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ETS2 is a prostate basal cell marker and is highly expressed in prostate cancers aberrantly expressing p63

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Abstract

Background: Rare prostate carcinomas aberrantly express p63 and have an immunophenotype intermediate between basal and luminal cells. Here, we performed gene expression profiling on p63-expressing prostatic carcinomas and compared them to usual-type adenocarcinoma. We identify ETS2 as highly expressed in p63-expressing prostatic carcinomas and benign prostate basal cells, with lower expression in luminal cells and primary usual-type adenocarcinomas.

Methods: A total of 8 p63-expressing prostate carcinomas at radical prostatectomy were compared to 358 usual-type adenocarcinomas by gene expression profiling performed on formalin fixed paraffin embedded tumor tissue using Affymetrix 1.0 ST microarrays. Correlation between differentially expressed genes and TP63 expression was performed in 5239 prostate adenocarcinomas available in the Decipher GRID. For validation, ETS2 in situ hybridization was performed on 19 p63-expressing prostate carcinomas and 30 usual-type adenocarcinomas arrayed on tissue microarrays (TMA).

Results: By gene expression, p63-expressing prostate carcinomas showed low cell cycle activity and low Decipher prognostic scores, but were predicted to have high Gleason grade compared to usual-type adenocarcinomas by gene expression signatures and morphology. Among the genes over-expressed in p63-expressing carcinoma relative to usual-type adenocarcinoma were known p63-regulated genes, along with ETS2, an ETS family member previously implicated as a prostate cancer tumor suppressor gene. Across several cohorts of prostate samples, ETS2 gene expression was correlated with TP63 expression and was significantly higher in benign prostate compared to usual-type adenocarcinoma. By in situ hybridization, ETS2 gene expression was high in benign

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Alba Torres and Mohammed Alshalalfa had equal contribution.

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basal cells, and low to undetectable in benign luminal cells or usual-type adenocarcinoma. In contrast, ETS2 was highly expressed in 95% (18/19) of p63-expressing prostate carcinomas.

Conclusions: ETS2 is a predominantly basally-expressed gene in the prostate, with low expression in usual-type adenocarcinoma and high expression in p63-expressing carcinomas. Given this pattern, the significance of *ETS2* loss by deletion or mutation in usual-type adenocarcinomas is uncertain.

Keywords

ETS2; gene expression; in situ hybridization; p63; prostate basal cells; prostatic carcinoma

1 | INTRODUCTION

Prostate carcinomas aberrantly expressing p63 protein are a rare and molecularly distinct subclass of prostate cancer.[1–4] Though generally indolent, the study of these unusual tumors provides unique insights into prostate cancer biology. We have previously demonstrated that p63-expressing prostate cancers express the ΔNp63 isoform of p63,[3,4] though it is unknown whether it is transcriptionally active in these cells. These tumors have an immunophenotype intermediate between prostatic basal and luminal cells, in contrast to usual-type adenocarcinomas which generally have a luminal phenotype.[3] Although they express androgen receptor and markers of prostatic origin such as NKX3.1, p63-expressing tumors do not appear to rearrange *ERG* at a detectable frequency (an alteration seen in half of usual-type adenocarcinomas) and many lack *GSTP1* hypermethylation (an alteration seen in the vast majority of usual-type adenocarcinomas).[3] To better molecularly characterize these tumors, we compared gene expression profiling of formalin fixed paraffin embedded (FFPE) p63-expressing tumors to usual-type adenocarcinomas. We find that p63-expressing carcinomas show transcriptomic profiles consistent with their indolent clinical behavior, despite their apparently higher Gleason grade by gene expression signatures and morphology. In addition, these tumors highly express a number of p63 target genes, suggesting active p63-mediated transcription. Though they show low expression of most *ETS*-family genes consistent with the known lack of *ERG* gene rearrangements in this tumor type, p63-expressing carcinomas show relatively high expression of ETS2, a gene which has previously been implicated as a prostate cancer tumor suppressor gene.

2 | MATERIALS AND METHODS

2.1 | Patient and tissue selection

After IRB approval, a total of 19 p63-expressing tumors at radical prostatectomy were retrieved from the Johns Hopkins pathology database. A subset of eight of the p63-expressing tumors were selected for transcriptome profiling on the basis of adequate available tumor tissue. These tumors were also included in a previously published tissue microarray (TMA) study for immunophenotyping,[3] which included all 19 cases of p63-positive tumor sampled in quadruplicate and a separate nodule of usual-type adenocarcinoma sampled for six cases where it was present for sampling. For comparison by gene expression, we used 358 usual-type adenocarcinomas from a previously published

