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Clinical Studies

Cancer Research

The Ability of Biomarkers to Predict Systemic Progression in Men with High-Risk Prostate Cancer Treated Surgically Is Dependent on *ERG* Status

R. Jeffrey Karnes[1], John C. Cheville[2], Cristiane M. Ida[3], Thomas J. Sebo[2], Asha A. Nair[3], Hui Tang[4], Jan-Marie Munz[3], Farhad Kosari[3], and George Vasmatzis[3]

Abstract

The objective of this study was to assess the relationship of the tumor protein levels of TOP2A and MIB-1 and *ERG* status with cancer-specific outcomes in men with high-risk prostate cancer treated by radical prostatectomy (RP). A 150-pair case-control study was designed from RP patients who developed systemic progression (SP) within 6 years of RP (cases) and men who were free of disease at least 8 years after RP (controls). The cases and controls were matched on conventional prognostic clinical parameters. TOP2A and MIB-1 levels were assessed by immunohistochemical methods, and *ERG* status was assessed by quantitative reverse transcription-PCR. The prognostic abilities of TOP2A and MIB-1 were significantly better in *ERG* (−) patients, and TOP2A was superior to MIB-1. In receiver operating characteristic analysis, the TOP2A and MIB-1 scores exhibited AUCs of 0.81 and 0.78 for *ERG*(−) patients, versus 0.67 and 0.68 for *ERG*(+) patients, respectively. Clinical parameters attained an AUC of 0.65 in *ERG*(−) patients and 0.54 in *ERG*(+) patients. When both markers were incorporated into a model for *ERG*(−) patients, the AUC increased to 0.83, with TOP2A showing a stronger association with SP than MIB-1. The time to SP was significantly associated with TOP2A; higher 5-year SP rates were observed in patients with higher TOP2A protein levels. In addition, although patient numbers are small, the response to adjuvant androgen deprivation therapy is associated with *ERG* status, showing more significant treatment effect in *ERG*(+) patients. *Cancer Res; 70(22); 8994–9002. ©2010 AACR.*

Introduction

In men with prostate cancer that undergo radical prostatectomy (RP), the clinicopathologic features predictive of the development of metastases [systemic progression (SP)] and death from prostate cancer consist of Gleason score (GS), tumor-node-metastasis (TNM) stage, surgical margin status, and preoperative serum prostate-specific antigen (PSA) levels (1–4). The vast majority of men with GS 6 prostate cancer and a substantial number of men with high GS (GS >7) prostate cancer are cured by surgery. However, in men with high-GS cancer and other adverse pathologic features such as extraprostatic extension, regional lymph node involvement, and positive margins of resection, more than 10% (5) develop SP and die from prostate cancer following RP. For these patients, contemporary clinical and pathologic features provide no additional prognostic information (6). The lack of prognostic data for these high-risk prostate cancer patients prevents individualized postoperative adjuvant therapy based on the risk of SP. In addition, there are no data available to predict a durable response to adjuvant androgen deprivation therapy (ADT).

We recently reported a prognostic model for high-risk prostate cancer that is based on the expression of several genes as well as DNA ploidy (6, 7). This gene panel model was developed and internally validated in men with high-risk prostate cancer treated by RP; it consisted of the DNA ploidy and mRNA levels of cadherin-10 and DNA topoisomerase 2α (*TOP2A*) and the status of gene fusion of common recurrent transmembrane protease serine 2 (*TMPRSS2*) and erythroblastosis virus E26 transforming sequence (*ETS*) transcription factors *ERG, ETV1*, and *ETV4*. The ETS fusion status was based on the mRNA levels of these genes. This panel achieved an area under the curve (AUC) of 0.79 in receiver operating characteristic (ROC) statistical analysis in predicting SP in an independent validation set. In this model, the strongest predictor of outcome was *TOP2A*, which achieved an AUC of 0.71.

ERG overexpression in prostate cancer was first observed by Vanaja and colleagues (8) in 2003. Subsequently, Tomlins and colleagues (9) identified a prostate cancer–specific gene fusion of *TMPRSS2-ERG* that explained the overexpression of *ERG*. The role of this fusion in prostate

Authors' Affiliations: [1]Department of Urology, [2]Laboratory Medicine and Pathology, [3]Department of Molecular Medicine and Center for Individualized Medicine, and [4]Department of Health Sciences Research, Mayo Clinic, Rochester, Minnesota

Note: R.J. Karnes, J.C. Cheville, and G. Vasmatzis contributed equally to this publication and share corresponding authorship.

Corresponding Authors: George Vasmatzis, John C. Cheville, or R. Jeffrey Karnes, Mayo Clinic, 200 First Street Southwest, Rochester, MN 55905. Phone: 507-266-4617 or 507-284-2511; Fax: 507-284-1599; E-mail: vasmatzis.george@mayo.edu, cheville.john@mayo.edu, or karnes.r@mayo.edu.

