

Aberrant Cytoplasmic Expression of p63 and Prostate Cancer Mortality

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Abstract

Protein expression of p63 is used to differentiate prostate cancer from benign mimickers. Recent studies suggest that it may also distinguish aggressive prostate cancer with down-regulated expression occurring in men with more advanced disease. We conducted a prospective study among 298 men ages 51 to 84 years who were diagnosed with prostate cancer in the Physicians' Health Study in 1983 to 2004 and whose tissue was available for immunohistochemical staining. We used Cox proportional hazards regression to evaluate the association of p63 protein expression with fatal prostate cancer. We correlated p63 expression with tumor cell proliferation (Ki-67) and apoptosis (TUNEL staining). The predominant location of tumor p63 staining occurred in the cytoplasm, an uncommon departure from the strong nuclear staining usually observed in nonneoplastic basal cells. Increasing ex-

pression of cytoplasmic p63 (tertiles) was associated with prostate cancer mortality ($n = 19$ deaths); the hazard ratios (95% confidence intervals) were 1.0 (reference), 4.0 (0.9-18.9), and 5.9 (1.3-27.5; $P_{\text{trend}} = 0.03$). The positive trend remained significant ($P = 0.047$) after multivariable adjustment for age, year of diagnosis, and Gleason score. Higher tertiles of cytoplasmic p63 were also associated with reduced levels of apoptosis ($P_{\text{trend}} = 0.0408$) and increased cellular proliferation ($P_{\text{trend}} = 0.0026$). We found aberrant expression of p63 in the cytoplasm to be associated with increased prostate cancer-specific mortality up to 20 years after diagnosis. The mislocalized expression was associated with reduced apoptosis and higher proliferative activity and may suggest an oncogenic role in prostate cancer progression and survival. (Cancer Epidemiol Biomarkers Prev 2009;18(2):595-600)

Introduction

Expression of the p63 gene, a member of the p53 family (1), is down-regulated in adenocarcinoma of the prostate compared with normal prostate and is used as a basal cell marker in the diagnosis of prostate cancer (2). Differences in p63 expression are associated with cancer progression or a poor prognosis for several cancer sites, including overexpression in the ovaries and oral squamous cell carcinoma (3, 4), down-regulated expression in the upper urinary tract and prostate (5-7), and aberrant cytoplasmic expression in lung adenocarcinoma (8).

The p63 gene is critical to embryonic development of the epidermis and its derivative structures including the prostate gland (2, 9-11). The p63 protein is normally expressed in basal cells of epithelial structures, including

the prostate epithelium, and is involved in epithelial differentiation and proliferation (2). The role of this transcription factor in carcinogenesis is complex, as it encodes two classes of proteins with opposing tumor suppressor and oncogenic functions including transcription activation, apoptosis, and cell proliferation (2, 12-17). In adenocarcinomas, p63 tends to be underexpressed (18), and in prostate cancer specifically, negative immunohistochemical staining of p63 is a clinically useful tool for identifying benign mimickers (2). Recent studies have also identified p63 as important in signatures of advanced disease, with lower expression associated with disease progression and the development of lethal prostate cancer (6, 7). We undertook this study to further evaluate the role of p63 in distinguishing fatal disease in men with prostate cancer followed up to 20 years.

Materials and Methods

Study Population. This study was nested within the Physicians' Health Study (PHS) I and II randomized trials of aspirin and nutritional supplements for the primary prevention of cancer and cardiovascular disease among U.S. male physicians (described in detail elsewhere; refs. 19, 20). Briefly, PHS I began in 1982 among 22,071 physicians ages 40 to 84 years without a history of cardiovascular disease or cancer at baseline and PHS II

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Table 1. Clinical characteristics by tertiles of p63 percent-positive area of men diagnosed with prostate cancer in PHS, 1983-2007

