Frequent Discordance Between *ERG* Gene Rearrangement and ERG Protein Expression in a Rapid Autopsy Cohort of Patients With Lethal, Metastatic, Castration-Resistant Prostate Cancer

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BACKGROUND. *ERG* rearrangements in localized prostate cancer can be detected with high sensitivity and specificity by immunohistochemistry (IHC). However, recent data suggest that ERG IHC may be less sensitive for *ERG* rearrangements in castration-resistant prostate cancer (CRPC). Thus, we sought to examine ERG protein expression in a cohort of rapid autopsy patients with lethal metastatic CRPC (mCRPC).

METHODS. A tissue microarray (TMA) of tumor sites from these patients was evaluated for ERG, prostate-specific antigen (PSA), and androgen receptor (AR) expression by IHC and correlated with *ERG* rearrangement status by fluorescent in situ hybridization (FISH). IHC was scored as the product of tumor cell staining intensity (0–3) and percentage of cells positive (0–100) (overall product score range = 0–300).

RESULTS. All 16 (100%) *ERG* rearrangement negative (ERG^{neg}) patients were also negative for ERG tumor cell expression (i.e., IHC product score = 0). Of the 10 *ERG* rearrangement positive (ERG^{pos}) patients, two (20%) were completely negative for ERG tumor cell expression, while eight (80%) had weak ERG expression (median IHC product score = 5–110). Of these

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eight *ERG*^{pos} patients, five (63%) had at least one tumor site without any detectable ERG expression. For a given *ERG*^{pos} patient, ERG expression varied both between and within tumor sites; AR and PSA expression also varied between tumor sites, and there was no significant correlation between ERG and AR or PSA expression.

CONCLUSIONS. These data reveal frequent discordance between ERG IHC and *ERG* FISH in *ERG*^{pos} patients from this unique cohort of heavily treated lethal mCRPC. *Prostate 74:1199–1208, 2014.*

KEY WORDS: ***TMPRSS2-ERG*; androgen receptor (AR); immunohistochemistry (IHC); fluorescent in situ hybridization (FISH); rapid autopsy**

INTRODUCTION

Recurrent gene fusions involving the ETS family of transcription factors have been identified in nearly half of human prostate cancers [1–4]. When they do occur, these gene fusions are an early, clonal event in the pathogenesis of prostate cancer [2,4,5], which has helped illuminate its multifocal nature and subsequent clonal metastatic dissemination [6,7]. *TMPRSS2-ERG* is the most common ETS gene fusion in prostate cancer, occurring in more than 40% of all localized and metastatic tumors [8,9]; it is produced by rearrangement of chromosome 21, which brings *ERG* expression under androgen control via androgen receptor (AR)-mediated *TMPRSS2* transcriptional regulation. *ERG* gene rearrangements can be detected reliably by fluorescent in situ hybridization (FISH) [8], and in localized prostate cancer, immunohistochemistry (IHC) with an anti-ERG antibody has high sensitivity and specificity for the *ERG* gene fusion product, which demonstrates strong nuclear expression in *ERG* rearrangement positive (*ERG*^{pos}) tumor foci [10,11]. Recent literature suggests that ERG IHC, however, may be comparatively less sensitive for *ERG* rearrangements in castration-resistant prostate cancer (CRPC) [12,13], as well as prostate cancer with high-grade neuroendocrine features (i.e., small cell carcinoma of the prostate) [14,15].

Rapid ("warm") autopsies provide invaluable opportunities for the procurement of primary and metastatic tissue samples from patients with advanced, treatment-resistant tumors [16,17]. At the University of Michigan Health System, we have performed rapid autopsies on a large cohort of patients with heavily treated metastatic CRPC (mCRPC), and samples from these patients' tumors have played an essential role in advancing our understanding of lethal prostate cancer, including novel mechanisms of androgen signaling dysregulation [9,18–20]. Because *TMPRSS2-ERG* is androgen-regulated in *ERG*^{pos} prostate cancer, ERG protein expression may not be a faithful reporter of *ERG* rearrangement status in advanced, heavily treated tumors, which often demonstrate a reactivated but dysfunctional AR signaling axis [20]. Thus, we sought to examine the spectrum of ERG protein expression at various tumor sites, as well as the concordance between ERG IHC and *ERG* FISH, in a large cohort of rapid autopsy patients with lethal mCRPC.