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Table 1. The distribution of the clinical and pathologic features of all patients in this study was balanced between cases and controls (when patients were split by ERG status SV invasion became unbalanced)

A. Clinical and pathologic features of the patients in the case-control study

Feature	Case (n = 141)	Control (n = 117)	P
Age at surgery			
Mean (SD)	63.8 (7)	64.1 (6.4)	0.7
Median	65	65	
Range	(47–77)	(50–76)	
Preoperative PSA (ng/mL)			
Median	11.4	11.3	0.996
Q1, Q3	1.3, 23.3	1.6, 21.1	
Range	1.3–143	1.6–119	
Gleason score			
7	87 (61%, 1.18)	63 (54%, 0.79)	0.25
8+	54 (39%, 2.18)	54 (46%, 1.35)	
Pathologic stage, 1997 TNM			
$T_{2a}N_0$	8 (6%)	11 (9%)	0.25
$T_{2b}N_0$	21 (15%)	21 (18%)	
$T_{3a}N_0$	31 (22%)	30 (26%)	
$T_{3b4}N_0$	50 (36%)	27 (23%)	
T_xN_+	30 (21%)	28 (24%)	
Margin Positive	88 (63%)	78 (67%)	0.53
Adjuvant Hormonal Treatment	55 (39%)	46 (39%)	0.99
Adjuvant Radiation Treatment	16 (11%)	15 (13%)	0.77

B. Clinical and pathologic features according to *ERG* status

	ERG(+)		*ERG*(–)	
	Cases, n = 56	Controls, n = 56	Cases, n = 83	Controls, n = 57
GS 7	39 (70%)	33 (59%)	46 (55%)	26 (46%)
GS 8+	17 (30%)	23 (41%)	37 (45%)	31 (54%)
SV invasion negative	28 (50%)	29 (52%)	46 (55%)	44 (77%)
SV invasion positive	28 (50%)	27 (48%)	37 (45%)	13 (23%)
Nodal status negative	44 (79%)	38 (68%)	66 (80%)	48 (84%)
Nodal status positive	12 (21%)	18 (32%)	17 (20%)	9 (16%)
Margin status negative	15 (27%)	16 (29%)	36 (43%)	21 (37%)
Margin status positive	41 (73%)	40 (71%)	47 (57%)	36 (63%)

NOTE: In B, the numbers in parentheses are the percentage of corresponding patients compared with all patients in that category (n). Unknown *ERG* status, n = 6.

cancer development and progression is unknown, but it seems to be an early event in prostate carcinogenesis, and although there is contradictory data, the majority of studies have identified an association of the *TMPRSS2-ERG* fusion with higher GS, higher stage, and more aggressive tumor behavior (10–13). At this time, it is unclear if prognostic molecular markers are shared between *ERG*(+) and *ERG*(–) cancers or if these are two unique cancer subtypes that have different molecular pathways for development and progression.

The objective of this study was to assess the prognostic ability of TOP2A protein levels detected by immunohistochemistry and the widely accepted proliferative marker MIB-1 relative to *ERG* overexpression in men with high-risk prostate cancer treated by RP. In addition, the associations of TOP2A protein levels, *ERG* overexpression, and response to adjuvant ADT were analyzed.

Materials and Methods

One hundred fifty men who developed SP (defined by biopsy-proven or radiographic-documented development of metastasis) or died with metastatic prostate cancer within 6 years following RP were identified (cases) using the Mayo Clinic Radical Prostatectomy database between the years 1994 and 2004. These cases were matched by a computerized score on GS, pTNM stage, margin status, preoperative serum PSA, age, and year of surgery with 150 men that did not develop SP or die with at least 8 years of follow-up (controls).

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Based on these data, an initial set of 300 samples, consisting of matched cases and controls, was defined. Tissues were acquired from the tissue bank and reviewed by a pathologist (J.C.C.) blinded to the case-control status. Of the 300 samples, 141 cases and 117 controls were selected by the pathology review and had sufficient tissue of the highest Gleason pattern available for experimental analysis. The median follow-up from RP to SP or last follow-up was 2.4 years for cases and 13.2 years for controls. The clinical and pathologic features of these subjects were balanced for all clinical parameters and had similar frequencies in adjuvant treatment (Table 1A).

TOP2A protein levels and MIB-1 were evaluated using standard immunohistochemical (IHC) techniques. H&E tumor sections from each patient were reviewed and blinded to the case-control status (C.M.I. and J.C.C.). All H&E slides were reviewed for primary and secondary Gleason patterns, and the block with the highest primary or secondary Gleason pattern and greatest tumor content of that pattern was selected for immunohistochemical analysis of TOP2A and MIB-1 as well as for assessment of *ERG* mRNA levels. Tumors were not assessed for tertiary patterns. After immunostaining, the areas with the highest percent of positive staining tumor cells ("hotspot areas") were visually encircled, and this area could include a tertiary pattern.

Immunostaining using monoclonal antibodies to TOP2A protein (clone 3F6, Novacastra; 1:100) and to Ki-67 antigen (clone MIB-1, Dako; 1:300) were done. The IHC stain was detected using the Dako Advance polymer-based detection system (Dako). Both proteins (TOPO2A and Ki-67) localize to the cell nucleus, resulting in a distinct and dark nuclear staining pattern. No cytoplasmic staining was identified. For each batch of immunostaining reaction, normal human tonsil section was used as a positive control. The immunostained slides were analyzed blinded to the case-control status, and the most intensely staining foci were circled, ranging from 2.4 to 110 mm^2. In most of cases, the circled tumor area was approximately the same for both TOP2A and MIB-1 analyses. Each slide was scanned using a Slide Scanner (Bacus Laboratories, Inc.) as previously described (14). The system captures digital images at 480- to 752-pixel resolution at ×40 magnification. Computer-assisted IHC stain quantification was performed using IHC Score Software (Bacus Laboratories, Inc.) to obtain measurements of the TOP2A and MIB-1 total nuclear area as well as immunoreactive nuclear area of the invasive tumor component within the circled foci. TOP2A and MIB-1 immunostaining was expressed as the percentage of invasive tumor total nuclear area that stained positive [labeling index (LI)]. After digital imaging analysis, slides were visually reassessed in relation to the TOP2A and MIB-1 LIs to ensure appropriate quantification.