Characteristics	Tertiles of p63 percent positivity			P
	1 (0-1.76)	2 (1.77-3.89)	3 (3.90-53.79)	
	(n = 99)	(n = 100)	(n = 99)	
	Mean (SE) or %			
Median (interquartile range) p63 percent positivity	1.1 (0.8-1.4)	2.5 (2.0-3.0)	6.6 (4.9-9.1)	
Age at diagnosis (y)	65.8 (0.6)	67.2 (0.6)	65.9 (0.6)	0.83*
Body mass index (baseline; kg/m ²)	24.6 (0.3)	24.4 (0.3)	24.6 (0.3)	0.99*
Follow-up time (y)	10.0 (0.4)	9.7 (0.4)	8.0 (0.4)	<0.001*
PSA at diagnosis (ng/mL)	11.2 (3.4)	7.4 (3.6)	14.8 (3.6)	0.49*
Missing (%) [†]	7.1	17.0	17.2	
Gleason score				
4-6	29 (29.3)	23 (23.0)	38 (38.4)	0.05 [‡]
7	57 (57.6)	61 (61.0)	41 (41.4)	
8-10	13 (13.1)	16 (16.0)	20 (20.2)	
Clinical stage				
T ₁ , N _X /N ₀	45 (45.5)	47 (47.0)	58 (58.6)	0.15 [‡]
T ₂ , N _X /N ₀	47 (47.5)	45 (45.0)	33 (33.3)	
T ₃ /T ₄ or N ₁ /M ₁	4 (4.0)	4 (4.0)	1 (1.0)	
Missing (%)	3.0	4.0	7.1	
Prostate cancer death				
Yes	2 (2.0)	8 (8.0)	9 (9.1)	0.04 [§]
No	97 (98.0)	92 (92.0)	90 (90.9)	

*F test for trend.

[†]Forty-nine percent of men with missing PSA (n = 20) were diagnosed in pre-PSA era.

[‡]Global χ^2 test.

[§] χ^2 test for trend across tertiles.

began in 1997 among 14,641 physicians ages ≥ 50 years. Follow-up information and mortality data are 97% complete on all participants.

Case Identification. A prostate cancer diagnosis was based initially on self-report and then confirmed through a review of medical records and pathology reports by an End Point Committee of physicians (M.J. Stampfer is a member). Deaths were identified from the National Death Index and postal system, and next-of-kin and medical records were reviewed to adjudicate causes of death, including prostate cancer.

Tissue Microarrays. For this study, we obtained archival formalin-fixed, paraffin-embedded tissue specimens for men who had a radical prostatectomy or transurethral resection of the prostate between 1983 and 2004. Two study pathologists (M.A.R. and S.P.) conducted a systematic re-review of all tissue specimens for standardized Gleason grading and to identify the dominant prostate cancer nodule with the highest Gleason score pattern from each specimen. A manual tissue arrayer was used to construct three high-density tissue microarrays in which at least three tumor tissue cores from the targeted areas were transferred, arraying 0.6 mm cores per case.

Immunohistochemistry

p63. Sections (5 μ m) of each tissue microarray were mounted on charged slides and subjected to microwave treatment for antigen retrieval and incubated with a 1:600 dilution of the 4A4 mouse monoclonal antibody (Lab Vision), which binds to all isoforms of p63. A semi-automated image analysis system with high reproducibility (Chromavision) was used to measure protein

expression of p63 (21). Using scanned digital images of the cores (22), the percent of positively stained area for p63 was scored on a scale of 0% to 100%. A study pathologist (M.B.) targeted those areas with histologically recognizable prostate cancer to focus on protein expression of p63 from tumor tissue only and was blinded to clinical outcomes.

Ki-67. Sections (5 μ m) were used for the Ki-67 antigen, which was used as a proliferation marker using a rabbit polyclonal antibody (Vector) diluted 1:1,500 with a citrate-based antigen retrieval. The Ki-67 score was assessed as the number of stained nuclei over the total number of tumor nuclei using the Ariol instrument SL-50 (Applied Imaging) after selection of the tumor areas of each core for full quantitative image analysis.

Apoptosis. Based on 5 μ m sections, the TUNEL assay was used to identify the percent of tumor cells undergoing apoptosis using the Apoptag Peroxidase *In situ* kit (Chemicon International) according to the instructions of the manufacturer. The Apoptag score was assessed as the number of positive cells out of the total number of tumor cells independently by two study pathologists (M.F. and A.F.). The whole area of each tumor core was evaluated for the score. Due to fixation and technical artifacts, the total number of specimens with reliable staining for apoptosis data was reduced to n = 227 for analyses.

