Low-Level TOP2A Amplification in Prostate Cancer Is Associated with HER2 Duplication, Androgen Resistance, and Decreased Survival

Amanda J. Murphy, Caroline A. Hughes, Ciara Barrett, Hilary Magee, Barbara Loftus, John J. O’Leary, and Orla Sheils

Abstract
HER2 and TOP2A genes, located on 17q, can be coamplified in cancer. Overexpression of both genes has been reported in high-grade, androgen-resistant prostate cancer. Both genes have not been compared in a single prostate cancer study and the frequency of TOP2A amplifications in prostate cancer is unknown. Using tissue microarrays, we did immunohistochemistry and fluorescence in situ hybridization for HER2 and TOP2A in 100 prostate cancers (41 localized and 59 advanced) and 42 cases of benign prostatic hyperplasia (BPH). Amplification was defined as a target/centromere signal ratio of ≥1.5. HER2 immunohistochemistry was scored from 0 to 3+. Percentage nuclei staining for topoisomerase IIα (topoIIα) was recorded; overexpression was defined as ≥5% cells staining. Eighteen (31%) advanced prostate cancers showed topoIIα overexpression; 12 (26%) showed TOP2A low-level amplification; 9 (16%) expressed HER2; and 6 (13%) showed HER2 low-level amplification. No high-level amplification of either gene (target/centromere signal ratio of ≥3.0) was detected. TOP2A coexpression and coamplification were seen in 75% and 66% of HER2-positive cases, respectively. Localized prostate cancer or BPH showed no gene amplification or topoIIα overexpression. Gene amplification or overexpression correlated with high stage and Gleason score. The presence of TOP2A amplification in advanced cancer was associated with androgen resistance and decreased survival by multivariate analysis. This is the first study to document low-level TOP2A amplification in prostate cancer and an association with reduced survival. TOP2A amplification may occur with or without HER2 duplication and is often associated with topoIIα expression. Therapies directed against topoIIα (and HER2) in such patients may improve survival. [Cancer Res 2007;67(6):2893–8]

Introduction
The HER2 oncogene encodes a tyrosine kinase receptor with homology to epidermal growth factor receptor-1 (1, 2). HER2 is overexpressed by many tumors, usually secondary to HER2 amplification. In patients with breast cancer, HER2 amplification is associated with poor clinical outcome, but response to trastuzumab, a monoclonal antibody to HER2 protein (3). Because of the availability of this treatment modality, there is increasing interest in the amplification status of HER2 in other cancers, including prostate cancer.

The TOP2A gene is located adjacent to HER2 on chromosome 17q. TOP2A encodes topoisomerase IIα (topoIIα), an enzyme involved in DNA replication that is the target of a number of chemotherapeutic agents (4). Coamplification of HER2 and TOP2A has been described in breast and bladder cancer (5, 6). In breast cancer, TOP2A amplification occurs in a subset of HER2-positive tumors and is associated with increased sensitivity to topoIIα inhibitor chemotherapy (7).

Studies of HER2 expression in prostate cancer have yielded conflicting results. Recent studies using the 0 to 3+ scoring criteria have shown low rates of HER2 expression in prostate cancer, with expression mainly found in high-grade, androgen-insensitive tumors (8, 9). TopoIIα expression in prostate cancer has also been shown to be associated with increased Gleason score and androgen resistance (10, 11). The expression patterns of both genes have not been compared in a single study; it is not known if tumors that overexpress topoIIα coexpress HER2.

HER2 amplifications have been described in a percentage of HER2-positive prostate cancers (12, 13). The frequency of TOP2A amplification in prostate cancer and the existence of an association with HER2 amplification have not been reported to date. In light of this, we compared amplification and expression patterns of both genes in prostate cancer using tissue microarrays (TMA).

Materials and Methods

Case Selection and Review
One hundred patients with prostate cancer and 42 patients with benign prostatic hyperplasia (BPH) were randomly selected from all patients who underwent prostate surgery at the Adelaide and Meath Hospital during the period 1999 to 2003. Archival paraffin blocks from each case were retrieved from the pathology archives for TMA sampling. Chart review was done for each case. Patients with prostate cancer were divided into two groups: (a) patients treated with radical prostatectomy (n = 41) and (b) patients unsuitable for curative resection treated with hormonal therapy and palliative transurethral prostate resection (TURP; n = 59).