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Johns Hopkins radical prostatectomy (RP) cohort with available gene expression microarray data on the same platform as described below.[5,6]. This cohort was originally selected to test for prognostic biomarkers and tissues were also sampled on 16 individual TMAs. For examination of ETS2 expression by in situ hybridization, we used a separate TMA cohort of 30 usual-type adenocarcinomas from 2014. This cohort was selected for its relatively recent cases, as we have previously observed attenuation of RNA in situ hybridization signal with extended FFPE tissue block storage.[7] For all cases on TMA, tumor tissue from the dominant tumor nodule and benign tissue were separately sampled in quadruplicate utilizing 0.6 mm cores. Tissues were subsequently punched with a 1 mm punch for gene expression microarray profiling. For model evaluation in prospective samples, we evaluated de-identified expression profiles from 5239 prospective RP tumor samples available for research in the Decipher GRID®. Finally, for comparison to RNA sequencing, we utilized 25 radical prostatectomy cases from Johns Hopkins with available fresh frozen usual-type adenocarcinoma tissue and paired benign prostate tissue for which RNA-sequencing had previously been performed.[8]

2.2 | Preprocessing and expression profiling using Human Exon 1.0ST arrays or RNA sequencing

RNA extraction from FFPE tissues, amplification, labeling and hybridization to Affymetrix Human Exon 1.0 ST microarrays was performed in a CLIA-certified clinical laboratory using the Decipher® prostate cancer classifier (GenomeDx Biosciences, San Diego, CA) as described previously.[9–11] The SCAN algorithm was used for individual patient profile pre-processing and normalization. The RNA sequencing protocols and datasets used were previously described.[8] Reads were aligned using RSEM and gene expression measures for ETS2 and TP63 were extracted as transcripts per million (TPM).

2.3 | Detection of ETS2 by in situ hybridization

Chromogenic in situ hybridization for ETS2 RNA was performed with the RNAscope® FFPE kit 2.5 from Advanced Cell Diagnostics (ACD, Hayward, CA) as previously described for other ETS family members.[12] ETS2 (NM_004454.2) probes were utilized. All cases were qualitatively scored by a blinded surgical pathologist, using a 0–3+ intensity scoring system to assess for distinct red punctae present in tumor cells (see Figures 4 and 5).

2.4 | Immunohistochemistry

Multiplex CK903/p63/AMACR immunostaining was performed using the Ventana HQ kit on the Ventana Discovery Ultra automated immunostainer (Ventana/Roche, Tucson, AZ) for cytokeratin 903 (1:50 dillution; C34903; DAB detection; Enzo Life Sciences, Farmingdale, NY), p63 (1:50 dilution; CM163A; DAB detection; Biocare Medical, Pacheco, CA) and Alpha-Methylacyl-CoA Racemase (:50 dilution; Z2001L; Red; Zeta Corporation; Tucson, AZ).

2.5 | Statistical analysis

Statistical analyses were performed in R v3.2.2, and all tests were two-sided using a 5% significance level. Fisher’s exact test was used to study the association between categorical

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variables. Area Under Curve (AUC) was used for model performance evaluation. Pearson's correlation was used to find correlated genes to TP63. Wilcoxon rank sum test was used to evaluate differences in mean expression between groups. Kruskal Wallis test was used to analyze differences between ETS2 expression by RNAseq between groups. Spearman's correlation was used to examine correlation of p63 and ETS2 expression in RNAseq.

3 | RESULTS

We compared gene expression data from 8 p63-expressing carcinomas and 358 usual-type adenocarcinomas previously profiled. Initial comparisons by previously published gene expression classifiers revealed that p63-expressing carcinomas had low scores using the Decipher gene expression prognostic classifier compared to usual-type adenocarcinoma (Figure 1A) and relatively low cell cycle gene expression using genes in the CCP classifier (Figure 1B).[13] However, using a previously published gene expression signature that distinguishes Gleason score ≤6 from Gleason score ≥8 tumors,[14] p63-expressing carcinomas showed gene expression consistent with relatively higher Gleason grades (Figure 1C). Taken together, these apparently conflicting data confirm our previous clinical observations that p63-expressing carcinomas are relatively indolent tumors which may paradoxically morphologically appear to have high Gleason grade (frequently pattern 4 with poorly formed glands, see Figure 5), thus Gleason grading should be avoided in this tumor subset as it is misleading.[2]

We next compared the gene expression profiles of 8 p63-expressing carcinomas with 358 usual-type adenocarcinomas from a previously published Johns Hopkins cohort. A total of 104 differentially expressed genes were identified (Figure 2A). To prioritize candidates for further analysis, genes were ranked by two criteria (Figure 2B, Supplementary Table S1). First, for each gene we examined its correlation with TP63 gene expression across the GRID radical prostatectomy cohort (n = 5239) using the Pearson correlation coefficient. Second, we examined the area under the curve (AUC) for receiver operator characteristic (ROC) for each gene in terms of predicting whether a tumor was a p63-expressing carcinoma when 8 p63-expressing carcinomas were pooled with the 358 usual-type adenocarcinomas from Johns Hopkins. MIR205 was second only to TP63 itself (Figure 2C) among the differentially expressed genes with the highest correlation to TP63 expression and the highest AUC (Figure 2D). As *MIR205* is a gene directly regulated by ΔNp63α in urothelial carcinoma cell lines,[15] this provided some validation of our approach and strongly suggests that p63 is transcriptionally active in these unusual prostate tumors with aberrant p63-expression.