Clinical and Demographic Characteristics. We collected baseline questionnaire data on demographics such as age, height, and weight in 1982 for PHS I participants and in 1997 on new PHS II participants. We collected information on clinical stage and prostate-specific antigen (PSA) at diagnosis through medical records. Clinical

stage of disease was determined through a review of medical records and pathology reports and categorized according to the tumor-node-metastasis staging system (2002 American Joint Committee on Cancer). If data were missing from medical records, self-reported stage and diagnostic PSA were supplemented from information collected through follow-up questionnaires. Tumor grade was assigned by a study pathologist (M.A.R.), who conducted a systematic re-review of all tissue specimens to determine a pathologic Gleason score and grade for each patient. Assignment of tumor grade and stage was done while blinded to p63 levels and mortality information.

Statistical Analyses. The current study is based on 298 men with sufficient tumor tissue available for immunohistochemical analysis ($n = 270$ from prostatectomy and $n = 28$ from transurethral resection of the prostate). The percentage of area that stained positive for p63 was assessed as a continuous marker and in tertiles based on the distribution in the study population. The means of continuous variables, such as age, year of diagnosis, and PSA, were compared across tertiles of p63 expression using ANOVA and the F test for trend for statistical significance. The percent of cells positive for Ki-67 and proportion of cells undergoing apoptosis were log-transformed and analyzed across tertiles of p63-positive area as linear variables using ANOVA and the linear F

test for trend ($P < 0.05$). The duration of survival was calculated as the interval from time of cancer diagnosis to either a prostate cancer death (time to event) or censored at time of death from other causes or at end of follow-up (March 31, 2007). Cox proportional hazards regression was conducted to compute the hazard ratio and 95% confidence interval (95% CI) for survival duration. Multivariate hazard ratios were adjusted for age (years), year of diagnosis, PSA levels at the time of diagnosis (continuous, log-transformed), Gleason score (4-6, 7-10), and clinical stage [dichotomized-localized (T_1 - T_2 , N_0 / N_X , and M_0 / M_X) versus extraprostatic disease (T_3 - T_4 or N_1 or M_1)]. We used the SAS program package version 9.1 (SAS Institute) to carry out statistical analyses with a significance level of .05. This study was approved by the Institutional Review Board at Partners Healthcare.

Results

Men had a mean age at diagnosis of 66.3 years and a mean body mass index of 24.5 kg/m² (Table 1). Ten percent of the tissue samples were collected from transurethral resection of the prostate ($n = 28$). Most had clinically localized disease (92%), but pathologic staging at the time of surgery revealed 22% ($n = 66$) with extraprostatic disease, 6% ($n = 18$) with positive seminal vesicles, and 3% ($n = 9$) with lymph node invasion (data