MATERIALS AND METHODS

A tissue microarray (TMA) comprised of rapid autopsy material from 30 patients with mCRPC has been previously described [18]; these patients received multimodal therapy, including a combination of radical prostatectomy, hormone deprivation (i.e., bilateral orchiectomy and/or first-generation anti-androgen therapy), radiation, and/or chemotherapy. Briefly, the TMA includes samples from all metastatic tumor sites, as well as primary tumor within the prostate (when present at the time of autopsy; i.e., no prior radical prostatectomy), and each tumor site is represented in triplicate cores. In the current study, ERG, AR, and prostate-specific antigen (PSA) IHC was performed using a BenchMark ULTRA automated stainer and the ultraView Universal DAB Detection Kit (Ventana Medical Systems, Oro Valley, AZ). The following primary antibodies were used: ERG (EPR3864; pre-dilute, Ventana Medical Systems); PSA (polyclonal; pre-dilute, Ventana Medical Systems); and, AR (AR441; pre-dilute, Cell Marque, Rocklin, CA). All available TMA tissue cores were evaluated for ERG, AR, and PSA protein expression by two study pathologists (A.M.U. and R.M.), and cores without tumor were excluded from further analysis. ERG endothelial cell expression was used as an internal positive control for ERG IHC. Staining intensity was scored as negative (0), weak (1), moderate (2), or strong (3), and the percentage of positive tumor cells was recorded (0–100). For ERG and AR, only nuclear expression was scored. For each evaluable core, an IHC product score (range 0–300) was calculated as the product of the staining intensity and percentage of positive tumor cells. For a given patient and tumor site (e.g., lung from patient #1), if more than one core was evaluable, the final IHC product score was calculated as the

average of all evaluated cores. For TMA cores from ERG^{pos} patients with negative ERG tumor cell expression and without discernible ERG endothelial cell expression, whole tissue sections from parent tissue blocks were evaluated by ERG IHC to confirm positive ERG endothelial cell expression. Because protein expression in tissue sections might be subject to a number of fixation and methodological variables, particularly for rapid autopsy specimens [21], TMA ERG tumor cell expression was validated by whole tissue sections from at least two parent tissue blocks for each ERG^{pos} patient.

Statistical correlation between ERG, AR, and PSA tumor cell expression was assessed by calculating the Pearson correlation coefficients (PCC) and corresponding *P*-values for all possible IHC pairs (i.e., ERG and PSA, ERG and AR, and PSA and AR). All statistical analyses were performed using R (version 3.0.2). Heat map plots were generated using GENE-E (Broad Institute, Cambridge, MA).

RESULTS

We previously reported the clinicopathologic characteristics of and the frequency and mechanism of *TMPRSS2-ERG* rearrangements in a rapid autopsy cohort of patients with lethal mCRPC [9,18]. ERG protein expression, however, has not been systematically assessed in this cohort; thus, we sought to evaluate ERG tumor cell expression by IHC using a tissue microarray (TMA) containing a range of tumor sites. Using a dual-color, break-apart *ERG* FISH method, our previously published data demonstrated that 10 (33%) of the patients in this cohort were positive for *ERG* rearrangement (ERG^{pos}), while 20 (67%) were negative for *ERG* rearrangement (ERG^{neg}) [9]. Currently, we evaluated ERG tumor cell expression in these patients by assigning each TMA core an IHC product score (range 0–300; see Materials and Methods). Twenty-six (87%) patients were evaluable for ERG tumor cell expression by IHC. Consistent with the high specificity of ERG IHC for the detection of *ERG* rearrangements in prostate cancer [10], all 46 tumor sites from ERG^{neg} patients were negative for ERG expression (i.e., IHC product score = 0) (Fig. 1K). Twenty-eight tumor sites from ERG^{pos} patients were scored for ERG expression, and the number of sites per patient ranged from one to four. Two (20%) of the ERG^{pos} patients (patients #2 and #22), both with high-grade neuroendocrine features, were completely negative for ERG tumor cell expression across all sites. For the remaining eight ERG^{pos} patients, ERG tumor cell expression was weak (median site IHC product score = 5–110) (Fig. 1E). Five (63%) of these patients had at least one tumor site without any detectable ERG protein expression (Fig. 1H), and the maximum IHC product score for any site ranged from 5 to 32. Thus, altogether, seven (70%) of the ERG^{pos} patients had completely negative or weak ERG expression at all tumor sites. The remaining three ERG^{pos} patients demonstrated weak to moderate ERG tumor cell expression at all sites (maximum IHC product score = 100–220) (Fig. 1B).

To further validate our TMA results, ERG IHC was performed on whole tissue sections from at least two parent tissue blocks for each ERG^{pos} patient, including any TMA site with negative ERG tumor cell expression but without ERG endothelial cell expression (i.e., no internal positive control); seven [7] cores without an internal positive internal control were identified, and all demonstrated retained ERG endothelial cell expression on the evaluated corresponding parent tissue sections. Furthermore, whole-section ERG IHC was concordant with the corresponding TMA results in all cases except one (patient #1), for which the discordant results are noteworthy (Fig. 2). The TMA results for this patient demonstrated weak or negative ERG tumor cell expression at all four metastatic tumor sites (liver, lymph node, soft tissue, and pancreas). The corresponding whole tissue sections, on the other hand, revealed remarkable intra-site heterogeneity of ERG tumor cell expression, ranging from negative to patchy and weak to diffuse and strong (Fig. 2). ERG endothelial cell expression, on the other hand, was moderate to strong throughout these sections. Interestingly, for this patient, the areas of TMA core sampling (as revealed by holes in the tissue sections) were concentrated in areas of weak ERG tumor cell expression (data not shown), providing a plausible explanation for the observed discordance between TMA and whole-section ERG IHC. Also concordant with the TMA results, two ERG^{pos} patients (#2 and #22) with high-grade neuroendocrine features were negative for ERG tumor cell expression in whole tissue sections. A third ERG^{pos} patient (#24) with high-grade neuroendocrine features demonstrated negative whole-section ERG tumor cell expression at two metastatic tumor sites (liver and lymph node) but patchy and weak ERG tumor cell expression at another site (lung) (Fig. 3); intriguingly, as opposed to the diffuse high-grade neuroendocrine features in the liver and lymph node, the metastatic tumor cells in the lung section exhibited a predominantly poorly differentiated acinar morphology. Overall, these data demonstrate that, in the majority of the heavily treated ERG^{pos} mCRPC patients studied herein, ERG tumor cell expression, as detected by IHC, is predominantly weak, with considerable inter- and intra-site heterogeneity.