Because most other differentially expressed genes showed dramatically lower correlation with TP63 expression in usual-type adenocarcinoma, we next focused on the genes that had the highest AUC for predicting p63-expressing carcinoma status. Three genes showed an AUC = 1 in this analysis: *ETS2*, *CROT* and *CYP4B1*. Though relatively little is known about *CROT* and *CYP4B1* in prostate cancer, *ETS2* was of particular interest as members of the *ETS* gene family undergo frequent genomic rearrangements leading to their over-expression in prostate cancer.[16–18] Indeed, ETS2 shows a similar DNA binding motif to ERG, the most frequently rearranged *ETS* gene family member in usual-type prostate carcinoma.[19] Given that *ERG* rearrangements have not been documented in p63-expressing

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prostate carcinomas, the dramatically higher expression of ETS2 in this subset of tumors relative to usual-type adenocarcinomas was intriguing (Figure 2E) and suggested the possible hypothesis that p63-expressing carcinomas are driven by alternative *ETS* family genes.

However, because we have previously shown that p63-expressing carcinomas have a partially basal phenotype,[3] we first sought to determine whether ETS2 might itself be a primarily basally-expressed gene in the prostate. To do this, we examined RNA-seq data from 25 usual-type adenocarcinomas and their paired benign samples. Strikingly, median ETS2 gene expression was markedly higher in benign prostate tissue compared to usual-type adenocarcinoma, a pattern that held true for nearly all paired samples (Figures 3A and 3C). Consistent with the microarray data above, TP63 expression was correlated with ETS2 expression in benign and tumor samples (Figure 3B). These data suggested that ETS2 might be predominantly expressed in prostate basal cells which are sampled abundantly in benign prostate and are largely absent from tumor samples taken from usual-type adenocarcinomas.

To confirm our hypothesis that ETS2 is a primarily basally-expressed gene, we used chromogenic RNA in situ hybridization to localize ETS2 expression across a spectrum of prostate tissues. In benign prostate tissues from 30 radical prostatectomies containing usual-type adenocarcinoma, ETS2 was predominantly expressed in high molecular weight keratin- and p63-positive prostatic basal cells, with much weaker to undetectable expression benign prostatic luminal cells (Figure 4). Among 30 usual-type adenocarcinomas, ETS2 expression was uniformly lower than in surrounding basal cells of entrapped benign glands, though occasionally somewhat higher than levels seen in the adjacent benign luminal compartment (Figure 4, left panels). By contrast, ETS2 expression was equal to or higher than entrapped benign basal cells across 95% (18/19) of p63-expressing carcinomas examined (Figure 5), though there was some inter-tumoral heterogeneity in absolute levels of ETS2 expression that could be due to differing RNA preservation between samples.

4 | DISCUSSION

P63-expressing prostate carcinomas are rare tumors with generally indolent behavior, yet they may inform our understanding of the biology of basal-luminal cell differentiation in the prostate. Here, we have performed the first gene expression analysis of p63-expressing carcinomas, which have an intermediate basal-luminal phenotype, comparing them to usual-type adenocarcinomas which have a largely luminal phenotype. Consistent with their documented indolent clinical behavior, p63-expressing carcinomas show relatively low scores on prognostic and proliferative gene classifiers, a finding that contrasts with morphologic and gene-expression data demonstrating paradoxically high Gleason grade in these rare cases. These data support our group's prior suggestion that these variant tumors be excluded from Gleason grading[2] and reinforce the importance of recognizing these tumors at the time of diagnosis to avoid inappropriately high Gleason grading that may be inconsistent with the true low grade behavior of these neoplasms.

In differential gene expression analysis comparing p63-expressing carcinomas to usual-type adenocarcinomas, we were surprised to find that in addition to genes known to be directly