ERG mRNA levels were assessed as previously described (7). Briefly, quantitative PCR was performed for *ERG* expression on the identical block used for the IHC analysis. H&E sections were reviewed and the areas of tumor circled. Sections (10 μm) were taken from each block and prepared under RNase-free conditions and deparaffinized in xylene. Tumor was scraped from the slide based on the circled template from the H&E slide and placed in 1.5-mL tubes containing digestion buffer (RecoverAll kit, Ambion). Total RNA was isolated according to the RecoverAll RNA isolation procedure and treated with DNase using Ambion Turbo DNA-free Kit according to the manufacturer's instructions (Ambion). The amount of RNA was measured with the Quant-iT RiboGreen kit (Invitrogen). Reverse transcription was done using the Superscript III First Strand Synthesis System (Invitrogen) and 500 ng of RNA in a 40-μL reaction volume.

Quantitative PCR was done on each sample by adding 12.5 ng of total RNA equivalent cDNA to a 20-μL reaction volume

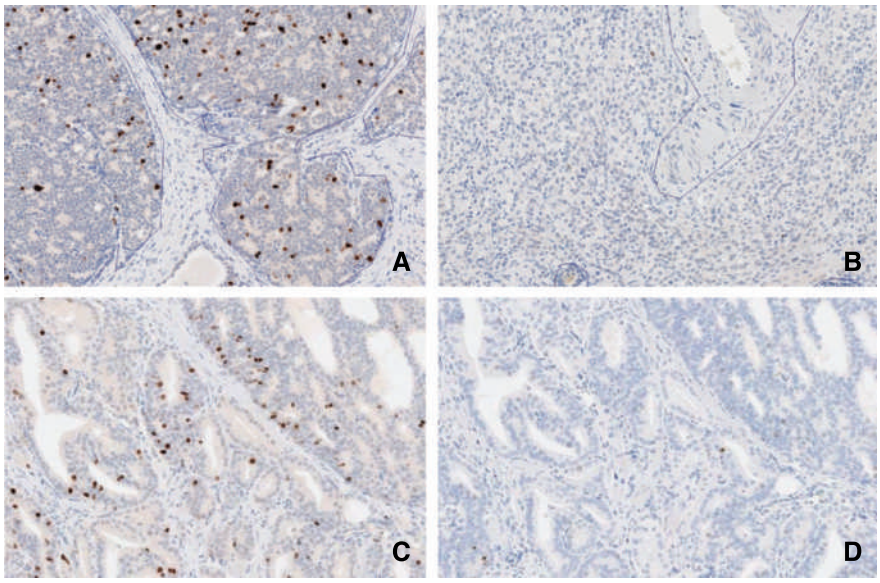

Figure 1. A and B, TOP2A immunostaining quantitation. A, *ERG*(–) Gleason 4 + 4 adenocarcinoma from a patient that died from metastatic prostate cancer (case) showing a TOP2A LI of 4.5% compared with (B) a *ERG*(–) Gleason 5 + 5 adenocarcinoma from a patient that was alive and free of disease 7 y after RP (control) showing TOP2A LI of 0.4% (×100). C and D, MIB-1 versus TOP2A LIs in an *ERG*(–) Gleason 4 + 4 adenocarcinoma from a patient that was alive and free of disease (control): high MIB-1 LI of 6.24% (C; 50×) versus low TOP2A LI of 0.15% (D; ×50).

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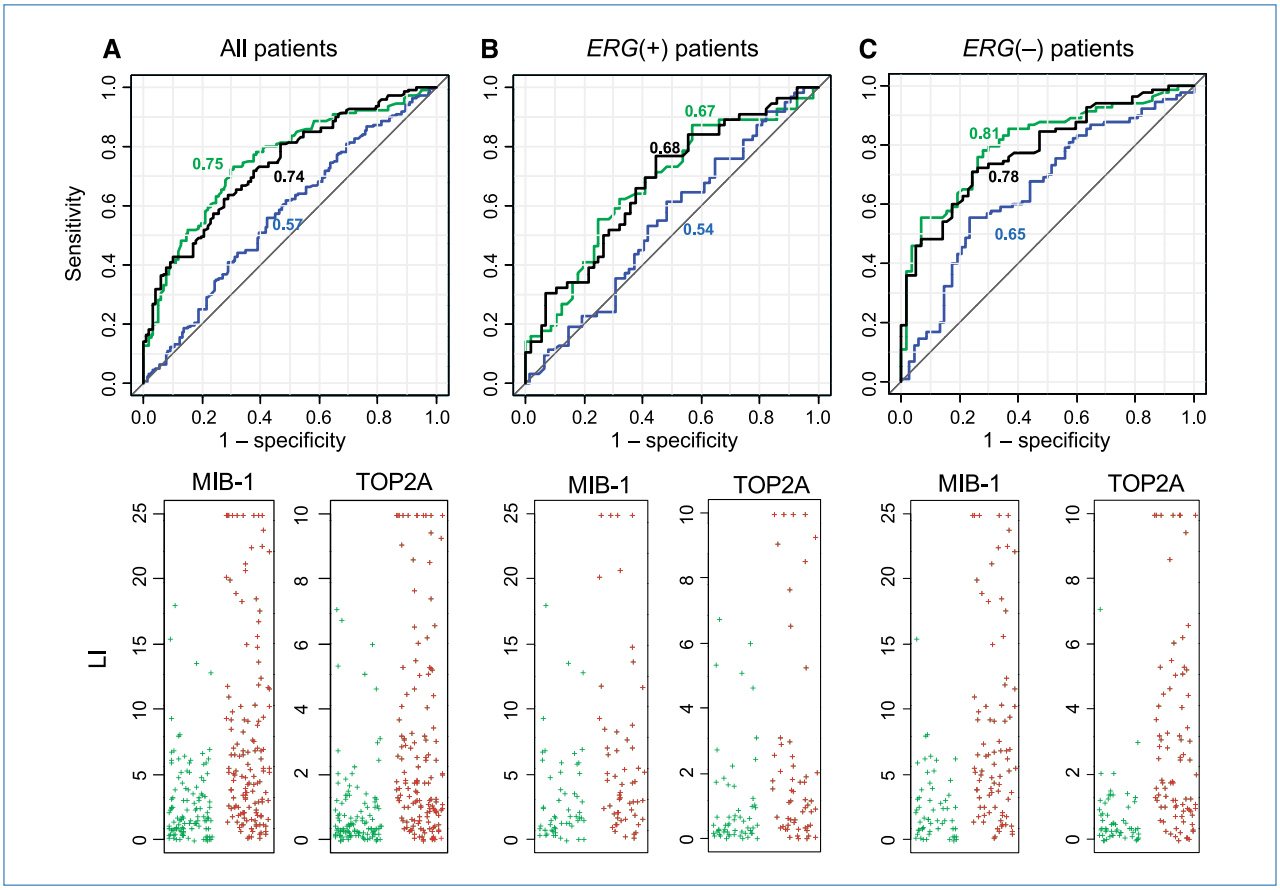

