The influence of testosterone suppression on HER2 immunoexpression in prostatic neoplastic tissue

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Abstract. During initial risk assessments, the metastatic potential of prostate cancer (PCa) may not be fully considered. The tumor's multicentric origin, which is associated with genetic mutations, may explain existing treatment limitations. Investigating human epidermal growth factor receptor 2 (HER2) expression in patients with different stages of PCa may therefore increase understanding of the mechanisms associated with the development of castration resistance. The present study examined the association between HER2 expression and the histologic features of PCa subjected to radical prostatectomy (RP) and evaluated the role of testosterone suppression in HER2 expression. In group 1, specimens from individuals who underwent RP without prior neoadjuvant androgen deprivation therapy (ADT) were included (n=42). In group 2 (PCa with ADT), specimens from individuals who underwent RP and received neoadjuvant cyproterone acetate during distinct periods (200 mg daily for 1-24 months) were included (n=150; cohort derived from a previous study). Immunohistochemical expression of HER2 was associated with prognostic factors such as perineural invasion, extra-prostatic disease, T stage, serum prostate-specific antigen (PSA), angiolymphatic invasion and surgical margins. Univariate regression analysis indicated that perineural invasion, PSA, International Society of Urological Pathology, angiolymphatic invasion, margin, T stage and neoadjuvant ADT was associated with HER2 expression. Ordinal regression analysis indicated a significant effect of neoadjuvant ADT alone on HER2 expression (P<0.001). In addition, regression analysis indicated a significant effect of neoadjuvant ADT alone on HER2 expression (odd ratio=0.01; 95% CI, 0.00, 0.02; P<0.001). HER2 was expressed in PCa samples but was not associated with known prognostic factors. The use of short-acting ADT and the consequent blockage of testosterone effect may suppress the expression of HER2 in PCa cells.

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Introduction

The human epidermal growth factor receptor 2 (HER2) oncogene encodes a transmembrane protein (17q12-21.32) with tyrosine kinase activity, which acts as a growth factor (1). HER2 has been detected with variable expression in a wide variety of malignant tumors and has been found to be an adverse prognostic marker in breast (20%) and ovarian (33%) adenocarcinomas (2,3). Overexpression of the HER2 protein and amplification of the HER2 gene, or both, occurs in approximately 25% of breast cancers and is associated with aggressive behavior (4).

Although unequivocal data on HER2 overexpression are not available for prostate cancer (PCa), evidence suggests that it may be crucial for disease progression and aggressiveness (5). A recent study supporting these findings was a comprehensive immunohistochemical (IHC) evaluation of 2,525 samples, which revealed positive associations between HER2 staining, PCa aggressiveness, and recurrence (6). In addition, high levels of HER2 have been correlated with tumor growth in LAPC-4 androgen-independent PCa cells (7). Furthermore, HER2-dependent signaling may support the development of castration-resistant PCa (CRPC) through androgen ligand-independent mechanisms (8).

However, there are no available data on the influence of castration on HER2-dependent signaling in patients with castration-sensitive PCa. Investigating the expression of HER2 in patients undergoing hormonal therapy during distinct periods could also increase our understanding of the

mechanisms associated with the development of castration resistance.

The metastatic potential of PCa may not be fully understood during the initial risk assessment (9). The tumor's multicentric origin, associated with genetic mutations, may explain treatment pitfalls (10,11).

Our research had two objectives: To correlate HER2 expression with the histologic features of PCa subjected to radical prostatectomy (RP) and to evaluate the role of testosterone suppression in HER2 expression.

Materials and methods

Patients. RP specimens were obtained from patients who were consecutively treated at two different institutions from 1998 to 2011 (Santa Casa of São Paulo Hospital and Centro Universitário FMABC Hospital). The local ethics committee approved the study (84427718.0.0000.0082 and 06937412.0.1001.0082).

Formalin-fixed paraffin-embedded (FFPE) tissue blocks of tumor samples were identified and divided into two groups. group 1 included specimens from individuals who underwent RP without prior neoadjuvant androgen deprivation therapy (ADT) (n=42). group 2 (PCa with ADT) included specimens from individuals who underwent RP after receiving neoadjuvant cyproterone acetate during distinct periods (200 mg daily for 1-24 months) (n=150; cohort derived from a previous study) (12).