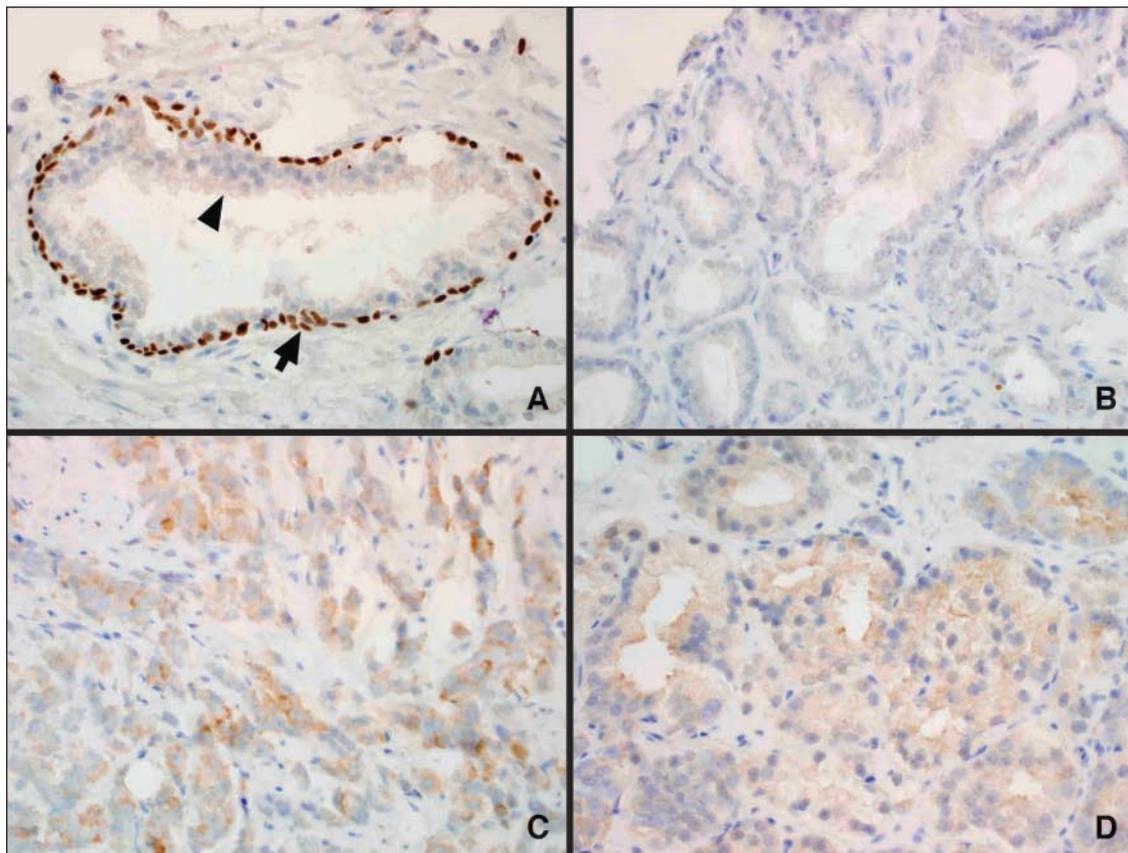


Figure 1. A. Normal prostate gland showing p63 staining of basal cells (arrow) and lack of staining of luminal cells (arrowhead; $\times 40$). B. Prostate adenocarcinoma showing no cytoplasmic or nuclear staining for p63 ($\times 40$). C and D. Prostate adenocarcinoma showing cytoplasmic staining for p63 ($\times 40$).

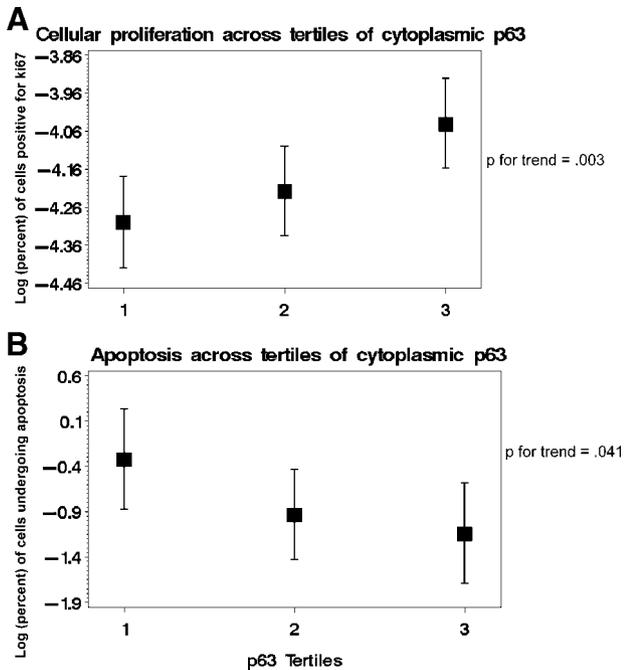


Figure 2. Log (%) of Ki-67-positive cells (A) and log (%) of cells undergoing apoptosis (TUNEL assay; B) across tertiles of cytoplasmic p63.

not shown). Nearly a third of participants had low-grade disease (Gleason 4-6) and the mean PSA at diagnosis was 11.1 ng/mL.