Figure 2. ROC analysis for TOP2A, MIB-1, and the clinical model in three populations: all patients, *ERG*(+) patients, and *ERG*(–) patients. The corresponding scatter plots for TOP2A and MIB-1 below the ROC curves show the controls in green and the cases in red. A, analysis for all patients: the AUC was 0.75 for TOP2A (green line), 0.74 for MIB-1 (black line), and 0.57 for the clinical model (blue line). B, analysis for *ERG*(+) patients: the AUC was 0.67 for TOP2A (green line), 0.68 for MIB-1 (black line), and 0.54 for the clinical model (blue line). C, analysis for *ERG*(–) patients: the AUC was 0.81 for TOP2A (green line), 0.78 for MIB-1 (black line), and 0.65 for the clinical model (blue line).

for each gene using SYBR Green PCR Master Mix (Applied Biosystems, ABI) on an ABI 7900HT real-time PCR machine using the manufacturer's default cycling conditions. Primers for quantitative PCR were designed by using Primer Express software (ABI) to amplify a 70- to 85-bp fragment of the Affymetrix target sequence from previous microarray experiments (7). ΔCts were obtained by subtracting the Ct of the normalizing gene from the Ct of the test gene of the same reverse transcription reaction. The normalizing gene, 40S ribosomal protein S28 (*RPS28*), was chosen by assessment of the Affymetrix data from previous experiments for a gene exhibiting the most stable expression across all prostate samples, normal and cancer. For technical reasons, the *ERG* status could not be determined for 6 patients.

Statistical methods

The R package was used for all statistical calculations. Case and control distributions of the clinical and pathologic parameters were compared using χ^2 and *t* tests. ROC curve areas were estimated for clinical and pathologic parameters (TOP2A and MIB-1 LIs) for all patients and stratified by the *ERG* status using the somers2 and ROC functions. SEs for AUCs were computed by the Rank Correlation for Censored Data (rcorr.cens) test (15). Interaction terms were computed using logistic regression glm function in R package. Multivariate logistic regression modeling was also performed by the glm function in R package. IDI statistics (16) was used to evaluate performance improvements of a model by adding a new parameter. The clinical parameter model that included GS, pTNM stage, preoperative PSA, and margin status was also developed by glm. The pTNM stage parameter was modeled as a discrete variable, where $T_{2a}N_0 = 0$, $T2bN_0 = 1$, $T_{3a}N_0 = 2$, $T_{3b4}N_0 = 3$ and $T_xN_+ = 4$. The percent survival curves related to SP were generated by the survfit function, and statistical differences were computed by the survdiff function. All studies were carried out under Mayo Institutional Review Board–approved protocols.

Results

TOP2A and MIB-1 LIs in relation to outcome and *ERG* status

The immunostaining quality of TOP2A and MIB-1 was comparable, and staining was localized to the tumor cell

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Table 2. Logistic regression modeling and statistical significance for MIB-1, TOP2A, and SVstat for *ERG* (–) patients

Models	Variables	Coefficients	*P*	AUCs
1	TOP2A	0.88	0.00004	0.81
2	MIB1	0.28	0.00004	0.78
3	TOP2A + MIB1	0.66, 0.13	0.007, 0.075	0.83
4	SVstat	1.44	0.0002	0.66
5	TOP2A + SVstat	0.83, 1.25	0.0001, 0.004	0.83
6	TOP2A + MIB-1 + SVstat	0.61, 0.13, 1.31	0.011, 0.058, 0.003	0.85

nuclei (Fig. 1). The evaluation of the staining was restricted to the two highest Gleason patterns, 4 and 5. Mean LIs ± SD for cases and controls were 3.45 ± 5.18% (range, 0.02–30.74%) and 0.92 ± 1.34% (range, 0.01–7.09%) for TOP2A (Fig. 1A for high TOP2A; Fig. 1B and D for low TOP2A) and 7.99 ± 8.86% (range, 0.11–43.58) and 3.25 ± 3.33% (range, 0.04–18.05) for MIB-1, respectively (Fig. 1C). For both, a significant difference was noted for cases and controls ($P < 0.001$) when the association of all relevant clinical parameters with case-control status was insignificant (Table 1A). In addition, TOP2A and MIB-1 LIs were statistically correlated (Spearman's coefficient $r = 0.77$).