Because ERG protein expression in ERG^{pos} prostate cancer is regulated by androgen signaling (via AR

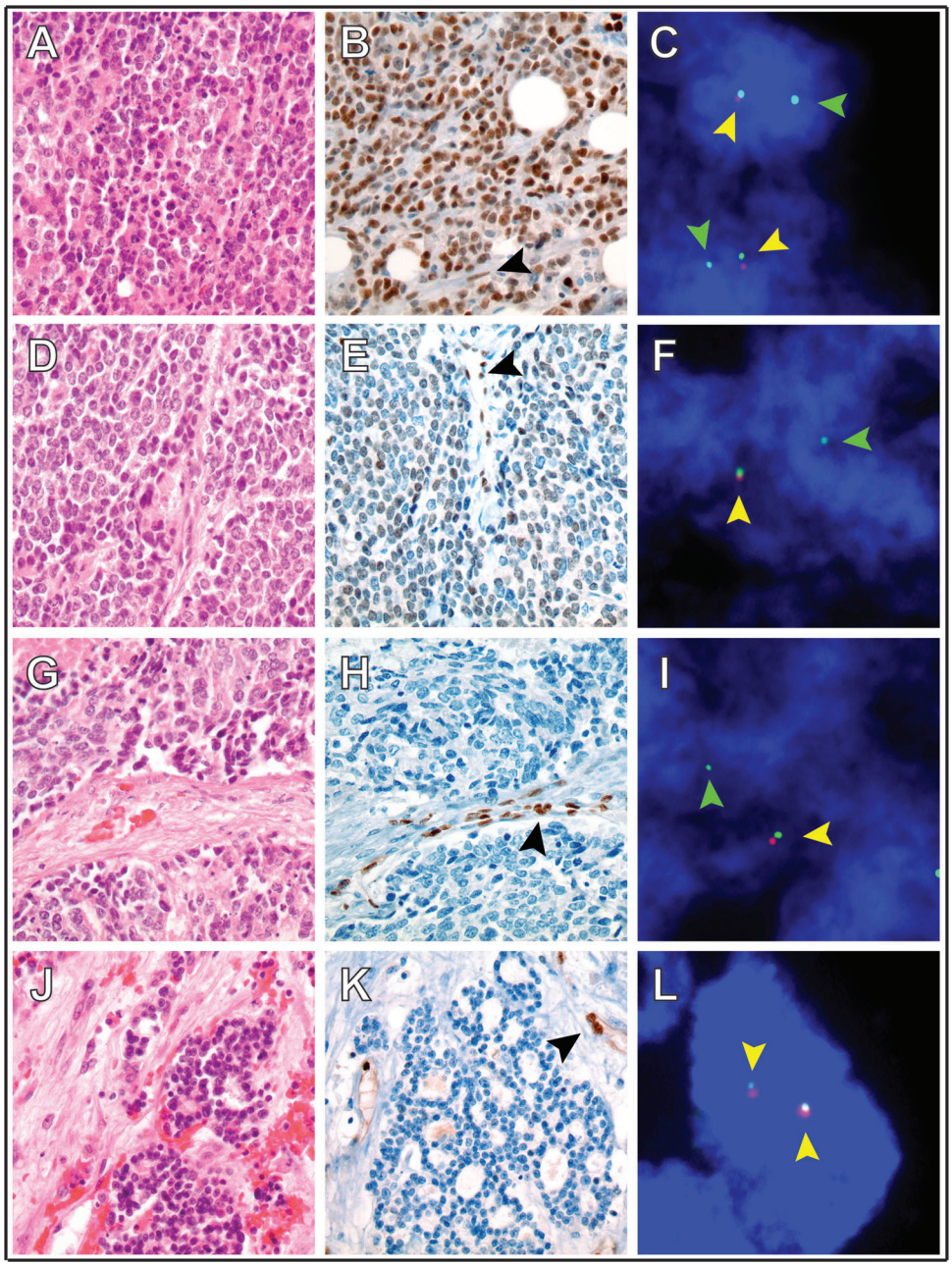

Fig. 1. Discordance between ERG IHC and *ERG* FISH in a rapid autopsy cohort of lethal mCRPC. (**A**, **D**, **G**, **J**) H&E, (**B**, **E**, **H**, **K**) ERG IHC, or (**C**, **F**, **I**, **L**) *ERG* FISH in (**A**–**I**) ERG^{pos} or (**J**–**L**) ERG^{neg} patients. In ERG^{pos} patients, ERG tumor cell expression ranges from (**B**) diffuse and moderate to (**E**) patchy and weak to (**H**) negative, and ERG IHC is negative in ERG^{neg} patients (**K**). Strong ERG endothelial cell expression (black arrowheads) provides an internal positive control for IHC staining in cases with negative ERG tumor cell expression. Dual-color, break-apart FISH method to determine *ERG* rearrangement status: wild type *ERG* allele = yellow signal (colocalized signals; yellow arrowhead), and rearranged *ERG* allele = single green signal (green arrowhead). H&E and ERG IHC = 400× magnification.

response elements in the *TMPRSS2* promoter) [3], one possible explanation for the predominantly weak and heterogeneous ERG tumor cell expression observed in our cohort is dysregulated androgen signaling. Previously, we reported on the