The following clinical data set was collated for each patient: age, date of initial diagnosis, preoperative prostate-specific antigen (PSA), clinical (group 2) or pathologic (group 1) stage, combined Gleason score, date of radical prostatectomy (group 1) or TURP (group 2), time to PSA recurrence or clinical evidence of disease progression, and survival time or time to last follow-up. Clinical data for both groups are summarized in Table 1. In group 2, the presence or absence of hormone resistance was recorded; this was defined as increasing PSA or other evidence of disease progression despite hormonal treatment.

Age and date of diagnosis were recorded for all patients with BPH.

Note: This work was presented at the U.S. and Canadian Academy of Pathology 95th Annual Meeting in Atlanta, GA, and the corresponding abstract was published in Modern Pathology. 2006;19(Suppl 1):S15A.

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doi:10.1158/0008-5472.CAN-06-2962
showed the greatest correlation with both androgen resistance and Gleason cases of BPH. To verify the presence of tumor in prostate cancer cases and benign glands in tissue microarray, tissue sections from each TMA were stained with H&E to confirm tissue morphology and to assess the presence of tumor. At least 1-mm intervals. A 4-μm section from each TMA was stained with H&E to confirm tissue morphology and to assess the presence of tumor. For each case, three 0.6-mm-diameter tissue cores were taken from a single paraffin block and sent to a VP2000 automated processor (Vysis) as follows: slides were deparaffinized in xylene for three 5-min intervals, followed by two 1-min intervals in 95% ethanol. Sections were treated in 0.2 mol/L HCl for 20 min at room temperature, pretreatment reagent (Vysis) for 30 min at 80°C, and protease solution (Vysis) for 40 min at 37°C. Slides were immersed in formalin, passed through graded alcohols (70%, 85%, and 95%) and air dried.

DNA denaturation and probe hybridization. Sections were denatured at 72°C for 5 min in 70% formamide 2× SSC solution. The double-stranded TOP2A probe was denatured by heating at 72°C for 5 min. Denaturation was not required for the single-stranded HER2 probe. After probe application, slides were coveredslipped and incubated on a thermal cycler for 18 h at 37°C. Posthybridization, slides were washed in 0.2× SSC 0.3% NP40 solution for 2 min at 73°C and counterstained with 4,6-diamidino-2-phenylindole (DAPI) solution (Vysis).

Fluorescent microscopy and scoring. Sections were examined by one pathologist (A.J. Murphy) with an epifluorescence microscope equipped with dual and single filters to detect the Spectrum Orange/Spectrum Green/DAPI fluorochromes. Tissue spots were scanned using a 40× objective to account for possible heterogeneity. Initially, the signals in 20 nuclei were counted to obtain a target/centromere signal ratio. In all cases with HER2 expression, TOP2A overexpression, or where the initial target/centromere ratio was ≥1.2 or ≤0.8, the number of signals in 60 nuclei was counted. Based on the previous reporting of low-level amplification of HER2 in prostate cancer (13) and of TOP2A amplification in breast cancer (7), amplification was defined as a ratio of the number of target gene signals to the number of CEP17 signals of ≥1.5. The presence of chromosome 17 aneusomy was also assessed. As in previous breast cancer studies (14), disomy 17 was defined as an average of 1.75 to 2.25 CEP17 signals per cell, whereas cases with <1.75 or >2.25 CEP17 signals per cell were defined as having aneusomy 17.

Statistical Analysis
Statistical analysis was done using SPSS for Windows, Version 12.0. The following tests were done: Fisher’s exact test for association with clinicopathologic variables, Kaplan-Meier survival analysis, univariate analysis using the log-rank test, and multivariate analysis using Cox regression.

Results
HER2 status in prostate cancer and BPH. Interpretation of HER2 immunohistochemistry based on the TMA was possible for 97 cancers and 41 BPH cases. Two cancers (2%) showed HER2 3+ reactivity, three cancers (3%) showed 2+ staining, and four cancers (4%) showed 1+ staining. The remaining 88 cancers (91%), including all localized cancers, showed 0 staining. All nine cancers that showed HER2 reactivity were locally advanced cancers of Gleason score 8 or more. Follow-up was available on eight of these patients; seven had developed androgen-resistant tumors. Two cases of BPH (5%) showed 1+ staining for HER2. The remainder showed 0 staining.