TOP2A protein levels achieved an AUC of 0.75 (95% CI, 0.69–0.81; Fig. 2A, red curve), whereas MIB-1 LIs achieved an AUC of 0.74 (95% CI, 0.68–0.8; Fig. 2A, black curve). To show the improvement over clinical parameters in this group of patients (for whom clinical parameters were matched), we fit a logistic regression model that included GS, pTNM stage, preoperative PSA, and margin status (see Materials and Methods). This model exhibited an AUC of 0.57, but none of the coefficients had significant *P* values (Fig. 2A, blue curve).

ERG overexpression was present in 41% of cases and 48% of controls. As the association of TOP2A LIs and SP varied for *ERG* status ($P = 0.005$ for interaction), we stratified the analysis by *ERG* status. The prognostic ability of TOP2A protein levels for SP was significantly better in men with *ERG*(–) cancers than in men with *ERG*(+) cancers, with an AUC of 0.81 (95% CI, 0.74 to 0.88) versus 0.67 (95% CI, 0.57–0.77; Fig. 2B and C, red curves). Similarly, when analysis was stratified by *ERG* status, the prognostic ability of MIB-1 LIs for SP was also significantly better in men with *ERG*(–) cancers than in men with *ERG*(+) cancers, with an AUC of 0.78 (95% CI, 0.71–0.85) versus 0.68 (95% CI, 0.58–0.78; Fig. 2B and C, black curves).

To better characterize the predictive value of TOP2A and MIB-1 LIs, we tested TOP2A (model 1) and MIB-1 (model 2) alone and in combination (TOP2A + MIB-1, model 3). Interestingly, among men with *ERG*(–) cancers, multivariate modeling (model 3) showed a *P* value of 0.007 for TOP2A, and the contribution of MIB-1 was of marginal significance with a *P* value of 0.075 (Table 2). The AUC of the multivariate model was 0.83 (95% CI, 0.76–0.9; Fig. 3A, green curve). The change in AUCs from models 1 and 2 to model 3 was tested for

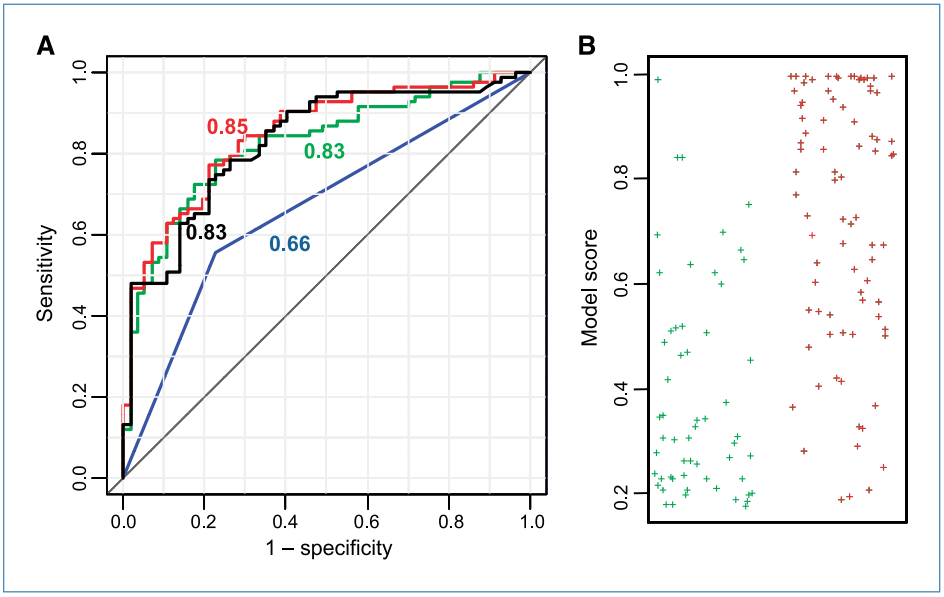

Figure 3. ROC analysis for the multivariate model combining SVstat, TOP2A, and MIB-1 in *ERG*(–) patients. A, the final combined model (red curve) achieved an AUC of 0.85 (95% CI, 0.79–0.91). This model was significantly better than the SVstat model (blue curve), the combined [TOP2A + SVstat] model (black curve), and the combined [TOP2A + MIB-1] model (green curve). B, the plot indicates the normalized model score of the final combined model, showing the controls in green and the cases in red.

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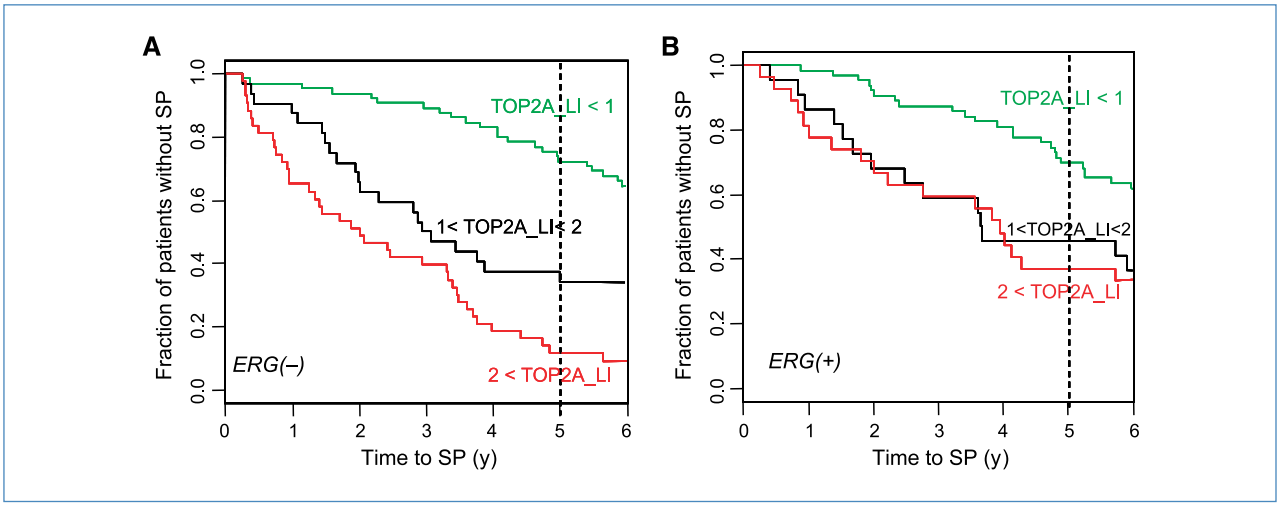