expression of AR, as well as the androgen-regulated prostate-specific antigen (PSA), in lethal mCRPC patients [18]; by IHC, AR and PSA demonstrated variable protein expression both within and between tumor sites. However, in that prior study, *ERG* rearrangement status was not available, and thus, in order to correlate ERG, AR, and PSA protein expression in ERG^{pos} tumors, we sought to reassess AR and PSA IHC in an analogous manner to ERG. For this study, fresh TMA slides were evaluated

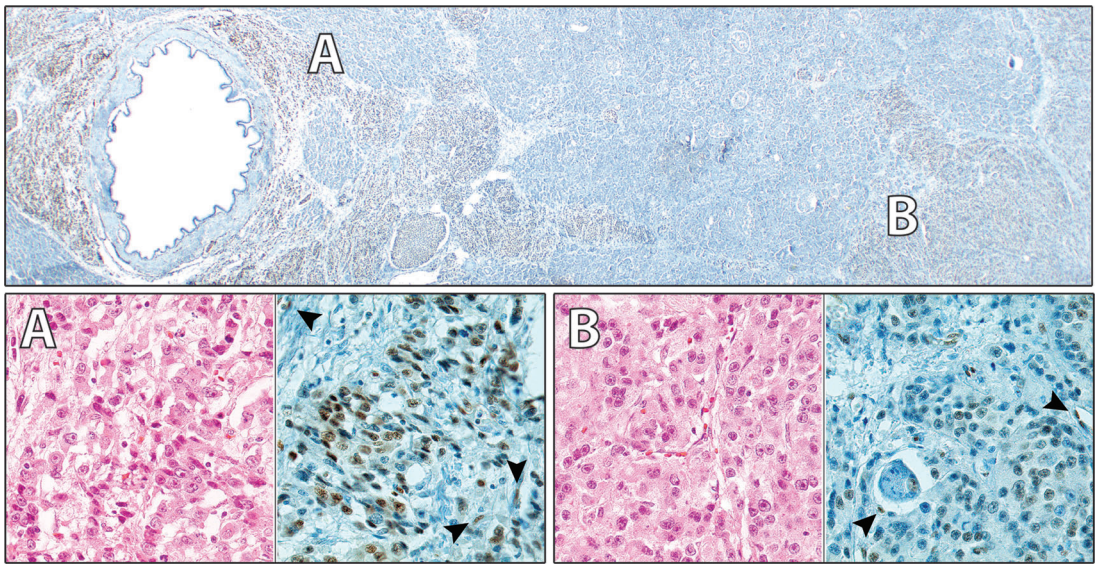

Fig. 2. Intrasite heterogeneity of ERG tumor cell expression in a case of lethal *ERG*^{pos} mCRPC. (Top panel) Low-power image of pancreas involved by *ERG*^{pos} mCRPC (patient #1) demonstrating significant intrasite heterogeneity of ERG tumor cell expression (ERG IHC; 20× magnification). Letters correspond to higher power images in bottom panel. (Bottom panel; **A** and **B**) High-power images of areas indicated by letters in the top panel, showing (**A**) diffuse and strong or (**B**) patchy and weak ERG tumor cell expression (left sub-panel = H&E, right sub-panel = ERG IHC; 400×). Strong ERG endothelial cell expression (black arrowheads) provides an internal control for IHC staining in areas with weak ERG tumor cell expression.