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regulated by p63, such as *MIR205*, *ETS2* is among the genes whose over-expression best distinguishes p63-expressing carcinomas from usual-type carcinomas. This finding was particularly intriguing as we have previously shown that *ERG* gene fusions, occurring in nearly half of usual-type prostate adenocarcinomas, do not occur at a measurable frequency among p63-expressing carcinomas, nor can ERG protein expression be detected in these tumors.[3] Based on RNA-seq and in situ hybridization data from benign prostate tissues, we find that ETS2 is highly expressed among benign basal cells in the prostate, and thus its high expression in p63-expressing carcinomas is likely part of a program of basal differentiation in these tumors. Based on these data, it is reasonable to hypothesize that *ETS2* may be a direct target of p63-transcription in prostatic epithelial cells, though the correlation coefficient between p63 and ETS2 expression was notably lower than that seen for known p63 target *MIR205* in our study. Intriguingly, a p63-family member, p53, can interact with ETS2.[20] In fact, p53 proteins harboring oncogenic missense mutations have an even stronger interaction with ETS2 than wild-type forms, binding to the tetra-dimerization domain of p53 and regulating gene transcription via ETS-binding site motifs.[20,21] This interaction likely contributes to gain-of-function phenotypes for mutant p53 in some contexts.[20,21] Thus it is also conceivable that an ETS2-p63 interaction might contribute to transcriptional changes in p63-expressing prostate carcinomas.

Our finding that ETS2 is highly expressed predominantly in prostate basal cells with lower expression in luminal cells and in usual-type prostatic adenocarcinomas is also interesting in light of a number of studies suggesting that *ETS2* may function as a prostate cancer tumor suppressor gene.[19,22,23] *ETS2* is located adjacent and telomeric to *ERG* on chromosome 21 and thus undergoes mono-allelic deletion in prostate cancers in which a *TMPRSS2-ERG* fusion is generated through genomic deletion of intervening sequence.[19] Interestingly, we found generalized low expression of the gene across all usual-type adenocarcinomas, thus it was difficult to discern whether a subset of tumors showed even lower expression consistent with ERG rearrangement by *ETS2* deletion. In humans, some data suggest that tumors with *TMPRSS2-ERG* fusions occurring through deletions are more aggressive than their counterparts where the rearrangement occurs through translocation, though the mechanism of this finding has been debated.[24,25] If true, this might suggest that *ETS2* is a tumor suppressor whose loss contributes to tumor progression in usual-type adenocarcinoma. This hypothesis is supported by in vitro data demonstrating a decrease in proliferation and invasion in prostate cancer cell lines ectopically expressing *ETS2*[19,22] and the finding that *ETS2* is infrequently also inactivated via mutation in advanced castration resistant prostate cancer.[19]

Along similar lines, a recent study in mice with concurrent *PTEN* deletion found that tumors arising in the context of *TMPRSS2-ERG* fusions by deletion (with *ETS2* loss) were more aggressive than those where the rearrangement was modeled by genomic insertions leaving *ETS2* intact.[23] Interestingly, these findings in mice were supported by human data suggesting that ETS2 expression is lower in lethal versus indolent prostate cancer cases in the Physicians' Health Study and Health Professionals Follow-up Study.[23] However, given increasing agreement that most prostatic adenocarcinomas likely originate from luminal cells[26,27] and our finding of low to undetectable expression of ETS2 in luminal cells, it is unclear whether further decreases in ETS2 expression during tumorigenesis are likely to be

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biologically significant. Though consistent with what would be expected from a tumor suppressor, the fact that ETS2 expression is lower in prostatic adenocarcinoma compared to benign prostate is actually due to the lower numbers of benign basal cells in adenocarcinoma, rather than loss of ETS2 expression within the luminal compartment during tumorigenesis. Accordingly, the finding that ETS expression is lower in indolent compared to lethal prostate tumors could simply be the result of increased amounts of intervening benign prostate tissue in indolent, low Gleason grade cases compared to lethal cases with predominantly higher Gleason grade.

There are a few limitations of this study that merit discussion. First, we have only examined ETS2 RNA expression throughout this work, as validated antibodies to detect ETS2 protein expression in tissue have not been developed. It is conceivable that there is a discordance between protein and RNA expression, though it seems likely that most of our findings would still hold at the protein level. In addition, many of the usual-type adenocarcinoma samples utilized in this study (the 358 Johns Hopkins cases and the larger prospective GRID cohort) are skewed toward relatively high-risk disease as they were selected for prognostic studies (in the case of the former) or sent for further clinical molecular testing (in the case of the latter). Therefore, they may not be entirely representative of usual-type adenocarcinoma in the way that a consecutive case series might be, though the sheer size of the prospective cohort likely mitigates this weakness to some extent. Finally, the in situ hybridization studies were performed on tissue microarrays, which limits ability to discern whether ETS2 expression showed intratumoral heterogeneity in the tumors examined.