HER2 amplification status was evaluable in 93 cancers and 40 BPHs using FISH. Six (6.5%) tumors showed low-level HER2 amplification, with copy number ratio ranging from 1.5 to 2.19. In no case with high-level HER2 amplification (copy number ratio ≥3.0) was detected. The HER2-amplified tumors comprised both 3+ cases, two of three 2+ cases, none of the 1+ tumors, and 2 of 83

### Table 1. Patient and tumor characteristics in the two groups

<table>
<thead>
<tr>
<th></th>
<th>Group 1 (n = 41)</th>
<th>Group 2 (n = 59)</th>
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<td><strong>Age (y)</strong></td>
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<tr>
<td>T4</td>
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</table>

### Tissue Microarray Construction
Three TMAs were assembled using a manual tissue arrayer: TMA-1 contained 41 localized prostate cancers (group 1), TMA-2 contained 59 advanced prostate cancers (group 2), and TMA-3 contained 42 BPH cases. For each case, three 0.6-mm-diameter tissue cores were taken from a single archival paraffin block and inserted into the appropriate TMA block, spaced at 1-mm intervals. A 4-μm section from each TMA was stained with H&E to verify the presence of tumor in prostate cancer cases and benign glands in cases of BPH.

Immunohistochemistry
Immunohistochemistry for topolⅡa was done on an OptiMax (BioGenex, San Ramon, CA) automated immunostainer. After pressure cooker antigen retrieval in Trilogy solution (Cell Marque, Rocklin, CA), slides were incubated with primary antibody (DakoCytomation, Glostrup, Denmark) at 1:250 for 30 min followed by visualization using the Envision+ detection kit (DakoCytomation Denmark). Immunohistochemistry for HER2 was done using the HercepTest kit (DakoCytomation Denmark) according to the manufacturer’s instructions. All slides were counterstained with hematoxylin. Positive controls were included for each case: internal and external breast cancer controls (DakoCytomation Denmark) according to the manufacturer’s instructions. Primary antibody was omitted from negative controls.

Immunohistochemical Evaluation
HER2 immunohistochemistry was evaluated by two pathologists (A.J. Murphy and C. Barrett). Cases were scored as showing 0, 1+, 2+, or 3+ staining according to the criteria detailed in the HercepTest package insert (Dako). The percentage nuclei showing topolⅡa staining was evaluated by one pathologist (C. Hughes) and was validated using an Aiol image analysis microscope as previously described (11). TopolⅡa overexpression was defined as ≥5% positive nuclear staining. This was the cutoff point that showed the greatest correlation with both androgen resistance and Gleason score using Fisher’s exact test.

Fluorescence In situ Hybridization
Fluorescence in situ hybridization (FISH) was done using dual colored probes containing a Spectrum Orange–labeled probe for HER2 (PathVysion HER2 DNA Probe kit, Vysis, Downers Grove, IL) or TOP2A (LSI TOP2A/CEP17 Probe, Vysis) and a Spectrum Green–labeled probe for centromere 17 (CEP17). Slide preparation and pretreatment. Sections of 4 μm were cut onto silanized slides (DAKO), dried in an oven at 56°C overnight, and pretreated on a VP2000 automated processor (Vysis) as follows: slides were deparaffinized in xylene for three 5-min intervals, followed by two 1-min intervals in 95% ethanol. Sections were treated in 0.2 mol/L HCl for 20 min at room temperature, pretreatment reagent (Vysis) for 30 min at 80°C, and protease solution (Vysis) for 40 min at 37°C. Slides were immersed in formalin, passed through graded alcohols (70%, 85%, and 95%) and air dried.

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tumors scoring 0 by immunohistochemistry. Five of these six tumors were androgen resistant; one tumor that had scored 0 on HER2 immunohistochemistry was androgen sensitive. The mean HER2/CEP17 ratio increased progressively from tumors scoring 0 to those scoring 3+ (Table 2). No case of localized prostate cancer or BPH showed HER2 amplification.

**TOP2A status in prostate cancer and BPH.** As previously reported (11), topoIIα immunohistochemistry was available for 99 tumors and 41 BPHs. Based on the cutoff point of ≥5% positivity, 18 tumors (18%) showed topoIIα overexpression. All were locally advanced tumors, and all were of Gleason score ≥8 except for one Gleason 7 tumor. Follow-up was available for 17 of these patients; all had developed androgen-resistant tumors. No localized tumor or BPH case showed topoIIα overexpression.

FISH for TOP2A copy number was evaluable in 88 cancers and 40 BPH cases; low level TOP2A amplification was present in 12 (14%) cancers (copy number ratio 1.57 to 2.15). All of these were advanced, androgen-resistant cancers that had shown topoIIα overexpression. No case of localized prostate cancer or BPH showed TOP2A amplification, and none of the advanced cancers showed high-level TOP2A amplification.