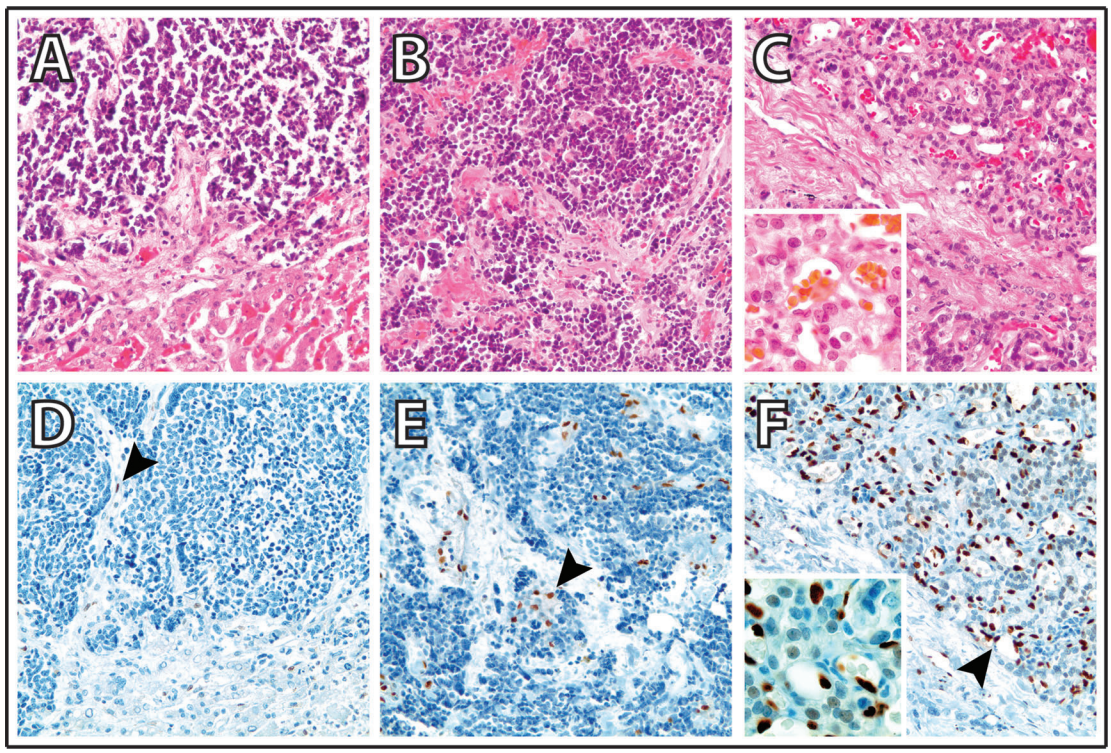

Fig. 3. Intersite heterogeneity of ERG tumor cell expression in a case of lethal *ERG*^{pos} mCRPC. (**A**–**C**) H&E or (**D**–**F**) ERG IHC of (**A**, **D**) liver, (**B**, **E**) soft tissue, or (**C**, **F**) lung in an *ERG*^{pos} patient with mCRPC (patient #24). Tumor cells in the liver and soft tissue (**A**, **B**, **D**, **E**) exhibited high-grade neuroendocrine morphology and were negative for ERG expression, whereas tumor cells in the lung (**C**, **F**) demonstrated a poorly differentiated acinar morphology and had patchy and weak ERG expression. Strong ERG endothelial cell expression (black arrowheads) provides an internal control for IHC staining in areas with negative or weak ERG tumor cell expression. 200× magnification. Inset in C and F = 1,000× magnification.

for AR and PSA tumor cell expression by IHC using current staining protocols and available antibodies (see Materials and Methods). Thirty-two tumor sites from ERG^{pos} patients were scored for AR expression, and the number of sites per patient ranged from two to five. Overall, maximum AR IHC product scores ranged from 0 to 290 (median = 165); five (50%) of the ERG^{pos} patients had a maximum score greater than 200, while two (20%) had a maximum score less than 100. One (10%) ERG^{pos} patient, with high-grade neuroendocrine features, was negative for AR expression at all three tumor sites. Another ERG^{pos} patient, also with high-grade neuroendocrine features, was negative for AR expression at three of five tumor sites, while the remaining two sites exhibited relatively robust expression (median IHC product score = 200). In all, four (40%) ERG^{pos} patients had at least one tumor site without AR protein expression, and the maximum AR IHC product score for any site in these patients ranged from 0 to 205. Thirty tumor sites from ERG^{pos} patients were scored for PSA expression, and the number of sites per patient ranged from two to four. Overall, maximum PSA IHC product scores ranged from 0 to 300 (median = 130); four (40%) of the ERG^{pos} patients had a maximum PSA IHC score greater than 200, while two (20%) had a maximum score less than 100. One (10%) ERG^{pos} patient, with high-grade neuroendocrine features, was negative for PSA expression at all three tumor sites. In all, three (30%) ERG^{pos} patients had at least one tumor site without PSA protein expression, and the maximum PSA IHC product score for any site in these patients ranged from 0 to 300. Thus, while the majority of patients with ERG^{pos} mCRPC demonstrated moderate AR and PSA expression, there was significant variability between tissue sites, and a proportion of patients had at least one negative tumor site.