In conclusion, we find that the rare subset of p63-expressing prostate carcinomas have gene expression profiles largely consistent with their indolent clinical behavior. Compared to usual-type adenocarcinomas, these tumors over-express genes such as MIR205 that are known targets of p63-mediated transcription, suggesting that p63 is both expressed and transcriptionally active in this rare subset of prostatic neoplasms. Finally, we find that ETS2 is a gene over-expressed in p63-expressing carcinomas and benign basal cells compared to benign luminal cells or usual-type adenocarcinoma, consistent with the basal differentiation seen in this rare tumor type. Given our finding that ETS2 is predominantly a basally-expressed gene in the prostate, its potential role as a tumor suppressor gene in luminally-differentiated prostate cancer cells is uncertain.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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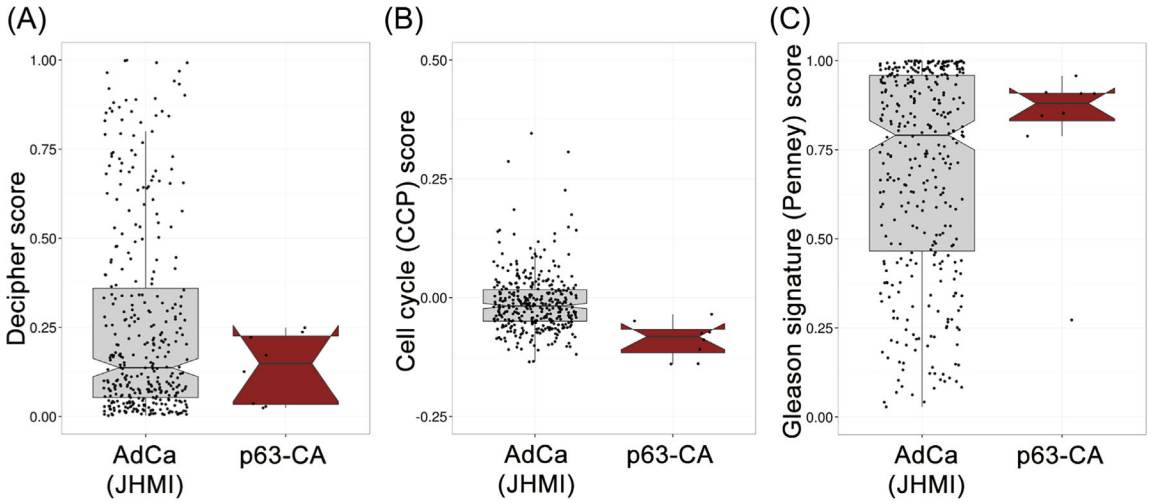

FIGURE 1.

Performance of gene expression prognostic signatures on p63-expressing prostate carcinomas compared to usual-type adenocarcinomas. Gene expression profiling was performed on 8 p63-expressing carcinomas and compared to 358 usual-type adenocarcinomas. A, p63-expressing carcinomas show lower Decipher scores compared to usual-type adenocarcinomas. B, p63-expressing carcinomas show lower cell-cycle proliferation (CCP) scores compared to usual-type adenocarcinomas. C, p63-expressing carcinomas show relatively high Gleason score by gene expression signature compared to usual-type adenocarcinomas

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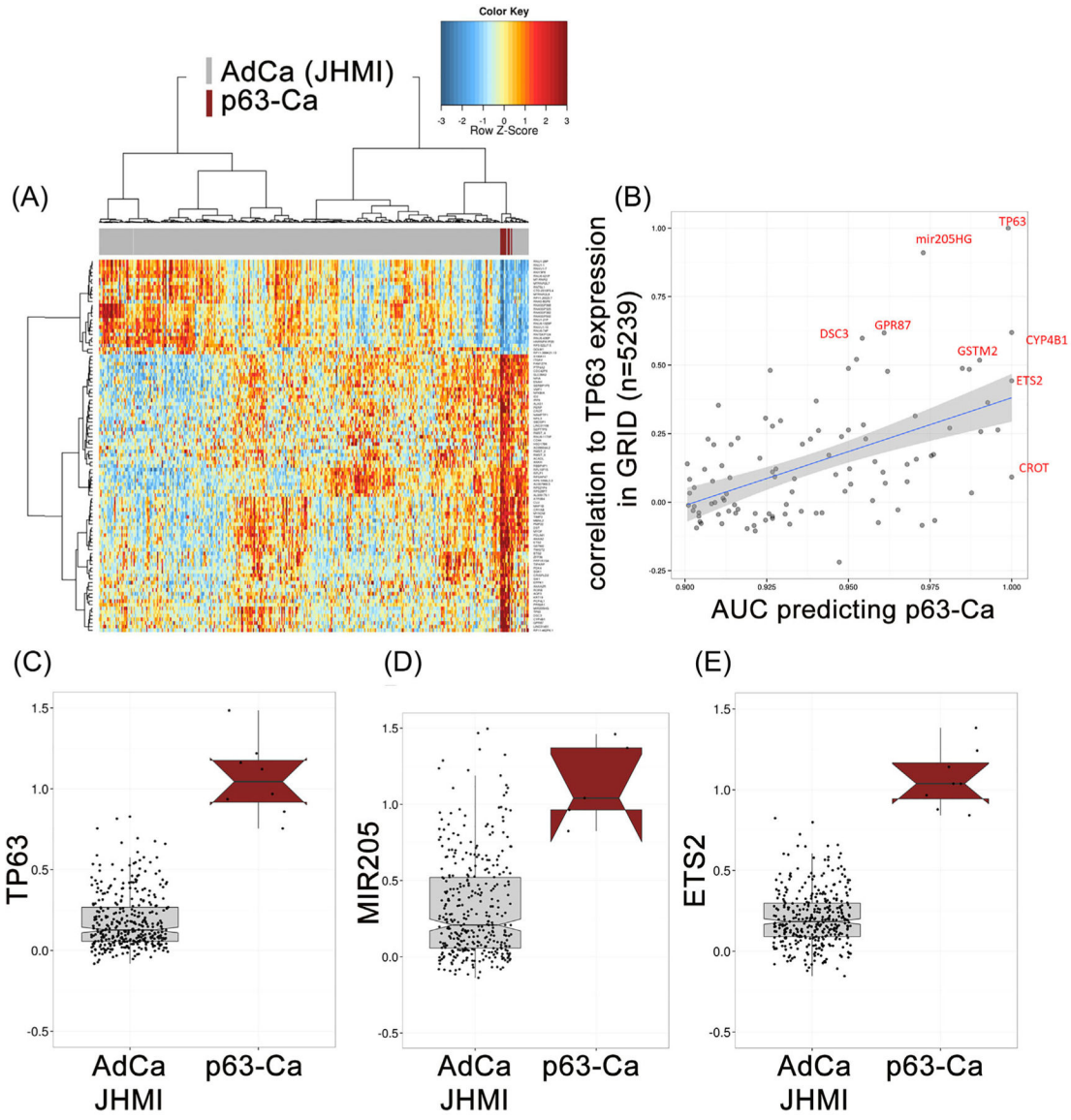