We observed predominantly cytoplasmic staining for p63-positive tumor cells (only two men had concomitant nuclear staining), which is a rare expression pattern for a protein that is normally absent in prostate adenocarcinoma and that usually exhibits strong nuclear staining in basal cells of benign prostate glands (Fig. 1). p63 percent positivity reflects the proportion of examined area that stained positive for this protein and a mean of 4.3% of the area (median = 2.5%) displayed p63 immunoreactivity (Table 1) with a maximum score of 54%. The low scores were expected given the underexpression of p63 in adenocarcinoma of the prostate and its use as a negative marker in the

diagnostic workup of prostate cancer. Although we observed no significant differences in age, PSA levels at diagnosis, or pathologic stage across tertiles of p63 percent positivity (Table 1), we found an increasing proportion of prostate cancer deaths in higher tertiles of cytoplasmic p63 expression ($P_{\text{trend}} = 0.04$) and a borderline significant difference in Gleason scores across p63 tertiles ($P = 0.05$). In the same histologic specimens, higher levels of cytoplasmic p63 were associated with a significantly higher frequency of Ki-67-positive cells ($P_{\text{trend}} = 0.0026$; $n = 279$), a marker of cellular proliferation, and lower proportion of cells undergoing apoptosis ($P_{\text{trend}} = 0.0408$; $n = 227$; Fig. 2).

Over the 20-year follow-up period, we observed 19 prostate cancer deaths, which occurred an average of 9.3 years after diagnosis (SD = 4.0; Table 1). In univariate analyses, we observed a significant positive association between the percent of area staining positive for p63 (as a continuous measurement) and fatal prostate cancer, with an ~7% increase in prostate cancer mortality for each additional percent of cytoplasmic p63 positivity [relative risk (RR), 1.07; 95% CI, 1.04-1.10; $P < 0.0001$]. The association persisted after we excluded the two men with colocalized nuclear p63 (RR, 1.10; 95% CI, 1.05-1.16; $P = 0.0001$). We also observed a significant trend with increasing tertiles of p63 positivity [RR, 1.0 (T_1 -reference), 4.0; 95% CI, 0.9-18.9 (T_2) and RR, 5.9; 95% CI, 1.3-27.5 (T_3); $P_{\text{trend}} = 0.028$; Table 2]. As expected, univariate Cox proportional hazards models were significant for fatal prostate cancer for important clinical characteristics, such as age and PSA at diagnosis (Table 2). The increasing risk associated with higher tertiles of p63 cytoplasmic immunoreactivity [RR, 1.0 (T_1 -reference), 2.7; 95% CI, 0.6-13.0 (T_2) and RR, 4.8; 95% CI, 1.0-22.9 (T_3)] yielded a borderline significant trend ($P_{\text{trend}} = 0.047$) after multivariable adjustment for age, year of diagnosis, and Gleason score. The trend persisted after further adjustment for clinical stage ($P_{\text{trend}} = 0.04$; data not shown).

Discussion

We found a positive association between cytoplasmic expression of p63 in prostate tumor tissue at the time of diagnosis and fatal prostate cancer. This association remained significant after adjustment for age, year of diagnosis, Gleason score, and stage. The low percent

Table 2. Univariate and multivariate Cox proportional hazards models for prostate cancer mortality in PHS, 1983-2007

Clinical variable at diagnosis	n	Univariate		Multivariate*	
		Hazard ratio (95% CI)	P	Hazard ratio (95% CI)	P
Age at diagnosis, y	298	1.16 (1.08-1.26)	0.0001	1.15 (1.06-1.24)	<0.001
Gleason score [4-6 (ref), 7-10]	298	3.3 (0.76-14.29)	0.11	2.3 (0.5-10.3)	0.26
Log (PSA at diagnosis), per 1-unit increase	257	3.5 (1.92-6.22)	<0.0001	2.9 (1.46-5.58)	0.002
p63 percent, per 1% increase	298	1.07 (1.04-1.10)	<0.0001	1.06 (1.03-1.09)	<0.001
p63 percent, tertiles					
Low	99	1.0 (Reference)		1.0	
Medium	100	4.0 (0.9-18.9)	0.08	2.7 (0.6-13.0)	0.21
High	99	5.9 (1.3-27.5)	0.02	4.8 (1.0-22.9)	0.05
Test for trend			0.028 [†]	0.047 [†]	

*Adjusted for age at diagnosis (continuous), year of diagnosis (continuous), and Gleason at prostatectomy/transurethral resection of the prostate (4-6, 7-10).