We next sought to examine possible correlation between ERG, AR, and PSA IHC in ERG^{pos} and ERG^{neg} patients in our cohort. For ERG^{pos} patients, negative, weak, or moderate ERG expression was observed with corresponding variable AR and PSA expression (Figs. 4 and 5); in these patients, there was no significant correlation between ERG and AR or PSA expression (Fig. 5). Interestingly, while some tumor sites exhibited negative ERG expression despite moderate AR expression (Fig. 4G), the opposite pattern was not observed (i.e., no sites with negative AR expression were positive for ERG expression). Finally, in ERG^{neg} patients, AR and PSA were strongly and significantly correlated (PCC = 0.76; P-value < 0.01), and similarly, there was a trend toward significant, moderate correlation between AR and PSA expression in ERG^{pos} patients (PCC = 0.57; P-value = 0.07) (Fig. 5). Thus, in this cohort of highly treated lethal ERG^{pos} mCRPC, there were differences in the correlation between AR and two androgen-regulated gene products (i.e., ERG from *TMPRSS2-ERG* and PSA).

DISCUSSION

Our data demonstrate profound discordance between ERG IHC and *ERG* FISH in ERG^{pos} patients from a unique cohort of lethal, heavily treated mCRPC. ERG tumor cell expression is negative or weak in the vast majority of ERG^{pos} patients, and when present, there is considerable inter- and intrasite heterogeneity. In addition, there is no significant correlation between ERG and AR expression in these tumors, despite known regulation of *TMPRSS2-ERG* by AR responsive promoter elements in clinically localized prostate cancer [3]. Taken together, these results suggest dysregulation of androgen signaling in lethal mCRPC. Intriguingly, recent exomic sequencing of a subset of this rapid autopsy cohort uncovered recurrent mutations in genes encoding essential AR transcriptional cofactors, including *FOXA1*, *MLL2*, *UTX/KDM6A*, and *ASXL1* [20].

Emerging data suggest low-level discordance between ERG IHC and *ERG* FISH in a subset (approximately 10–20%) of patients with metastatic prostate cancer [12,13]. Our results extend this finding to lethal mCRPC; however, while the ERG IHC results presented herein are quite striking, this rapid autopsy cohort represents a unique, heavily treated group of lethal mCRPC. Therefore, we believe these data should be applied cautiously, and additional studies investigating the effect of current hormone therapy and chemotherapeutic regimens on ERG protein expression in ERG^{pos} metastatic prostate cancer are warranted.

ERG IHC has poor overall concordance with *ERG* FISH in localized prostate cancer with high-grade neuroendocrine features [14,15]. Our TMA and whole tissue sections results expand this observation to lethal mCRPC with high-grade neuroendocrine features, as all three of the ERG^{pos} patients with high-grade neuroendocrine features in our cohort showed negative ERG tumor cell expression at the majority of tumor sites. AR and PSA expression in these tumors was also mostly negative or weak, although moderate expression of both proteins was detected at some sites. Interestingly, while tumor cells for one patient (#24) demonstrated diffuse high-grade neuroendocrine morphology without ERG expression at multiple sites, metastatic tumor cells in the lung exhibited a poorly differentiated acinar morphology and showed patchy and weak ERG expression (Fig. 3), supporting the idea that the androgen signaling pathway may be downregulated during progression from conventional acinar