FIGURE 2.

Differential gene expression analysis of p63-expressing carcinoma compared to usual-type adenocarcinomas. A, Heat map of hierarchical clustering of differential gene expression analysis comparing 8 p63-expressing carcinomas to 358 usual-type adenocarcinomas. There were 104 genes differentially expressed between the two groups and p63-expressing carcinomas cluster together (maroon bars). B, Each of the 104 genes from (A) were graphed according to their correlation with TP63 gene expression in a large prospective radical prostatectomy cohort (GRID cohort, $n = 5239$) and the AUC for that gene in distinguishing the original 8 p63-expressing carcinomas from the 358 usual type adenocarcinomas. Genes with a high AUC and/or high correlation are highlighted in red. C, RNA expression levels for p63 were higher in the p63-expressing carcinomas compared to 358 usual-type adenocarcinomas, as expected ($P = 0.004$; Wilcoxon test). D, RNA expression levels for miR205 were higher in the p63-expressing carcinomas compared to 358 usual-type

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adenocarcinomas ($P = 0.01$; Wilcoxon test). miR205 is known to be regulated by the p63 transcription factor.[15] E, RNA expression of ETS2 transcription factor was higher in the p63-expressing carcinomas compared to 358 usual-type adenocarcinomas ($P = 0.001$; Wilcoxon test)

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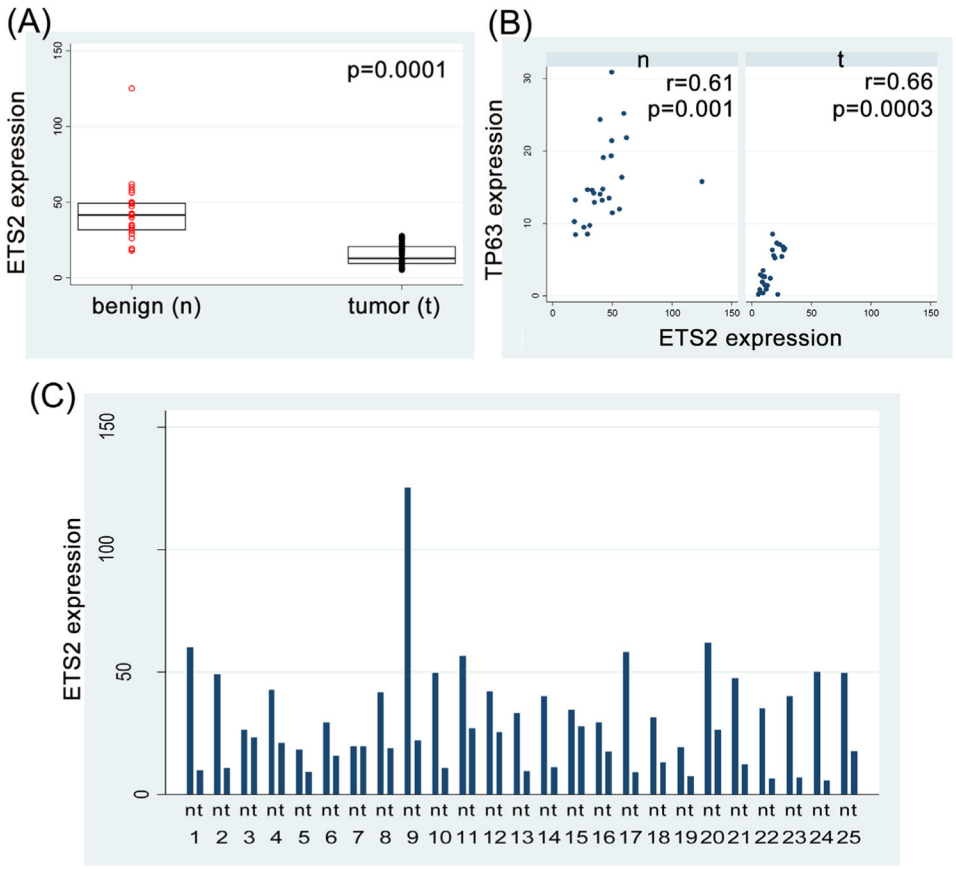