The patients in group 2 were those who were included in a study performed in 2014, which proposed hormonal therapy with neoadjuvant cyproterone before RP. The material was preserved in a paraffin block using the tissue microarray (TMA) technique for future studies. We chose to use this cohort because neoadjuvant cyproterone is not used today. Furthermore, it is not ethical to suppress testosterone for long periods of time in men who would undergo radical treatment.

All hematoxylin and eosin (H&E)-stained histological sections from the RP specimens were reviewed. An index tumor (highlighted on the slides) was defined as the focus with the highest Gleason pattern or the largest tumor (in case of a single pattern). Other prognostic factors evaluated were perineural invasion, extra-prostatic disease, T stage, serum prostate-specific antigen (PSA), angiolymphatic invasion, and surgical margins.

Immunohistochemistry. In group 1, four to ten tissue sections (4 μm thick) were collected from the index tumors and mounted on glass slides. In group 2, two to four tissue sections (6 μm thick) were mounted on glass slides from the TMA block, as previously described (12). Histological sections from breast carcinoma cases were used as reference patterns for the positive reactions. Non-neoplastic breast and prostatic tissues (from an internal sample) were used for negative reactions.

Anti-HER2 antibody A0458, a polyclonal rabbit anti-human c-erbB-2 oncoprotein antibody (Dako GmbH, Jena, Germany), was used (incubated at 1:600) for staining in tissue samples with distinct loss of basal cells (proven PCa). The sections without any previous confirmation of PCa were not tested. Antigen recovery was performed according to the HercepTest™ manual (Dako) (13). The diluted epitope recovery solution (1:10) was preheated in a tank at 85˚C and sections were dewaxed at room temperature and immersed in a preheated epitope recovery solution. They were heated to 97˚C and incubated for 40±1 min at 97˚C. They were then left in the tank until they reached a temperature of 85˚C. They were then removed from the tank and left on the table with the lid closed for subsequent cooling. After 10 min, the tissue sections were washed with diluted Dako wash buffer and soaked in this buffer for 5-20 min after epitope recovery and before staining.

All tissue sections were reviewed by two board-certified genitourinary pathologists (LHSS and MGC). All features were scored according to the Food and Drug Administration (FDA) and HercepTest™ manual interpretation (Dako) (13), which comprised intensity, percentage, and characteristics of the stain (from 0 to 3+), and then the calculation of a final expression score. The immunohistochemical expression of HER2 was correlated with prognostic factors. The Gleason score was reclassified according to the International Society of Urological Pathology standards for (14). T staging was assessed using the clinical tumor node metastasis (TNM) classification standard (15).

Statistics. The data were analyzed using STATA 14.0 (StataCorp LP). Frequency tables were selected for descriptive analyses. Chi-square and Fisher's exact tests were used to assess the frequency of responses between the groups. For continuous variables, we used the Mann-Whitney test. In addition, logistic regression and ordinal logistic regression were applied to investigate the effect of covariates on the expression of the HercepTest™. Statistical significance was set at $P<0.05$.

Results

Technical issues. A total of 192 men were included in this study. After analysis, 42 patients remained in group 1 and 104 in group 2. Due to unequivocal cancer tissue in the corresponding TMA section (remaining 104 samples), 46 samples were excluded from group 2. The proportion of non-interpretable samples for HER2 immunohistochemistry was 23.9%.

Immunohistochemistry. The demographic characteristics of the patients are shown in Table I. The mean age was 66 years (interquartile range, 61-80 years). The mean PSA level in the study was 11.78±12.4 ng/ml (±SD). group 2 presented higher PSA levels compared to group 1 (7.54±2.70 ng/ml vs. 13.49±14.27 ng/ml; P=0.0021).

