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ORIGINAL ARTICLE

Prostate cancer cell lines lack amplification: Overexpression of HER2

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Abstract

The potential overexpression of HER2 in prostate cancer cells has attended significant interest during the past few years, both as potential target for HER2 pathway focused therapy and as a mechanism involved in the progression to androgen independence. Conflicting results have been reported concerning HER2 status on clinical material, differences which generally have been attributed to methodological differences. Nevertheless, HER2 has been utilized for targeted therapy of prostate cancer in a number of preclinical studies and is still regarded as an exciting target molecule. In this study, the HER2 status of three widely used prostate cancer cell lines and corresponding xenografts has been analysed. By use of validated and FDA approved analytical staining techniques none of these cell lines or xenografts were shown to overexpress/amplify HER2, as demonstrated by immunohistochemistry and fluorescense in situ hybridization. These findings are important for the interpretation and understanding of the therapeutic effects when developing drugs targeting HER2 in prostate cancer cell lines and also emphasize the importance of using broad and validated analytical techniques.

Prostate cancer is the most common cancer in men and the second leading cause of death among men in Western countries. The survival rate for early stage prostate cancer is high but for relapsing tumors there is no adequate salvage therapy. Androgen blockade constitutes first-line therapy for metastatic prostate cancer. As a rule however, the disease progresses from an androgen-dependent (AD-PCa) to an androgen-independent state (AI-PCa) over time, thus rendering hormonal therapy less effective. The median survival of patients with AI-PCa is less than one year [1] and the treatment options are limited [2]. Hence, novel therapies for advanced prostate cancer are needed.

The HER2 oncoprotein is a transmembrane receptor with tyrosine kinase activity [3]. This protein is overexpressed in a subset of ovarian carcinomas, gastric adenocarcinomas, salivary gland adenocarcinomas, bladder carcinomas, pulmonary non-small cell carcinomas and prostate carcinomas [4]. In breast cancer, genomic amplification of the c-erbB-2 gene with a following overexpression of the HER2 receptor has been observed in 10–30% of the cases and is significantly associated with poor prognosis [5]. A humanised anti-HER2 monoclonal antibody, trastuzumab (Herceptin®), has been developed. Treatment with trastuzumab in combination with chemotherapy confers a survival benefit for patients with metastatic HER2 overexpressing breast carcinoma [6] and this finding has incited interest in using the drug for other cancers that may have similar overexpression.

Studies of HER2 protein expression in clinical prostate cancer are controversial since conflicting results have been reported with overexpression rates raging from 0–100% [7–9]. Immunohistochemical staining methods considered standard for HER2 detection in breast cancer with a FDA (U.S. Food and Drug Administration) approved and validated anti-HER2 antibody (DAKO) and a FDA approved

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and validated HER2 DNA Fluorescense In Situ Hybridization assay (PathVysion, Vysis) for detection of gene amplification, were used in a study by Calvo et al. No HER2 protein overexpression nor HER2 amplification in 25 specimens of AI-PCa was found [10]. This is of significant interest since HER2 overexpression has been discussed as potential factor in the progression from AD-PCa to AI-PCa [11]. Moreover, Craft et al. demonstrated that HER2 tyrosine kinase modulates the response of the androgen receptor to low doses of androgen suggesting a role for HER2 targeting in AI-PCa [11].

Several preclinical therapeutic studies utilizing HER2 as a target have been performed [12–15]. In a human xenograft model, trastuzumab induced tumor growth inhibition in AD-PCa tumors but had no effect in any of the AI tumors even though high expression of HER2 could be demonstrated by immunohistochemistry [13]. It was concluded that androgen dependence or the requirement for signalling through the androgen receptor, is a necessary element for Herceptin® response. Others have described responses of AI-PCa cell lines using HER2 as the target molecule [12]. Skrepnik et al. found moderate to high levels of HER2 expression in the androgen independent cell lines PC-3 and DU-145 as detected by immunohistochemistry [12]. Treatment with the HER2 specific oncotoxin AR209 effectively reduced tumors in athymic mice with PC-3 and DU-145 xenografts [12]. By use of the monoclonal antibody, 2C4, that hinders HER2 recruitment into erbB ligand complexes an efficient growth inhibition was demonstrated in several prostate cancer cell lines considered as not overexpressing HER2 [15]. The interpretation of the therapeutic outcome in these studies relies on the detected expression of HER2. A careful evaluation with standardized specific methodology is mostly warranted. Here, we report the analysis of the HER2 status in three widely used prostate cancer cell lines, PC-3, DU-145 and LNCaP and corresponding xenografts in athymic nude mice, using validated techniques considered standardized for the clinical evaluation of HER2 expression and amplification level.