FIGURE 3.

ETS2 and TP63 RNA expression in 25 tumor-benign tissue pairs from radical prostatectomy specimens. A, ETS2 gene expression is higher in benign prostate tissue (n) compared to tumor tissue (t) overall. B, TP63 and ETS2 gene expression are correlated in both benign prostate tissue (n) and tumor tissue (t). C, Examining each tumor-normal pair individually, ETS2 gene expression is uniformly higher in the benign (n) prostatic tissue, compared to the tumor (t) tissue

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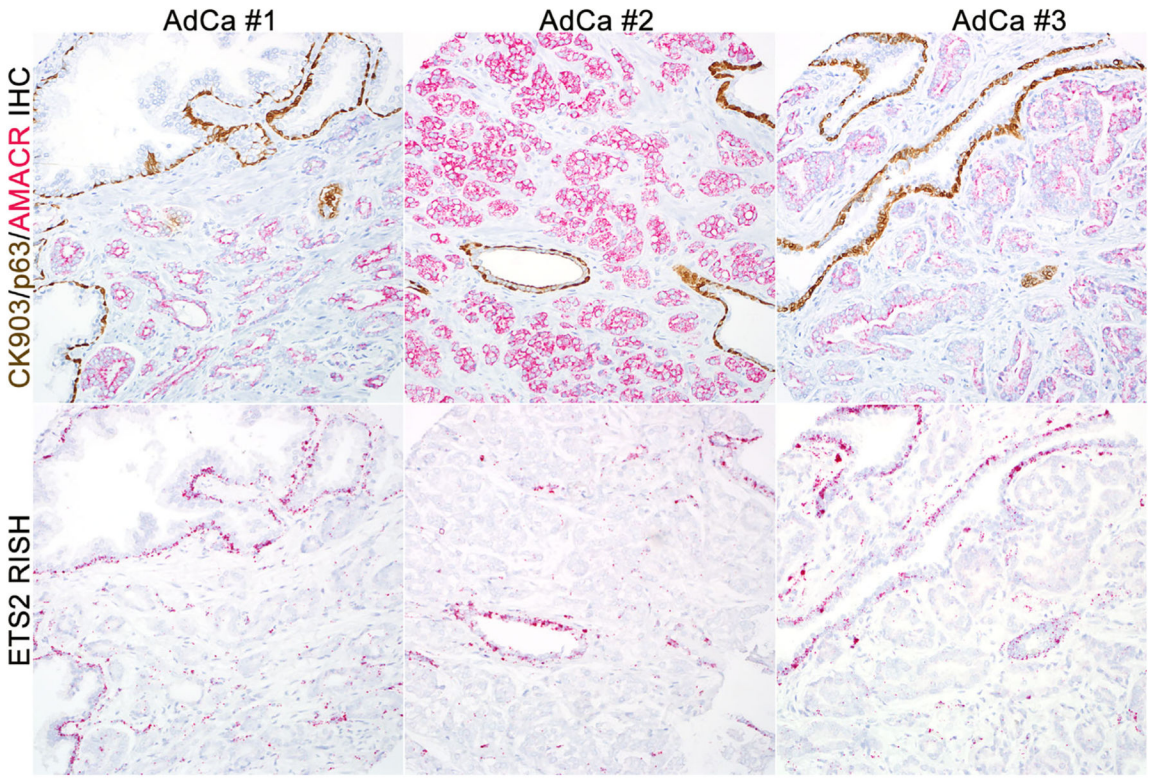

FIGURE 4.