Figure 4. Time to SP in relation to *ERG* status and TOP2A LI. The percentage of patients that developed SP was analyzed in relation to time to SP for the selected TOP2A LI cutoff points of 1% and 2%. A, patients with *ERG*(–) cancers could be stratified into three groups: slower progressors (TOP2A LI <1%; green), intermediate progressors (1% < TOP2A LI < 2%; black), and fast progressors (TOP2A LI >2%; red); $P < 0.015$. B, patients with *ERG*(+) cancer could be separated into two groups: slower progressors (TOP2A LI <1%; green) and intermediate/fast progressors (TOP2A >1%; red and black); $P < 0.001$.

significance using the IDI statistic proposed by Pencina and colleagues (16) The *P* values were 0.075 for the transition from model 1 to model 3 and 0.00007 for the transition from model 2 to model 3 (Table 2). Therefore, TOP2A significantly improved the MIB-1 model, whereas the contribution of MIB-1 to the TOP2A model was marginal, indicating that TOP2A had significantly better prognostic ability than MIB-1 in *ERG*(–) patients, as illustrated in Fig. 1E and F. The modeling analysis was also applied for men with *ERG*(+) cancers, and the predictive values of TOP2A and MIB-1 were not statistically different (data not shown).

The association of the clinical parameters with case-control status was reexamined separately for *ERG*(+) and *ERG*(–) cancers by logistic regression, and a significant association was found for pTMN stage in *ERG*(–) patients. Logistic regression models that included GS, pTMN stage, preoperative PSA, and margin status achieved AUCs of 0.54 and 0.65 for *ERG*(+) and *ERG*(–) cancers, respectively (Fig. 2B and C, blue curves); however, only the coefficient of the pTNM stage parameter had a significant *P* value in *ERG*(–) patients. As a significant association was found for pTMN stage in *ERG*(–) patients, the clinical parameters were further stratified by *ERG* status (Table 1B). There were no significant differences in the distribution of the clinical parameters except for seminal vesicle status (SVstat) in *ERG*(–) patients. When the SVstat parameter was modeled alone as a binary variable, it resulted in an AUC of 0.66 for *ERG*(–) patients ($P = 0.0002$).

To test if TOP2A also significantly added to SVstat, we tested SVstat alone (model 4, Table 2) and in combination with TOP2A (model 5, Table 2). The change in AUCs from models 4 to model 5 using the IDI statistic (16) was significant ($P < 10^{-9}$) with a corresponding AUC of 0.83 (Table 2). Therefore, TOP2A significantly improved the clinical model in *ERG*(–) patients. Additionally, when MIB-1 was added to model 5, the AUC for the TOP2A, MIB-1, and SVstat combined model (model 6, Table 2) was significantly improved to 0.85 ($P = 0.02$; Fig. 3A, red curve). A scatter plot of the model score of this combined model is shown in Fig. 3B.

TOP2A protein levels, *ERG* status, and association with time to systemic progression

For all patients in our study, the relationship between time to SP after RP, TOP2A LIs, and *ERG* status was examined. The time to SP was related to both TOP2A protein levels and *ERG* status (Fig. 4). We investigated two TOP2A LI cutoff points (TOP2A LIs of 1% and 2%), which, based on the scatter plot distribution (Fig. 2B and C), separated cases from controls. Subsequently, the percentages of patients that developed SP after RP (cases) with respect to *ERG* status were plotted. These TOP2A LIs clearly separated these patients into groups that significantly differed in the time to SP. It is noteworthy that curves relating percent of patients developing SP with time to SP generated from a case-control study design are not representative of the overall population of men with high-risk prostate cancer; therefore, they should not be confused with Kaplan-Meier survival curves. Nonetheless, when we generated such curves, a significant difference in the 5-year rate of SP based on both *ERG* status and TOP2A LIs was observed (Fig. 4).

The patients with *ERG*(–) cancers could be separated in three groups based on TOP2A LIs: slow progressors (TOP2A LI < 1%), intermediate progressors (1% < TOP2A LI < 2%), and fast progressors (TOP2A LI > 2%), as illustrated in Fig. 4A. There was a statistically significant difference in the time to SP between the three groups (slow to fast progressors, $P < 0.0001$; slow to intermediate progressors, $P < 0.001$; and intermediate to fast progressors, $P < 0.015$). When patients with *ERG*(+) cancer were separated into these three risk groups (see above), the difference between intermediate

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and fast progressors was not significant ($P = 0.7$), as illustrated in Fig. 4B. However, by using solely the TOP2A LI cutoff point of 1%, *ERG*(+) patients could be separated into two groups with significantly different times to SP ($P < 0.001$).

TOP2A protein levels, *ERG* status, and survival related to adjuvant ADT

To determine the effects of adjuvant ADT, we examined TOP2A protein levels and *ERG* status in patients that either received or did not receive ADT postoperatively and before SP (i.e., cases only). Although there were differences in time to SP based on tumor *ERG* status, these differences did not reach statistical significance (Fig. 5A and B). Of note, in patients with TOP2A LIs >2% and *ERG*(+) cancer, adjuvant ADT resulted in a significantly slower time to SP ($P = 0.015$). This effect was not observed in patients with *ERG*(−) cancer (Fig. 5C and D).