Materials and methods

Cell Lines, Culture Conditions and Xenografts

Three human metastatic prostate carcinoma cell lines LNCaP *(AD-PCa),* DU-145 (*AI-PCa*) and PC-3 (*AI-PCa*) were analysed and two human breast cancer cell lines, MDA-453 and BT-474 were used as controls. All cell lines were cultured in 5 ml dishes (Falcon) with RPMI 1640 (Sigma). 2 mM glutamine (Sigma), 10% fetal calf serum (Invitrogen) were added to the media. The cells were kept in a 37°C incubator with humidified air and 5% CO_2. The culture medium was changed two times a week. Cells were removed from the dishes by treatment trypsin (0,5 g)-EDTA (0,2 g) (Sigma, USA). In order to establish tumors for histopathological analyses, nude mice (Balb/c nu/nu, Boomholtgaard; Denmark) were inoculated subcutaneously between the fore and hind leg with approximately 20×10^6 tumor cells. When the tumor volume exceeded 60 mm^3, the tumors were resected and assayed for HER2 status. All cell lines were obtained from American Type Culture Collection (ATCC, USA).

Preparation of Paraffin Blocks

The various cell lines were harvested after tissue culture. First the entire material was centrifuged in a Shandon Cytoblock® cell block preparation system (Termo Shandon, Pittsburgh, USA) at 254 g (1500 rpm for 5 minutes) to create a cell pellet. The supernatant fluid was decanted and the pellet resuspended in 0.9% neutral phosphate buffered saline. After an additional centrifugation the pellet was further fixed with 10% neutral buffered formalin and gently transferred to a filter bag. After fixation for 24 hours in formalin, paraffin blocks were routinely prepared. For each cell line 4-μm-thick tissue sections were cut from the paraffin block and applied to positively charged slides. Sections from each cell block were deparaffinized and rehydrated in graded alcohols.

Immunohistochemistry

Immunostaining were performed on an autostainer using a minipanel consisting of three commercially available antibodies and manually using HercepTest (Dako).

Nexes autostainer. HER2 protein expression was evaluated immunohistochemically using two monoclonal antibodies, one for the extracellular domain (Ab17, dilution, 1:500;Neomarkers, Freemont, California, USA) and one for the cytoplasmic domain (CB11-Pathway, prediluted, FDA approved, Ventana Medical Systems, Tucson, Arizona, USA). In addition, one rabbit polyclonal antibody targeting cytoplasmic epitopes was used (A 485, dilution, 1:500; DAKO, Denmark). Immunoreactivity was enhanced by antigen retrieval treatment consisting of heating the slides in a microwave oven in a 10-mmol/L concentration of sodium citrate buffer, pH 7.3 two times for 10 minutes each time at 300 W and followed by cooling for 20 minutes at room

temperature. The sections were then stained using the Ventana Nexes Staining System. Briefly, after a 30-minute incubation at 37°C with the primary antibody, sections were incubated for another 10 minutes at 37°C with a secondary biotinylated antibody and then with avidin-peroxidase for another 10 minutes; 3',3-diaminobenzidine was used as the chromogen. All products needed for these steps are included in the DAB detection kit provided by the manufacturer (Ventana Medical Systems). Slides were counterstained in Mayer hematoxylin, dehydrated, and mounted. A multiblock control consisting of four cell lines representing 0, 1+, 2+ and 3+ staining served as the positive and negative controls for all immunohistochemistry stains.

HercepTest. The manufacturer's recommended method was followed when using the HercepTest kit (DAKO, FDA approved). The antigen retrieval method used in this kit involved immersing slides in a preheated (95°C) DAKO Epitope Retrieval Solution followed by heating in a water bath at 95°C for 40 minutes. This was followed by a 20-minute cool-down period at room temperature. Slides were incubated with the primary antibody (prediluted rabbit polyclonal antibody, A485) for 30 minutes at room temperature. The slides were then incubated with the DAKO Visualization Reagent (dextran polymer conjugated with horseradish peroxidase and goat antirabbit immunoglobulins) for 30 minutes. Diaminobenzidine was used as the chromogen, and the slides were counterstained with hematoxylin.