**Correlation of HER2 status with TOP2A status and CEP17 aneusomy.** Results of topoIIα immunohistochemistry were available for eight HER2-reactive tumors. Six (75%) showed overexpression of topoIIα. There was a significant association between HER2 expression and topoIIα overexpression (Fisher’s exact test, $P = 0.0009$). In the advanced tumor group, 11% of tumors expressed both HER2 and topoIIα, 21% overexpressed topoIIα only, 4% expressed HER2 only, and 64% expressed neither protein.

Five cases showing topoIIα overexpression and HER2 coexpression were evaluable by FISH; all five showed TOP2A amplification, and four showed HER2 amplification (Fig. 1). TOP2A amplifications were identified in seven additional cancers that showed no amplification of HER2. Amplification frequencies for the advanced tumor group (group 2) were as follows: 72% of tumors showed no amplifications, 2% showed amplification of HER2 only, 17% showed amplification of TOP2A only, and 9% showed amplification of both HER2 and TOP2A.

Aneusomy 17 was identified in 18 of 40 (45%) localized cancers, all of which had hypodisomy 17 (<1.75 signals per cell). A total of 25 of 52 (48%) advanced cancers had aneusomy 17; 7 showed polysomy 17 (>2.25 signals per cell), whereas 18 showed hypodisomy 17. All BPH cases showed disomy 17. There was no association between gene amplification and average number of CEP17 copies per cell.

**Correlation with clinicopathologic variables.** HER2 expression, topoIIα overexpression, TOP2A amplification, and HER2 amplification all correlated with high tumor stage and high combined Gleason score (Table 3). In addition, overexpression ($P = 0.007$) or amplification ($P = 0.036$) of TOP2A was significantly associated with androgen resistance (Fig. 2). There was no

### Table 3. Results from Fisher’s exact test for correlation between gene expression or amplification and clinicopathological variables (stage and grade)

<table>
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<th>TNM stage</th>
<th>Gleason score</th>
</tr>
</thead>
<tbody>
<tr>
<td>HER2 expression</td>
<td>$P = 0.010$</td>
</tr>
<tr>
<td>TopoIIα expression</td>
<td>$P = 0.000$</td>
</tr>
<tr>
<td>HER2 amplification</td>
<td>$P = 0.033$</td>
</tr>
<tr>
<td>TOP2A amplification</td>
<td>$P = 0.000$</td>
</tr>
</tbody>
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**Figure 1.** Gleason 5 + 4 tumor. A, 2+ HER2 staining; B, topoIIα overexpression; C, HER2; D, TOP2A amplification as seen as an excess of red (target gene) signals over green (centromere 17) signals with a ratio of ≥1.5.
association between aneusomy 17 or polysomy 17 and stage, grade, or androgen resistance.

Correlation with survival. Length of available follow-up ranged from 0 to 39 months, with a mean of 13.9 months. During this period, 34 patients with advanced (group 2) tumors had developed androgen resistance, and 19 patients with advanced tumors had died.

In patients with advanced tumors, the following variables were tested by univariate analysis for relation with survival: presence of TOP2A amplification, HER2 amplification, aneusomy 17, polysomy 17, HER2 expression, topolα overexpression, age, Gleason score, and serum PSA at diagnosis. The following variables were significantly related to survival: TOP2A amplification (P = 0.007), PSA at diagnosis (P = 0.019), and topolα overexpression (P = 0.036). Using multivariate analysis, TOP2A amplification (P = 0.047) and PSA at diagnosis (P = 0.022) retained significant association with outcome (Fig. 3).

Discussion

Since the early 1980s, there has been increasing interest and scientific research into antitumor therapy that targets tumor-specific molecular functions or surface antigens. The goal of such therapy is improved specificity and decreased morbidity compared with cytotoxic chemotherapy. Trastuzumab (or herceptin), a monoclonal antibody to HER2 protein, was one of the first targeted therapies to show a survival benefit in patients with breast cancers that overexpressed HER2 (15, 16). Recent studies have shown an additional survival benefit in HER2-positive breast cancers that show coamplification of TOP2A and HER2 genes when treated with topolα inhibitor therapy (7). The possibility of enhanced sensitivity to topolα inhibitors places new significance on the presence of TOP2A and HER2 coamplification in other tumors.