HER2 expression was observed in 85.7% of specimens in group 1 and only in 1% of group 2 (Table II) (Fig. 1). Fig. 2 shows the expression of HER2 over time. In group 2, HER2 expression was subdivided into periods of exposure to hormonal therapy with cyproterone. Even after short periods of exposure to therapy, its expression was completely suppressed. While the cancer was sensitive to hormone therapy, HER2 expression was not detected (Fig. 2).

When considering only patients without neoadjuvant ADT (group 1), univariate regression analysis showed an association between ISUP and HER2 expression (P=0.018). However, multivariate regression analysis showed that perineural invasion, PSA, ISUP, angiolymphatic invasion, positive

Table I. Baseline characteristics and outcomes for all patients.

Variable	Total N (%)	group 1 N (%)	group 2 N (%)	P-value
Perineural invasion				0.004
Yes	63 (43.15)	26 (61.90)	37 (35.58)	
No	83 (56.85)	16 (38.10)	67 (64.42)	
HER2 expression				<0.001
2+/3+	14 (9.59)	14 (33.33)	0 (0.00)	
0/1+	132 (90.41)	28 (66.67)	104 (100.00)	
ISUP				0.001
1-2	105 (71.92)	22 (52.38)	83 (79.81)	
3-5	41 (28.08)	20 (47.62)	21 (20.19)	
Angiolymphatic invasion				0.001
Yes	30 (20.55)	16 (38.10)	14 (13.46)	
No	116 (79.45)	26 (61.90)	90 (86.54)	
Surgical margins				<0.001
Yes	28 (19.18)	18 (42.86)	10 (9.62)	
No	118 (80.82)	24 (57.14)	94 (90.38)	
T stage				<0.001
T1-T2a	28 (19.18)	4 (9.52)	24 (23.08)	
T2b	35 (23.97)	3 (7.14)	32 (30.77)	
≥T2c	83 (56.85)	35 (83.33)	48 (46.15)	
Diabetes				0.105
Yes	7 (4.79)	4 (9.52)	3 (2.88)	
No	139 (95.21)	38 (90.48)	101 (97.12)	
Hypertension				0.001
Yes	39 (26.71)	19 (45.24)	20 (19.23)	
No	107 (73.29)	23 (54.76)	84 (80.77)	
Smoking				0.413
Yes	16 (10.96)	6 (14.29)	10 (9.62)	
No	130 (89.04)	36 (85.71)	94 (90.38)	
Ethnicity				<0.001
White	99 (67.81)	19 (45.24)	80 (76.92)	
Black	6 (6.16)	4 (9.52)	5 (4.81)	
Mixed ethnic ancestries	38 (26.03)	19 (45.24)	19 (18.27)	
Age (years)[a]	64.88±6.75 (66.00)	64.48±7.42 (64.50)	65.04±6.48 (66.00)	0.729
PSA (ng/ml)[a]	11.78±12.41 (8.55)	7.54±2.70 (7.15)	13.49±14.27 (9.70)	0.002

[a]Data are presented as the mean ± SD (median). HER2, human epidermal growth factor receptor 2; ISUP, International Society of Urological Pathology; PSA, prostate-specific antigen.

Table II. Distribution of HER2 expression in each group (P<0.001).

HER2 expression	group 1 N (%)	group 2 N (%)	Total N (%)
0	6 (14.29)	103 (99.04)	109 (74.66)
1+	22 (52.38)	1 (0.96)	23 (15.75)
2+	14 (33.33)	0 (0.00)	14 (9.59)
3+	0 (0.00)	0 (0.00)	0 (0.00)
Total	42 (100.00)	104 (100.00)	146 (100.00)

HER2, human epidermal growth factor receptor 2.