Evaluation of immunohistochemistry. All immunohistochemical stains were assessed using a semiquantitative scoring system following the criteria recommended by DAKO for the HercepTest and without knowledge of the results of the FISH tests. Overexpression of HER2 was defined as positive membranous staining in more than 10% of the tumor cells and tumors with cytoplasm staining without distinct cell membrane staining were scored as negative. The strength of staining was scored 1–3+ (weak, moderate and strong circumferential membranous staining) according to the instructions of the FDA approved DAKO HercepTest. Only 3+ staining was regarded as conclusively positive. The three antibodies stained on the Nexes system had previously been titered to similar staining intensities as the HercepTest with the help of multiblock cell line controls and semiautomatized IHC evaluation unit Ariol–50 (Applied Imaging).

Fluorescence In Situ Hybridization (FISH)

FISH analyses were performed using the PathVysion HER2 DNA Probe Kit (Vysis, Downers Grove, IL) according to the manufacturers' instructions and using reagents, probes, and positive controls purchased from the manufacturer. The slides were evaluated for HER2 gene copy number using an epifluorescence microscope (Zeiss Axioplan 2, Thornwood, NY). The PathVysion kit uses 2 directly labeled fluorescent DNA probes: LSI HER2, which is specific for the HER2 gene locus, and CEP 17, which is specific for the alpha satellite DNA sequence at the centromeric region of chromosome 17. The expected ratio of LSI HER2 to CEP 17 is 1.0 for normal or unamplified breast tissue specimens. A ratio of greater than 2.0 was considered amplified. Signals were counted for 60 tumor cell nuclei. Signal enumeration was performed following the criteria recommended by the manufacturer: overlapping nuclei were not counted, and split signals were counted as one chromosome component.

Results

Expression of HER2 by Immunohistochemistry

For immunohistochemical analysis, several types of antibodies have been used. Two mouse monoclonal (Ab17 and CB-11) and one rabbit polyclonal (A485) antibody targeting different structures of the receptor were investigated. Ab17 targets the extracellular domain of the receptor, whereas CB-11 and A485 target cytoplasmic epitopes. Staining was performed manually with A485 (FDA approved HercepTest) and automated using the Ventana Medical Systems Nexes autostainer with antibody CB-11 (FDA approved Pathway Test). In addition, the Nexes autostainer was used for antibody A485 and Ab17 (Table I). As seen in Table I and Figure 1e–1f, none the prostate carcinoma cell lines or xenografts overexpress the HER2 protein irrespective of the analytical technique used. For comparison, staining of the positive breast cancer controls MDA-453 and BT-474 are shown in Table I and Figure 1a–1d.

HER2 amplification by FISH

HER2 amplification was not detected in any of the prostate cancer cell lines studied or corresponding xenografts as seen in Table I. As expected, the positive breast cancer cell line controls MDA-453 and BT-474 show amplification of different degrees as depicted in Table I. Examples of the FISH analyses are shown in Figure 1.

Table I. In order to analyse the HER2 status, both manual and automatized (Ventana Nexes Autostainer) staining techniques with several well characterized commercially available antibodies were used. As seen, none of the prostate cancer cell lines neither overexpress nor amplify HER2. An identical pattern is seen for the corresponding xenografts of the prostate cancer cell lines. In contrast, the positive breast cancer cell line controls, MDA-453 and BT-474 overexpress and amplify HER2, as expected. For definitions of overexpression and amplification respectively, see materials and methods. N.D. =not determined.

	IMMUNOHISTOCHEMISTRY				FISH		
STAINING TECHNIQUE	Manual Staining	Ventana Nexes Autostainer			PathVysion		
ANTIBODY/PROBE	A485 (Herceptest)	A485	CB11 (Pathway)	Ab17	HER2/neu	C17	Ampl
CELLINES							
PC-3	0	0–1+	0	0	3,6	3,0	No
DU-145	0	0–1+	0	0	2,9	2,8	No
LNCaP	0	0–1+	0	0	3,1	2,3	No
MDA-453	2+	2+	2+	2+	12,3	1,8	Yes
BT-474	3+	3+	3+	3+	43,2	1,9	Yes
XENOGRAFTS							
PC-3	N.D.	0	0	0	3,3	2,8	No
DU-145	N.D.	0	0	0	2,3	2,0	No
LNCaP	N.D.	0	0	0	2,6	1,8	No