This is the first study to document TOP2A amplification in prostate cancer. We found that TOP2A was amplified in 14% of all cancers and 26% of advanced cancers. TOP2A amplification was associated with HER2 amplification, but occurred approximately twice as frequently (HER2 was amplified in 6.5% of cancers and 12% of advanced cancers). Only low-level amplification of either gene was detected. These results are in contrast to those from breast cancer studies, where TOP2A amplification only occurs in the setting of HER2 amplification, being found in 25% to 41% of HER2-amplified tumors (5, 17). In addition, most breast cancer cases with amplification of either gene show very high-level amplification, often with gene/CEP17 ratios or 10 or more (18). Deletion of TOP2A (defined as target/centromere ratio ≤0.75), which is found in 23% to 43% of HER2-amplified breast cancers (5, 16), was not identified in any of the cancers in our study. These
results suggest that the amplified region within the 17q locus and the extent of amplification can vary from tumor to tumor, with a predisposition for high-level HER2 amplification in breast cancer and an apparent predisposition for low-level TOP2A amplification in prostate cancer. In support of this theory, comparative genomic hybridization and FISH studies of upper gastrointestinal cancer and malignant peripheral nerve sheath tumors have identified 23 different genes from the 17q12-q21 region, including TOP2A and HER2, that may be variably amplified in these tumors (19–21). The factors that determine the amplicon boundaries in a particular tumor are not fully understood. In our study, we found a good correlation between gene expression and amplification; 80% of cases that overexpressed topolΩ showed TOP2A amplification, and 80% of HER2-positive tumors (2+ or 3+ staining) showed HER2 amplification. The correlation was not perfect; three cancers (3%) showed topolΩ overexpression without TOP2A amplification, whereas two cancers (2%) and one case of BPH (2%) showed 1+ HER2 staining but no HER2 amplification. Finally, two cases of prostate cancer (2%) showed HER2 amplification but 0 staining by immunohistochemistry. In the literature, there are reports of HER2-negative, HER2-amplified prostate cancers (13) as well as weakly positive cases of prostate cancer and BPH showing no HER2 amplification (22, 23). In addition, topolΩ overexpression without gene amplification has been reported in breast cancer (24). Although overexpression of topolΩ or HER2 is usually due to gene amplification, other transcriptional and post-transcription mechanisms may up-regulate gene expression in the absence of amplification. In addition, differences in antigen retrieval methods used for immunohistochemistry and FISH are likely to play a significant role as well as differing sensitivities of protein and mRNA to degradation during formalin fixation. The biological significance of HER2 amplification in the absence of HER2 expression is unclear; in our study, HER2 expression showed a stronger correlation with advanced stage and high Gleason score than HER2 amplification, but numbers of HER2-amplified tumors were low (n = 6).

The association of HER2 expression, topolΩ overexpression, and HER2 amplification with high Gleason score and advanced tumor stage is consistent with published data (8–13). Our group previously reported an association between topolΩ expression and androgen resistance in prostate cancer (11). In this study, we found a strong correlation between TOP2A amplification and androgen resistance as well as an association between TOP2A amplification and survival. TopolΩ expression was also associated with decreased survival by univariate analysis. These findings are reminiscent of the initial studies of TOP2A amplification and expression in breast cancer in which amplification or expression of TOP2A was associated with adverse tumor characteristics and decreased survival (25, 26). Subsequent studies revealed improved survival in such patients when treated with topolΩ inhibitor chemotherapy, compared with patients with cancers with no TOP2A amplification (7). Testing for TOP2A overexpression and amplification in advanced prostate cancer reveals a subset of patients likely to develop androgen-resistant, aggressive tumors. We speculate that these patients might benefit from more aggressive chemotherapy in addition to or instead of antiandrogen therapy. It is also possible these tumors may show increased sensitivity to topolΩ inhibitor therapy. In cases showing HER2 coamplification, treatment with hereceptin may also be beneficial. It is not clear from this study whether FISH or immunohistochemistry for TOP2A is more informative in relation to prognosis; topolΩ overexpression showed a higher level of association with androgen resistance, whereas TOP2A amplification showed a stronger correlation with survival. Because of the relatively low prevalence of HER2 and TOP2A gene aberrations, larger scale studies of advanced prostate cancer are advisable to confirm the findings herein and may indicate the most appropriate method for assessing TOP2A status.

In conclusion, this study documents low-level TOP2A gene amplification in prostate cancer. Increased TOP2A copy number is associated with adverse clinical features, including high stage, high Gleason score, HER2 amplification, androgen resistance, and decreased survival under multivariate analysis. Treatment options for advanced prostate cancer, particularly in the setting of androgen resistance, are limited. Randomized controlled trials are necessary to investigate any survival benefit from topolΩ inhibitor therapy in patients with TOP2A-amplified prostate cancer. In addition, large-scale meta-analysis may be required to confirm the prognostic significance of TOP2A amplification in prostate cancer.

Acknowledgments

Received 8/9/2006; revised 12/5/2006; accepted 1/17/2007.

Grant support: Meath Foundation, Adelaide, and Meath Hospital, Tallaght, Dublin.

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