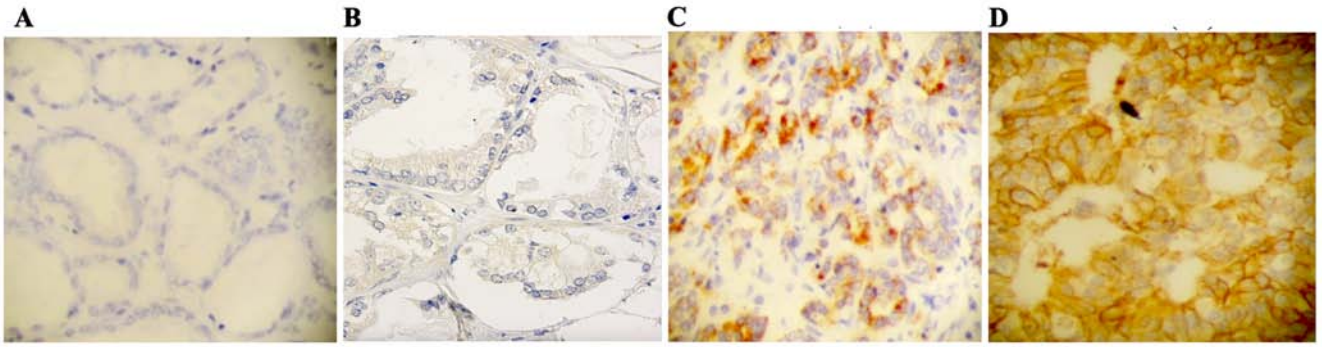

Figure 1. HER2 protein expression examined using anti-HER2 antibody A0485. (A) Absence of staining (0; negative control). (B) Faint/barely perceptible membrane staining detected in >10% of the tumor cells. Cells were stained in only part of the membrane (1+). (C) Weak to moderate complete membrane staining observed in >10% of tumor cells (2+). (D) Strong, complete membrane staining observed in >10% of the tumor cells (positive control; 3+). All magnifications are x200. HER2, human epidermal growth factor receptor 2.

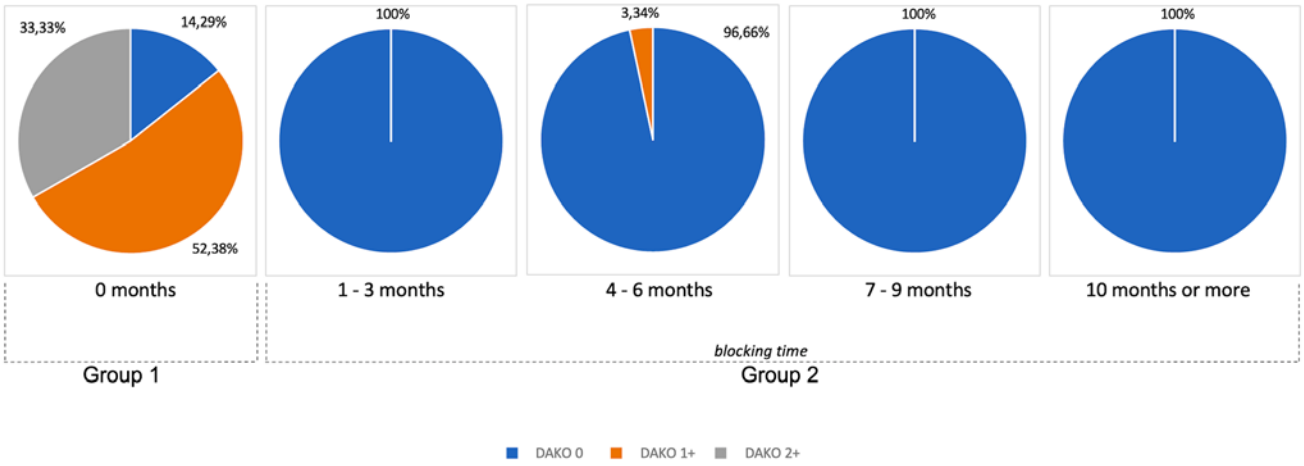

Figure 2. Expression of HER2 in patient group 1 and patient group 2 over time. In group 2, HER2 expression was subdivided into periods of exposure to neoadjuvant cyproterone acetate. A short period of neoadjuvant ADT suppressed HER2 expression. Dako 0, 1+ and 2+ refer to the graded relative intensities of staining. HER2, human epidermal growth factor receptor 2.

margin, and T stage had no significant effect on HER2 expression (P>0.05) (Table III).