ETS2 RNA in situ hybridization (RISH) in representative usual-type adenocarcinoma cases. Upper panels: Multiplex immunohistochemistry (IHC) highlights benign basal cells (brown) with p63 and high molecular weight keratin (CK903) expression. In contrast, tumor cells show absence of basal cells and positive AMACR (Alpha-Methylacyl-CoA Racemase, red) expression. Lower panels: ETS2 RISH is strongly positive in benign basal cells (red dots) and weak in tumor glands and luminal cells where only rare dots are present. All images captured at 20× magnification

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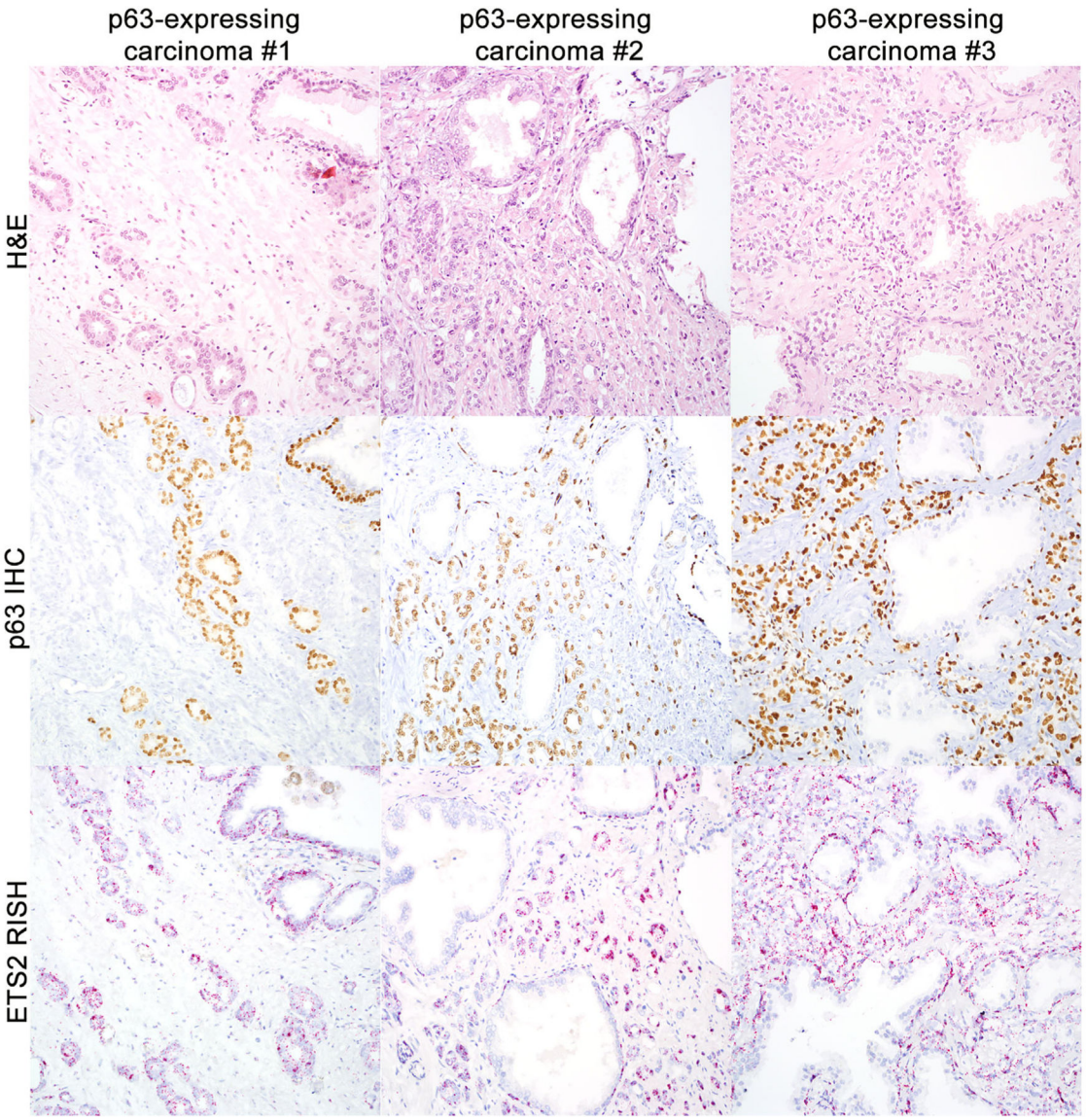

FIGURE 5.

ETS2 RNA in situ hybridization (RISH) in representative p63-expressing carcinoma cases. Upper panels: Hematoxylin and eosin (H&E) staining of representative p63-expressing tumor cases. Middle panels: Immunohistochemistry (IHC) for p63 protein is positive in p63-expressing carcinoma glands and intervening benign glands within benign basal cells. Lower panels: ETS2 RISH is strongly positive in benign basal cells (red dots) and p63-expressing tumor glands while in benign luminal cells, only rare dots are present. All images captured at 20× magnification