[†]Test for trend.

staining of p63 in our data is consistent with previous studies (23-26); however, the majority of staining occurred in the cytoplasm rather than the nucleus, the usual location for p63 expression in benign and neoplastic epithelial cells. In our data, higher levels of cytoplasmic p63 were also significantly associated with increased proliferative activity (Ki-67) and lower rates of apoptosis. Similar to the localization shift of tumor suppressor gene proteins that induces cellular events leading to carcinogenesis (27, 28), the cytoplasmic staining of p63, a transcription factor involved in transactivation, apoptosis, and proliferation that normally stains in the nucleus, may suggest an altered and potentially oncogenic function (8) for the mislocalized protein in prostate cancer progression and survival.

One of the strengths of our study is the use of a semiautomated, quantitative scoring system to determine objectively p63 expression on a continuous scale. We obtained sufficient tissue from the targeted areas, with three cores per patient (29), and evaluated differences in ranges below common threshold levels for p63 positivity (e.g., <5% and <10%). We found no suggestion of degradation or altered p63 expression of tissue over time; for tumors of similar grade and stage, there was no overall trend in the variation of p63 percent staining by time since tissue collection (data not shown). Another strength of our study is the diversity of prostate cancers represented in pre- and post-PSA periods of diagnosis and a standardized Gleason scoring system. The cohort of men with available tissue did not significantly differ from the overall cohort of men treated by prostatectomy in PHS with respect to demographics such as age, body mass index, smoking, and physical activity or clinical characteristics such as Gleason score or stage of disease ($P > 0.05$; data not shown). One disadvantage of this cohort is that it was restricted to men who were surgically treated, which may limit the generalizability of findings and does not address whether biopsy specimens can also yield prognostic data. Our study was limited to 19 deaths, but we detected a significant association even after adjustment for important clinical covariates.

We observed low p63 expression in our population (median = 2.5%), which is consistent with previous studies showing reduced levels of p63 in adenocarcinoma of the prostate (18, 23-26). Our findings are in contrast with two recent studies that reported an inverse association between p63 expression (as part of a genetic signature) and prostate cancer progression (6, 7). Bismar et al. generated a 12-gene signature for aggressive prostate cancer that included p63 based on its under-expression in metastatic cancer compared with benign tissue and localized disease, and the model was validated on a population of men followed for biochemical recurrence (6). In contrast to Bismar et al. who used PSA recurrence as an endpoint, our population was followed for the development of fatal disease, a more definitive outcome in terms of aggressive disease. Mucci et al. also reported lower levels of p63 staining with lethal prostate cancer in a Swedish watchful waiting cohort of men with T_{1a}-T_{1b} disease followed for up to 28 years (7). In our data, a low proportion of men presented with transurethral resection of the prostate-

diagnosed T_{1a}-T_{1b} disease (10.4%), yet the findings in these men also showed a positive association between cytoplasmic p63 immunoreactivity and prostate cancer mortality ($P = 0.06$; data not shown). Our results may reflect a chance finding, a recently reported rare phenomenon in prostatectomy cases (30), or a real shift from the nucleus to the cytoplasm that has functional significance for prostate cancer progression.

The nuclear localization of p63 is essential for its role as a transcription factor. Similar to p53, alterations in nuclear-cytoplasmic shuttling may lead to cellular mislocalization, which disrupts regulation of cell cycle checkpoints and apoptosis, contributing to the initiation or progression of cancer (27, 28, 30-36). The cytoplasmic sequestration of p53 is associated with metastasis and poor long-term survival in patients with inflammatory breast carcinoma and colorectal carcinoma (32, 33, 37, 38) and similar aberrant immunoreactivity of p63 in the cytoplasm is associated with higher lung cancer mortality (8). The localization shift may arise from disruptions in the nuclear transport pathway (28), such as those mediated by the murine double minute-2 gene (34, 39-42) where laboratory data show that p63-induced apoptosis is reduced when murine double minute-2 gene exports two isoforms of p63 (TAp63 α and TAp63 γ) from the nucleus to inhibit their transcription and proapoptotic activity (42). Our data linking higher levels of cytoplasmic p63 with reduced apoptosis and increased proliferation provide evidence for a potential mechanism of effect. The mislocalization and imbalance in p63 isoforms may alter p63 stability and function and thereby disrupt cell cycle arrest and apoptosis, which may have prognostic significance for cytoplasmic sequestration of p63 and the progression of prostate cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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