When comparing both groups, the univariate regression analysis indicated that perineural invasion, PSA, ISUP, angiolymphatic invasion, margin, T stage, and neoadjuvant ADT correlated with HER2 expression. Nevertheless, ordinal regression analysis, including all cited variables, indicated a significant effect on HER2 expression only for neoadjuvant ADT (P<0.001). Similarly, regression analysis indicated a statistically significant effect of neoadjuvant ADT alone on HER2 expression (OR=0.01; 95% CI: 0.00. 0.02; P<0.001) (Table IV).

Discussion

The epidermal growth factor receptor (EGFR) family consists of four members: EGFR/ErbB1, HER2/ErbB2, HER3/ErbB3, and HER4/ErbB4. These are activated by ligand binding (except for HER2), followed by dimerization and phosphorylation (16). HER2 is the preferred dimerization partner for EGFR, and both regulate cell proliferation, differentiation, angiogenesis, and survival (17). Nevertheless, the role of ErbB-2 vs. EGFR in androgen-stimulated proliferation is still not fully understood; this is partially due to the lack of suitable cell models (18). In the present study, we evaluated, for the first time, the effect of neoadjuvant ADT on HER2 expression.

According to our results, the expression of HER2 occurred at distinct levels in a significant number of cases and was not associated with any prognostic factors. Various immunohistochemical methods have been used to examine the relationship between HER2 expression and PCa. Significant heterogeneity in HER2 expression has been noted in these previous studies (19-21), which is partially explained by discrepancy between methods, lack of measurement standardization, and heterogeneity of PCa itself (22). An important example is the study by Sanchez *et al*, who used two different evaluation techniques: The standard and modified HercepTest™ (23). This approach was necessary to improve the quality of HER2 analysis in patients with PCa. HER2 overexpression was found to be related to tumor stage and Gleason score. Our decision to use the standard HercepTest™ as a means of immunohistochemical interpretation was based on the literature and availability of kits in our institution's laboratories.

The introduction of neoadjuvant ADT was sufficient to suppress HER2 expression (P<0.001). This suppression was so relevant that individuals who received neoadjuvant ADT

Table III. Univariate and multivariate ordinal logistic regression for HER2 expression in group 1.

	Univariate regression		Multivariate regression	
Parameter	Odds ratio (95% CI)	P-value	Odds ratio (95% CI)	P-value
Age	1.00 (0.93-1.09)	0.924	-	-
Perineural invasion				0.134
No	Reference	-	Reference	
Yes	0.81 (0.25-2.65)	0.731	0.33 (0.0-1.41)	
PSA	1.07 (0.87-1.33)	0.511	1.04 (0.81-1.34)	0.733
ISUP				
1-2	Reference	-	Reference	-
3-5	5.33 (1.33-21.27)	0.018	1.26 (0.30-5.33)	0.757
Angiolymphatic invasion				
No	Reference	-	Reference	-
Yes	3.38 (0.96-11.90)	0.058	3.87 (0.81-1.34)	0.090
Surgical margins				
No	Reference	-	Reference	-
Yes	2.76 (0.82-9.31)	0.102	2.19 (0.54-8.83)	0.271
T stage				
T1-T2A	Reference	-	Reference	-
T2B	1.00 (0.06-15.31)	0.999	0.86 (0.05-14.70)	0.918
>T2C	1.97 (0.25-15.28)	0.514	0.97 (0.10-9.36)	0.980
Diabetes				
No	Reference	-	-	-
Yes	1.40 (0.17-11.89)	0.755	-	-
Hypertension				
No	Reference	-	-	-
Yes	0.48 (0.15-1.60)	0.236	-	-
Smoking				
No	Reference	-	-	-
Yes	1.45 (0.30-7.07)	0.645	-	-
Ethnicity				
White	Reference	-	-	-
Black	2.23 (0.29-17.23)	0.443	-	-
Mixed ethnic ancestries	0.76 (0.22-2.61)	0.666	-	-

ISUP, International Society of Urological Pathology. HER2, human epidermal growth factor receptor 2.

had a 0.01 chance of HER2 expression compared to individuals who did not receive neoadjuvant ADT (OR=0.01; 95% CI, 0.00, 0.02; P<0.001). Similarly, Muniyan *et al* observed that a HER2 inhibitor blocked androgen-induced activation and cell growth (24). These results are consistent with previous observations that HER2 activation plays an essential role in regulating the androgen-stimulated proliferation of PCa cells (25). This pharmacological inhibition revealed that basal and androgen-induced ERK1/2 and p38 MAPK were significantly inhibited, which correlated with abolished cell growth. In our study, the suppression of HER2 caused by neoadjuvant ADT occurred as soon as one month after the initiation of therapy and was maintained thereafter. This suppression seemed to be maintained throughout the period that PCa was shown to be sensitive to hormone therapy.

