic cells, which grew in an uncontrolled manner during Herceptin therapy. HER-2 amplification and overexpression remained stable also during continued JIMT-1 culture and in the tumor xenografts, making it a suitable model for molecular trastuzumab resistance studies. The expression levels of HER-1, HER-3, and HER-4 were similar in JIMT-1 and SKBR-3, suggesting that their concentrations are unlikely to explain trastuzumab resistance.

The first straightforward hypothesis for trastuzumab resistance is that HER-2 protein could be structurally altered and thus not be able to bind trastuzumab on the cell surface. Our experiments showed that trastuzumab was able to immunoprecipitate HER-2 from the lysate of JIMT-1. Moreover, sequencing of the exons revealed no mutations, further indicating that there are no structural aberrations in the HER-2 gene and protein, which could explain trastuzumab resistance. Sequencing of the exons revealed no mutations, only a known single nucleotide polymorphism at exon 27.⁷

The basic functional phenomena associated with HER-2 receptor signaling are shedding of the external domain, heterodimerization, and tyrosine kinase phosphorylation. We showed that the ectodomain shedding takes place in JIMT-1 cells, although the concentration in mouse serum or culture medium was much lower than in two HER-2-overexpressing cell lines BT-474 and N-87.

The heterodimer HER-2-HER-3 is known to be the most potent intracellular signaling activator of the HER family (10, 11) and is considered as an oncogenic unit (12). When stimulating JIMT-1 and SKBR-3 cells with neuregulin, heterodimer formation took place both in JIMT-1 and in

SKBR-3 in the same manner. Weak but clearly detectable bands corresponding to HER-2-HER-3 heterodimers were found constitutively and after trastuzumab treatment. However, the sensitivity of the immunoprecipitation technique did not allow detailed quantitation.

Although functional aspects of the HER-2 receptor in JIMT-1 did not provide clear clues for resistance, we found that after exposure to trastuzumab, JIMT-1 cells showed no decreased phosphorylation of Akt, which is characteristic in trastuzumab sensitive cell lines (13). This phenomenon could not be explained by the tumor suppressor gene *PTEN*, the loss of which is known to lead to increased activity of Akt-1 pathway (14). With strong immunohistochemical staining using anti-*PTEN* antibody, the loss of *PTEN* is unlikely in JIMT-1 cells.

Results of the two main signal transduction pathways, Akt and Erk/mitogen-activated protein showed different effects in JIMT-1 cells on Herceptin exposure. Whereas the balance between phospho-Akt/total Akt remained stable, the ratio of phospho-Erk/total Erk was clearly decreased. This suggests that the MAPK pathway could in fact be inhibited in JIMT-1 cells although the trastuzumab had no inhibitory effects on cellular growth *in vitro* or in xenograft tumors. Thus, these experiments underline the link between inhibition of Akt phosphorylation and the lack of action of trastuzumab, at least in JIMT-1 cells. We anticipate that the present results may lead to more targeted studies aiming to identify molecular mechanisms that bypass the effect of trastuzumab and maintain the activity of the Akt pathway.

A straightforward experiment with a trastuzumab-resistance model is to study its sensitivity to drugs targeting other family members of the HER receptors. Using BT-474 cells as a control, we found that the new humanized antibody pertuzumab (2C4) was unable to inhibit growth of JIMT-1 cells *in vivo* or *in vitro*. Because pertuzumab is known to prevent heterodimerization of HER-2 and HER-3 (15), this phenomenon seems not to be critical in growth control of JIMT-1 cells. Blocking of HER-1 (EGFR) by a specific small-molecule inhibitor ZD1839 (Iressa) or all HER receptors by the pan-receptor tyrosine kinase inhibitor Ci1033 was found to have much smaller effects on JIMT-1 than on BT-474 cells. Thus, these results suggest that JIMT-1 is widely resistant to the inhibitor drugs of HER receptors. These results may direct forthcoming studies toward inhibitors of signaling pathways, especially the Akt-1 pathway.

As a cell line, JIMT-1 and its xenograft tumors carry phenotypic hallmarks of HER-2-positive breast cancer, that is, histologically representing a high-grade invasive ductal carcinoma lacking expression of estrogen and progesterone receptors. Moreover, the primary tumor and the xenograft were characterized by a high tumor-proliferation rate (Ki-67) and overexpression of p53, which are common features of HER-2-positive tumors (1). Characterization of the JIMT-1 cells by cytokeratin immunostainings indicated positivity both for luminal-type cytokeratins 8/18 and basal-type cytokeratins 5/14. This

⁷ <http://www.ensembl.org>

finding was confirmed in the patient's primary breast tumor, cultured JIMT-1 cells and xenografts. Thus, the tumor can be classified as belonging to the ~10% of invasive cancers forming the so-called progenitor cell or basal-like phenotype (16, 17). The microarray studies (16) have classified *HER-2*-positive tumors in general as an entity separate from basal-like tumors, but it is likely that these tumors, like JIMT-1, can occasionally carry *HER-2* amplification. As such, the cytokeratin 5-positive breast tumors are associated with poor prognosis (18) and perhaps with therapeutic resistance, but the latter aspect has not been firmly documented. Whether the cytokeratin 5/14 positivity defines a subgroup of tumors specifically resistant to Herceptin remains to be studied with large enough clinical tumor materials.

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