Discussion

Prostate cancer is the most common cancer in men in the Western countries. Even if there are curative options for the disease it has a bad prognosis once it has disseminated. The mechanisms underlying carcinogenesis of the prostate has been investigated by others and especially the transformation from androgen dependent to androgen independent disease has attended big interest. Carcinogenesis is a multistep process in which several families of genes are involved. The group of erbB genes which encode for the four transmembrane receptors, epidermal growth factor receptor (EGFR or erbB-1), HER2 (neu, erbB-2), HER3 (erbB-3) and HER4 (erbB-4) are involved in these processes and have been studied for many cancers including prostate cancer. In contrast to the other members of the erbB gene family, a ligand for the erbB-2 receptor has not been identified and might not exist. ErbB-2 has been extensively investigated in breast cancer with conclusive results with 10–30% showing overexpression of HER2 with an inverse correlation to estrogen receptor levels and prognosis. EGFR has also been vastly studied in prostate cancer and all four receptors should be regarded as an integrated family. In this study we investigated the expression of HER2 in three widely used prostate cancer cell lines, LNCaP, DU-145 and PC-3 by validated immunochemistry (IHC) and fluorescence in situ hybridization (FISH) technology.

In an early study [9], IHC staining of PC-3, LNCaP and DU-145 cells were all regarded as positive but it was noted that different subcellular compartments stained differently in the different cell lines. It was discussed that staining was positive in both androgen dependent and androgen independent LNCaP cells and that an increased expression was detected in these cells when exposed to dihydrotestosterone. Staining was foremost detected in the cytoplasm of the LNCaP cells. Only the PC-3 cell line showed predominately staining of the plasma membrane. In contrast, southern blot analysis of DNA samples from 10 prostate cancer tissues and the three prostate cancer cell lines did not show any HER2 gene amplifications. Watanabe et al. [16] investigated the HER2 expression in three cell lines; PC-3, DU- 145 and TSU Pr1, one specimen of primary prostate cancer and four specimens of BPH. Positive IHC reactivities were found in all cell lines and the antigen was detected mainly in the cytoplasm of the cells. HER2 mRNA was detected by reverse transcription (RT)-PCR in all cell lines as well as in the prostate cancer and BPH specimens. Amplifications of HER2 could not be detected either in cells or tissues with differential PCR or Southern blot. It was suggested that it is necessary to also detect mRNA of HER2 to determine an expression of HER2.

Several studies, including studies on xenografted tumors, have reported evidence for a role of HER2 in the proliferation and progression of prostate cancer into androgen independency. Craft et al. proposed that HER2 activates transcription of PSA and that HER2 and androgen act synergistically to superactivate PSA transcription [11]. This was particularly observed at low androgen concentrations, which lead to the suggestion that HER2 can restore androgen receptor function. Others have suggested that the role of HER2 is to interact with the other members of the EGF receptor family and that blocking of HER2 only will have an effect if the antibody used has an effect on a