We observed a higher percentage of HER2 expression in group 1 (85.7%). A significant impact of neoadjuvant ADT was noted; only 1% of group 2 patients presented with HER2 expression. In addition, the effect was noted regardless of the time of analysis (1-24 months). Even a short period of neoadjuvant ADT suppressed HER2 expression. The study results highlight an exciting correlation between HER2, PCa, and ADT.

Interestingly, Chen *et al* demonstrated that dual inhibition of EGFR/HER2 with ADT resulted in the apoptosis of PCa cells (26). This could be an alternative, especially for castration-resistant prostate cancer (CRPC). In another study,

Table IV. Univariate and multivariate ordinal logistic regression for HER2 expression in all groups.

	Univariate regression		Multivariate regression	
Parameter	Odds ratio (95% CI)	P-value	Odds ratio (95% CI)	P-value
Age	0.99 (0.94-1.04)	0.733	-	-
Perineural invasion				
No	Reference	-	Reference	-
Yes	2.15 (1.01-4.54)	0.046	0.31 (0.08-1.28)	0.106
PSA	0.88 (0.81-0.96)	0.003	0.96 (0.81-1.15)	0.694
ISUP				
1-2	Reference	-	Reference	-
3-5	3.81 (1.74-8.33)	0.001	1.73 (0.45-6.68)	0.425
Angiolymphatic invasion				
No	Reference	-	Reference	-
Yes	4.71 (2.04-10.86)	<0.001	3.43 (0.78-15.16)	0.104
Surgical margins				
No	Reference	-	Reference	-
Yes	7.54 (3.21-17.75)	<0.001	1.88 (0.50-7.10)	0.349
T stage				
T1-T2A	Reference	-	Reference	-
T2B	0.76 (0.14-4.07)	0.413	0.71 (0.06-8.98)	0.790
>T2C	5.04 (1.41-18.02)	0.013	1.35 (0.17-10.64)	0.776
Neoadjuvant ADT				
No	Reference	-	Reference	-
Yes	0.01 (0.00-0.01)	<0.001	0.01 (0.00-0.02)	<0.001
Diabetes				
No	Reference	-	-	-
Yes	2.76 (0.60-12.78)	0.193	-	-
Hypertension				
No	Reference	-	-	-
Yes	2.61 (1.20-5.67)	0.016	-	-
Smoking				
No	Reference	-	-	-
Yes	1.82 (0.63-5.24)	0.268	-	-
Ethnicity				
White	Reference	-	-	-
Black	3.79 (0.96-14.98)	0.057	-	-
Mixed ethnic ancestries	2.92 (1.30-6.58)	0.010	-	-

ADP, androgen deprivation therapy; HER2, human epidermal growth factor receptor 2; ISUP, International Society of Urological Pathology.

Di Lorenzo *et al* observed a significant association between HER2, high levels of PSA, and a high Gleason score in patients with metastatic CRPC, contributing to the hypothesis of the association between HER2 and PCa aggressiveness. PCa recurrence also correlated significantly with c-erB2 levels in 60% of cases (27).