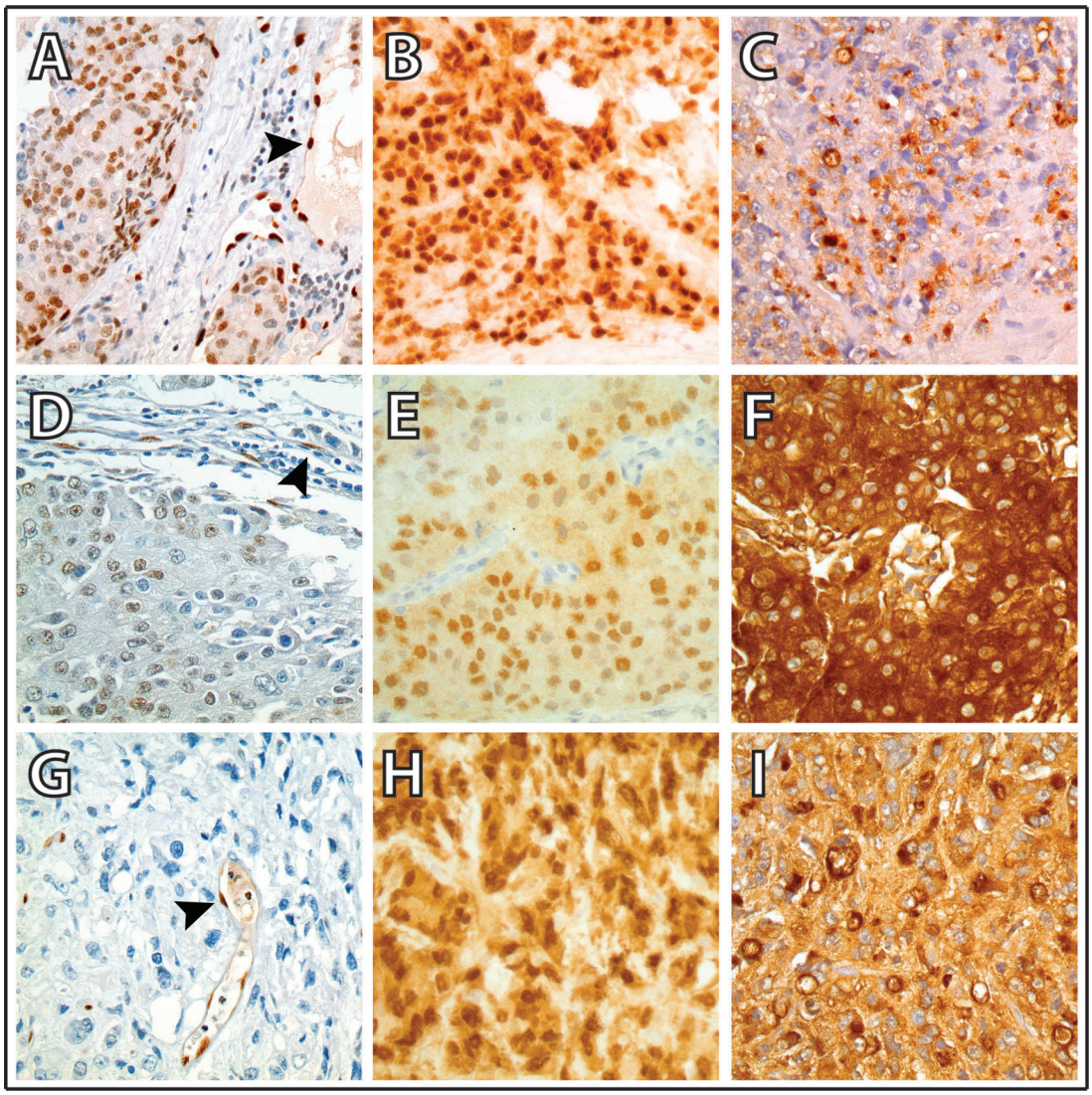

Fig. 4. Discordance between ERG and AR or PSA expression in lethal *ERG*^{pos} mCRPC. (**A**, **D**, **G**) ERG IHC, (**B**, **E**, **H**) AR IHC, or (**C**, **F**, **I**) PSA IHC in three patients (**A**–**C**; **D**–**F**; or, **G**–**I**) with *ERG*^{pos} mCRPC. ERG tumor cell expression is (**G**) negative, (**D**) patchy and weak, or (**A**) diffuse and moderate, and corresponding AR and PSA expression is variable (**B**, **C**, **E**, **F**, **H**, **I**). Strong ERG endothelial cell expression (black arrowheads) provides an internal control for IHC staining in areas with negative or weak ERG tumor cell expression. 400× magnification.

adenocarcinoma to high-grade neuroendocrine prostate cancer [22–26].

Our results suggest some practical implications from both a diagnostic and therapeutic perspective. First and foremost, our data confirm the high specificity of ERG IHC for the detection of *ERG* gene rearrangements in prostate cancer, as none of the *ERG*^{neg} patients showed ERG tumor cell expression at any site. Thus, the detection of ERG expression in prostate cancer cells is essentially diagnostic for *ERG* rearrangement. Second, *ERG* FISH may be the preferred method for detecting *ERG* gene rearrangements in certain specific instances, such as known metastatic prostate cancer and high-grade tumors with neuroendocrine features [27]. It is important to note, of course, that the utility of either modality (i.e., *ERG* FISH or ERG IHC) is limited to cases of prostate cancer with an *ERG* rearrangement, and therefore, a negative result does not exclude a diagnosis of prostate cancer.

Our results may also have important clinical consequences for patients with mCRPC, particularly in regards to selection of targeted therapeutics in the emerging era of personalized medicine [27,28]. Because ERG expression is regulated by androgen signaling in *ERG*^{pos} prostate cancer, it is clear that ERG expression (as detected by IHC) is affected by the androgen signaling status of these cells. Future studies may be indicated to evaluate ERG protein expression