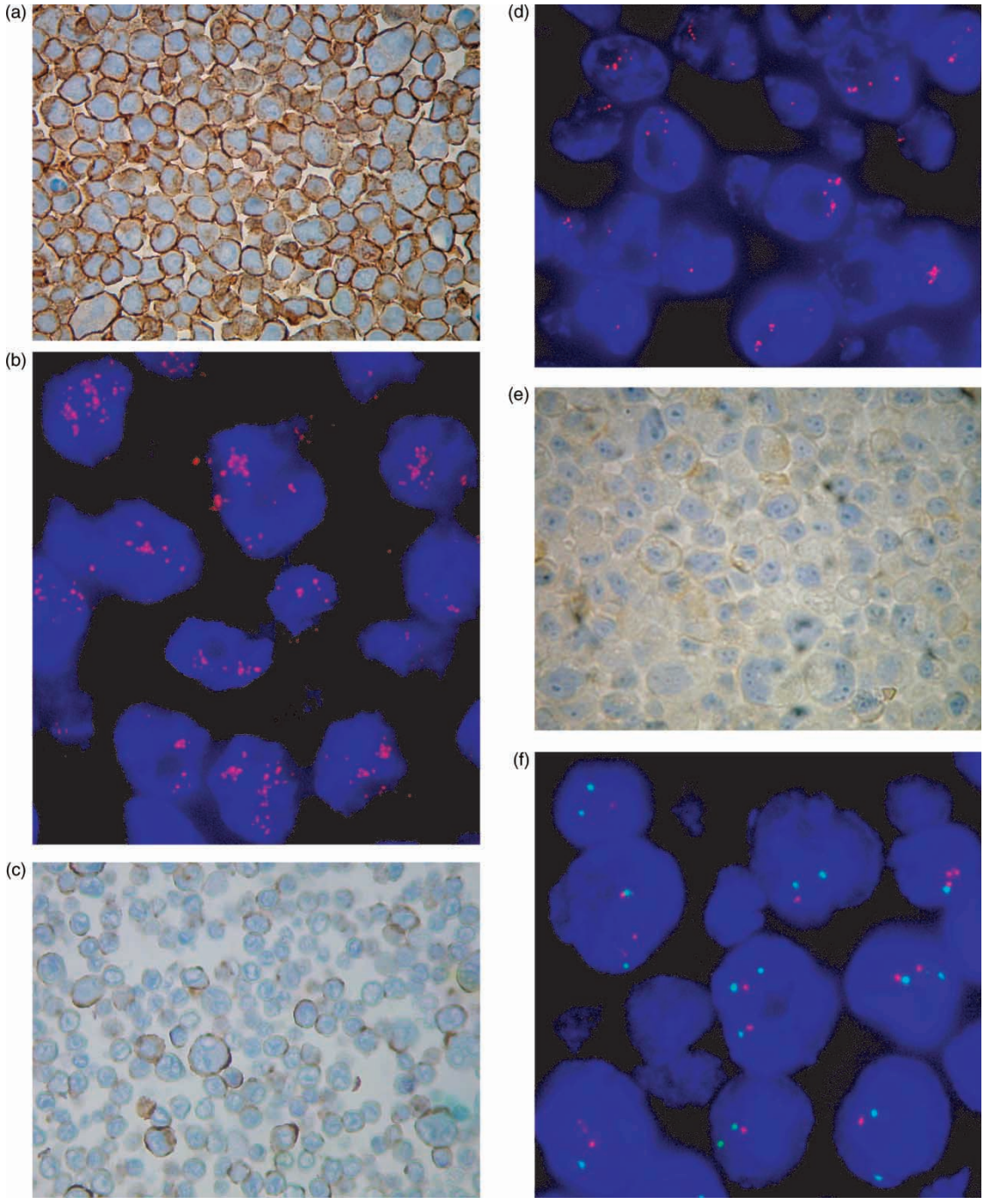

Figure 1. Examples of IHC (HercepTest) and FISH for HER2. Figure 1a demonstrates IHC for BT-474 cells (3+) and in Figure 1b FISH is shown for the same cell line (high amplification). The other positive breast cancer cell line control, MDA-453 (2+) is shown in Figure 1c–1d. In Figure 1e–1f, PC-3 cells are shown. These were found to stain negatively on IHC with no gene amplification, as demonstrated by FISH. Identical results were obtained for LNCaP and DU-145 cells (results not shown). In the FISH figures red dots show centromer 17 and green dots LSI HER2.

ligand-HER2 /- other HERreceptor complex formation [15,17]. Moreover, there are several studies demonstrating anti-tumoral effects in xenograft models following treatment with toxins coupled to anti-HER2 antibodies [12,14].

Similarly to in vitro studies on cell lines, results from studies on HER2 expression in specimens from patients with prostate cancer have varied significantly. Most likely these differences are a consequence of different analytical methodologies and technical set-up. As discussed by Calvo et al. [10] several steps in the analytical procedure including tissue acquisition, fixation, antigen retrieval, antibody used and scoring conventions may be differ-

ently performed. The present study clearly shows that the PC-3, DU-145 and LNCaP cell lines do not overexpress HER2 receptor protein or contain HER2 gene amplifications. For the corresponding xenografts, the results were identical, as seen in Figure 1. These findings are in line with the clinical study by Calvo et al. in which also validated IHC and FDA approved FISH techniques were used [10]. Furthermore we could not observe any differences in HER2 expression between the androgen-dependent LnCAP cells and androgen-independent PC-3 and DU-145 cells.

Several earlier studies have been using immunostaining methods that in our opinion were not always strictly validated. Some have even regarded weak staining of the cytoplasm as positive. In our study we used validated methods frequently used in clinical breast cancer analyses and approved by FDA.

HER2 pathway focused molecular therapy of prostate cancer could be selected on the same detection of overexpression/amplification of HER2 as in current breast cancer therapy. In a clinical setting this means utilizing validated and FDA approved analytical staining techniques. However, strict adherence to breast cancer decision protocols must be questioned, since the studies by Agus et al. [15] and Skrepnik et al. [13] showed that HER2 overexpression or amplification is not a prerequisite to obtain a therapeutic effect. Other molecular mechanisms for HER2 pathway activation such as mutation, increased transcription or post translational protein modification may be important in non-breast cancer. The finding that none of the prostate cancer cell lines studied here were found to overexpress/amplify HER2 was striking. This have implications for the interpretation and understanding of the therapeutic effects when developing drugs targeting HER2 in prostate cancer cell lines and also emphasizes the importance of using broad and validated techniques to assure comparable data.

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