Significant efforts have been made to determine whether neoadjuvant treatment improves clinical outcomes in PCa (27). For radiation therapy, numerous studies have shown benefits with the addition of neoadjuvant, concurrent, and adjuvant ADT in treating intermediate- and high-risk diseases (28,29). In contrast, the benefits of neoadjuvant therapy before RP (both ADT and chemotherapy) have not yet been determined. In addition, no significant improvement in progression-free survival and overall survival (OS) has been demonstrated in several trials (30). Despite this, neoadjuvant therapy before RP provides a unique opportunity to clarify the effects of treatment on the tumor microenvironment. Access to material from an old study in which patients underwent neoadjuvant hormone therapy offered a unique opportunity to study the effects of this type of treatment on HER2 expression; this is the reason why we included group 2 patients in this study. The

suppression of HER2 observed in our study may be one of the mechanisms related to the response of tumors to ADT.

Some studies have suggested that HER2 acts as a co-receptor in the cell response mediated by HER substrates (31-33). In addition, overexpression of HER2 could increase the rate of cell transformation, one of the pathways involved in castration-resistant prostate adenocarcinoma. The specific activation of HER2 induces many independent signaling pathways, such as phospholipase C (PLC), phosphatidylinositol 3-kinase (PI3K), the JAK-STAT pathway, mitogen-activated protein kinases (MAPKs), and proteins activated by stress (27). These pathways activate proto-oncogenes such as c-fos, c-jun, and c-Src, which could lead to cell proliferation even in the absence of testosterone.

Signaling of the PI3K pathway by HER2 also induces phosphorylation and inactivation of glycogen synthase kinase-3 (GSK3), resulting in increased nuclear levels of β-catenin, which in turn increases the activity of the androgen receptor (AR) and, consequently, stimulates the growth and survival of prostate cells. These findings delineate the mechanism by which HER2 and AR regulate the androgen pathway during prostate cell growth and survival (34).

In metastatic PCa, circulating levels of HER2 have often been used as predictive markers of progression (35,36). Jathal *et al* demonstrated that the failure of lapatinib in clinical trials of CRPC was due to its ability to significantly increase HER2 levels, which consequently led to increased protein synthesis rates. This resulted in the accumulation of excess HER2 in the plasma membrane, the formation of EGFR/HER2 dimers, and the transmission of signals to downstream targets that prevent loss of cell viability (36). Similarly, Tome-Garcia *et al* demonstrated that overexpression of the constitutively activated form of HER2 increases the metastatic potential of androgen-insensitive human PCa cell lines, but not of androgen-sensitive PCa cell lines (37). All these results can lead to the hypothesis that the moment of transformation of PCa in CRPC could be correlated with the moment of increased HER2 expression after inhibition by ADT.

Future studies will examine whether suppression of HER2 transcription results in the cellular transformation of PCa, mainly in CRPC. The data reported herein suggest a possible association between ADT and the inhibition of HER2 expression while the tumor was hormone-sensitive.

Our study has certain limitations. First, as in several other studies, we performed immunohistochemical analysis to evaluate HER2 expression in prostate specimens. However, while immunohistochemistry has an established track record for evaluating the expression of HER2 in breast cancer, it has not been used as definitively in PCa. The HerceptTest™ technique we used has specific instructions only for breast and gastric cancers, and not PCa. In addition, it did not show classic HER2 overexpression (3+) in any of the 146 cases studied. According to the HercepTest™ Interpretation Manual, the specific result 'HER2 +2' could be analyzed later with fluorescent *in situ* hybridization (FISH), which was not available in our laboratories (13). The use of FISH has also been suggested to solve the potential problem of inconsistent results whenever different antibodies are used in immunohistochemical testing. Another limitation was the use of medications for androgen deprivation. Despite these limitations, which could limit the clinical significance of our findings, our material is unique and provides valuable insights for research purposes, as well as suggesting possible directions for further research using different methods, such as FISH.

The data reported here suggest a possible association between testosterone-suppressing hormone therapy and inhibition of HER2 receptor expression.

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Availability of data and materials

The datasets used and analyzed during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

All authors contributed to data collection and analysis. GAP, FK, MGDC and LHSS drafted the manuscript. CLP, TFNL, MLW, NMC, MTM and SG edited the manuscript. All authors have read and approved the final version of the manuscript. GAP and FK confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Centro Universitario FMABC and Santa Casa of São Paulo Hospital (approval nos. 84427718.0.0000.0082 and 06937412.0.1001.0082, respectively). Written informed consent was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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