Discussion

This study is the first to indicate that prostate cancer prognostic biomarkers vary in their ability to predict adverse outcomes based on *ERG* status, suggesting subtypes of prostate cancer with unique molecular markers predictive of aggressiveness. Recently, Tomlins and colleagues (17) showed that *ETS* fusion-positive cancers can have unique transcriptional signatures compared with *ETS* fusion-negative prostate cancers; thus, it is not unexpected that these cancer types would differ in the expression of prognostic biomarkers. Our study showed that TOP2A protein levels had significantly better prognostic ability than MIB-1 in *ERG*(−) cancers. Furthermore, when both TOP2A and MIB-1 were entered into a prognostic model for this group of patients, an AUC of 0.83 was obtained in comparison with an AUC of 0.81 for TOP2A and 0.78 for MIB-1.

The findings of this study support previous studies, including our own (6), that identified *TOP2A* as a prognostic marker in prostate cancer at the mRNA level. Herein, immunohistochemically assessed TOP2A protein levels identified men at greatest risk of SP following RP. Additionally, in men that developed SP, TOP2A protein levels were able to stratify them into groups that differed significantly in the time to SP. Finally, although the numbers of patients in this study are small, in men with *ERG*(+) cancers and TOP2A LIs >2%, adjuvant ADT seemed to provide a benefit in delaying the time to SP. Additional data to support the role of *ERG* status in assessing response to treatment are suggested in a study by Attard and colleagues (18), which examined the effects of abiraterone acetate in 77 patients in a phase I/II clinical trial with PSA decline as the primary efficacy end point. Although conclusions from our study and that by Attard and colleagues are limited by study design and patient sample size, they suggest an association between *ERG* status and hormonal responsiveness. Therefore, subsequent well-designed retrospective cohort studies or prospective randomized controlled trials are warranted to further analyze the significance of these findings. In a recent review, Tomlins and colleagues also indicated the need for future investigation of *ETS* fusion status in relation to response to adjuvant therapy (19).

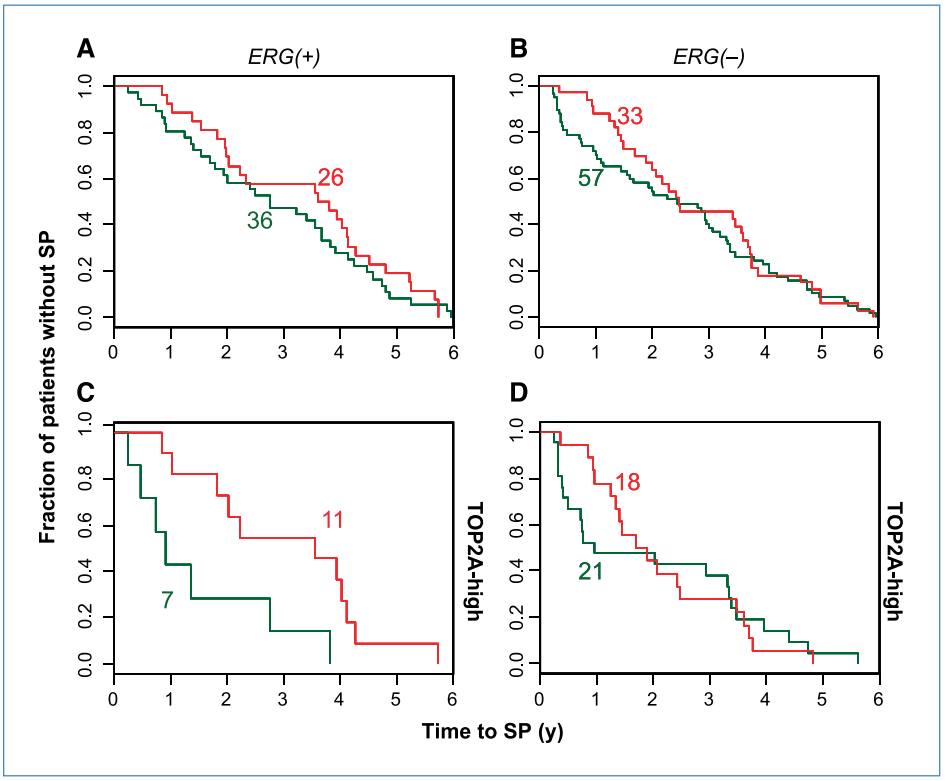

Figure 5. TOP2A LI and *ERG* status in relation to response to adjuvant ADT (cases only). Curves for the treated and nontreated groups are in red and green, respectively. The corresponding number of patients to each curve is indicated. The percentage of patients without SP was evaluated for time to SP according to *ERG* status. A and B, patients who developed SP (cases) regardless of TOP2A LIs. A, *ERG*(+) patients; B, *ERG*(−) patients. C and D, patients who developed SP (cases) with TOP2A LIs >2%. C, *ERG*(+) patients with TOP2A LIs >2% showed a significant difference in time to SP between the treated and nontreated groups ($P = 0.015$). D, *ERG*(−) patients with TOP2A LIs >2% did not show any significant difference between the treated and nontreated groups.

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Interestingly, when patients were reexamined for the distribution of clinical parameters based on case-control and *ERG* status, the presence of seminal vesicle (SV) invasion was higher in cases than in controls for *ERG*(–) patients, resulting in a significant increase in the AUC for the clinical parameters in this group of patients. The reason for this shift was unclear, but in our high-risk prostate cancer patient group, *ERG*(–) patients were more likely to have SV invasion and develop SP than *ERG*(+) patients, for whom the distribution of SV invasion was equal between cases and controls. When SV invasion was modeled alone, the AUC was 0.66 for *ERG* (–) patients. This finding is clinically relevant and will require external validation. When MIB-1 and TOP2A were added to a model incorporating SV status in *ERG*(–) patients, an AUC of 0.85 was attained.