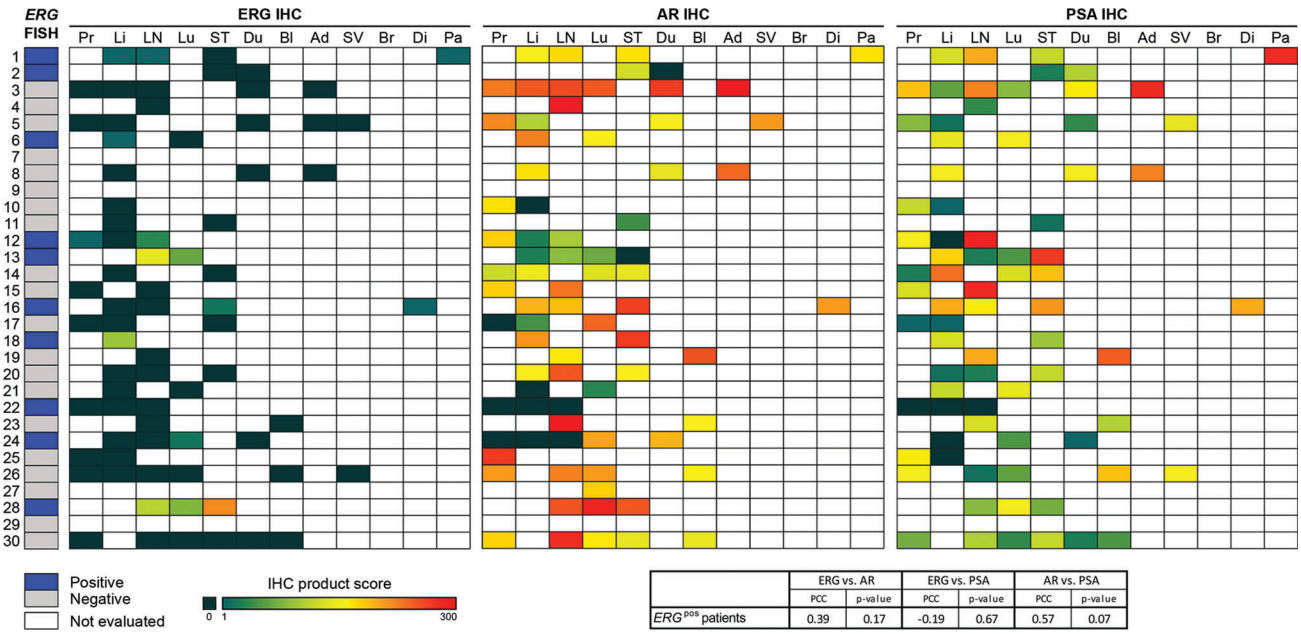

Fig. 5. Summary of ERG, AR, and PSA tumor cell expression in a rapid autopsy cohort of lethal mCRPC. A heat map displaying ERG, AR, and PSA IHC results of a TMA comprised of rapid autopsy material from 30 patients with lethal mCRPC; primary prostate tumor (when available at the time of autopsy; i.e., no prior radical prostatectomy) and all metastatic tumor sites were included in the TMA. (Previously, the *ERG* rearrangement status of each patient's tumor was determined by FISH [9].) In ERG^{neg} patients, ERG tumor cell expression is negative (i.e., IHC product score = 0; e.g., see patients #3, #26, and #30). In ERG^{pos} patients, ERG tumor cell expression is predominantly weak to moderate, and there is intersite heterogeneity (e.g., see patients #12, #13, and #28), with corresponding variable AR and PSA expression. ERG^{pos} patients with high-grade neuroendocrine features (see patients #2, #22, and #24) show mostly negative ERG, AR, and PSA tumor cell expression. There is no significant correlation between ERG and AR or PSA tumor cell expression in ERG^{pos} patients (see table at bottom). While AR and PSA show predominantly moderate tumor cell expression, there is significant intersite variability, and there is a trend toward moderate, significant correlation between AR and PSA in ERG^{pos} patients (see table at bottom). (Rows correspond to individual rapid autopsy patients [identifier listed to the left of the first column]. The first column [*ERG* FISH] represents the *ERG* rearrangement status, as detected by *ERG* FISH. All other columns represent the average ERG, AR, or PSA IHC product score at a particular tissue site for a given patient [tissue site identifier listed above the first row]: Pr, prostate; Li, liver; LN, lymph node; ST, soft tissue; Du, dura; Bl, bladder; Ad, adrenal gland; SV, seminal vesicle; Di, diaphragm; and Pa, pancreas]. *ERG* FISH: blue = positive, gray = negative. IHC product score [range 0–300]: negative [0] = dark green, weak and focal [1] = light green, and strong and diffuse [300] = red. White = not evaluated. PCC = Pearson correlation coefficient.)

as a biomarker for response to next-generation antiandrogen therapies in patients with ERG^{pos} CRPC. Finally, ERG protein expression in ERG^{pos} CRPC may also be a biomarker for the selection of patients to receive targeted ETS rearrangement-based therapies. PARP1, for example, is required for ERG-mediated transcription, and the utility of PARP1 inhibitor therapy for patients with ERG^{pos} mCRPC is currently being explored in clinical trials (e.g., NCT01576172) [29]. Presumably, targeted PARP1 therapy is dependent on ERG protein expression in tumor cells, and therefore, patients with ERG tumor cell expression (as determined by IHC in this trial) may benefit from PARP1 inhibitor therapy, while patients without ERG tumor cell expression may not. Future clinical trials targeting prostate cancer with ETS gene fusions will need to consider the potential discordance between *ERG* rearrangement status and ERG protein expression by IHC prior to selecting testing modalities for the trial.

CONCLUSIONS

In summary, herein we have characterized ERG protein expression in a unique cohort of rapid autopsy patients with heavily treated, lethal mCRPC. Further studies are warranted to delineate the exact mechanisms underlying the profound discordance between *ERG* rearrangement and ERG protein expression in these tumors.

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