Our study confirms the prognostic ability of MIB-1 in high-risk prostate cancer RP patients, and we show that the prognostic value is largely confined to men with *ERG*(–) cancers. TOP2A is a significantly better prognostic marker than MIB-1 in *ERG*(–) cancer, and both add prognostic information when incorporated into a model. This suggests that TOP2A and MIB-1 may not assess the same biological activity. The exact function of MIB-1 is unknown but is clearly associated with cell proliferation in both benign and malignant conditions. The function of TOP2A is well characterized, and in addition to proliferation, its expression may reflect a component of genetic instability with involvement of DNA repair mechanisms. We are currently investigating the functional role of TOP2A and its relationship to accumulated genetic abnormalities in prostate cancer, as well as the role of tumor sampling and heterogeneity for IHC staining. In men with *ERG*(+) tumors, both TOP2A and MIB-1 showed a weaker association with SP, indicating a need to identify additional molecular biomarkers for this group of patients.

As mentioned previously (6), the potential therapeutic role of TOP2A protein expands the types of adjuvant therapies available. TOP2A is the chemotherapeutic target of anthracyclines including doxorubicin, etoposide, and mitoxantrone. Moreover, *in vitro* studies have shown that the sensitivity of tumor cells to anthracyclines is dependent on the level of TOP2A protein expression. In addition to its value as a prognostic marker, TOP2A activity could also increase tumor sensitivity to cytotoxic chemotherapy, and as such, be a predictor of drug response. The role of TOP2A in response to anthracycline agents has been most extensively studied in patients with metastatic breast cancer, and it is suggested that the association of *HER2* to anthracyline response may be related to associated *TOP2A* gene amplification and protein overexpression (20–25). It is of note that we identified a significant correlation of *TOP2A* gene expression and TOP2A protein levels. There may be multiple explanations for the increased levels of mRNA and protein, including gene amplification at the DNA level; however, at this point, we have not examined our cases and controls for *TOP2A* gene amplification (26–28). Recently, Murphy and colleagues (26) showed that *TOP2A* gene amplification is present in prostate cancer and is associated with TOP2A protein expression. In addition, *TOP2A* amplification is significantly associated with high stage, GS, and decreased survival time. To the best of our knowledge, only one study has examined TOP2A protein expression in patients treated with an anti-TOP2A agent (30). In this series, 23 patients who had PSA recurrence after RP or radiation therapy were treated with mitoxantrone; as only 4 tumors had *TOP2A* assessed, no conclusions could be made.

As new agents emerge for the treatment of prostate cancer, including those that target TOP2A or with a different mechanism of inhibiting TOP2A resistance, it will be important to assess the effectiveness of these therapies in relation to TOP2A protein and *ERG* gene expression to identify patients that may potentially benefit most (29, 30). Moreover, if the status or level of a marker could guide treatment decision and be presurgically assessed in prostate biopsies, it could be readily translated into neoadjuvant treatment strategies.

It is important to note that there are several limitations particularly related to the assessment of the ERG fusion status through ERG transcript expression. The analysis by quantitative reverse transcription-PCR does not distinguish between the TMPRSS2-ERG fusion and the less common SLC45A3-ERG and NRDG1-ERG fusions, although 90% of the fusions related to ERG overexpression are the result of TMPRSS2-ERG. In addition, there are a small number of prostate cancers with the TMPRSS2-ERG fusion that do not overexpress ERG. Finally, the *ERG*(+) subtype in our study does not include the less common rearrangements involving the ETS transcription factors ETV1 and ETV4 because there is some evidence recently that they might not be functionally equivalent to ERG. In addition to the low homology at the protein level of ETV1 or ETV4 to ERG in the domains other than the DNA-binding domain, there is some recent evidence that the ERG and ETV1 transcription factors interact differently with the androgen receptor. ETV1, for example, has been shown to cooperate with androgen receptor in activating gene expression (31), whereas ERG seems to bind to androgen receptor and suppress androgen receptor–dependent transcription (32). In addition, further segregating *ERG*(–) patients for ETV1 and ETV4 will be limited by their low frequency. Finally, cutoff points of LI at 1% and 2% in *ERG*(–) were chosen to show that there is some association of TOP2A levels and survival and that choosing more than one clinical cutoff point might make a difference in a cohort study. Despite these limitations, the association of ERG with predictive biomarkers is important and clinically relevant, and future work is needed to determine the association of biomarkers with these less common ETS fusions in high-risk prostate cancer.

In summary, we identified an important role of TOP2A and MIB-1 protein levels in the prognosis of high-risk prostate cancer, which showed an association with *ERG* status. This underscores the importance of the prostate cancer–specific gene fusion of *TMPRSS2-ERG* with regard to the development of potential prognostic biomarkers. Additional studies are required to determine the significance of TOP2A protein levels and *ERG* status in relation to response to adjuvant therapy in men at high risk for systemic progression and death from

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prostate cancer. Furthermore, future efforts to identify biomarkers in *ERG*(+) prostate cancer are needed.

Disclosure of Potential Conflicts of Interest

Mayo Clinic has filed a patent application for parts of the technology described in this publication.

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The Ability of Biomarkers to Predict Systemic Progression in Men with High-Risk Prostate Cancer Treated Surgically Is Dependent on *ERG* Status

R. Jeffrey Karnes, John C. Cheville, Cristiane M. Ida, et al.

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