Published in final edited form as:

Int J Radiat Oncol Biol Phys. 2008 August 1; 71(5): 1309–1315. doi:10.1016/j.ijrobp.2007.12.010.

Protein Kinase A RI-alpha (PKA) Predicts Prostate Cancer Outcome: An Analysis of Radiation Therapy Oncology Group Trial 86-10

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Abstract

Purpose—The RI-alpha regulatory subunit of Protein Kinase A Type 1 (PKA) is constitutively overexpressed in human cancer cell lines and is associated with active cell growth and neoplastic transformation. This report examines the association between PKA expression and the end points of biochemical failure (BF), local failure (LF), distant metastasis (DM), cause-specific mortality (CSM) and overall mortality (OM) in men treated with radiotherapy, with or without short term androgen deprivation in RTOG 86-10.

Materials and Methods—Pretreatment archival diagnostic tissue samples from 80 patients were stained for PKA by immunohistochemical methods, out of a parent cohort of 456 cases. PKA intensity was scored manually and by image-analysis. Cox proportional hazards model for OM and Fine and Gray's regression models for CSM, DM, LF and BF were then applied to determine the relationship of PKA expression to the end points.

Results—Pretreatment characteristics of the missing and determined PKA groups were not significantly different. In univariate analyses, high PKA staining intensity was associated with BF (image-analysis: continuous variable, $p=0.022$), LF (image-analysis: dichotomized variable,

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Conflict of Interest: None

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p=0.011), CSM (manual, p=0.037; image-analysis: continuous, p=0.014) and DM (manual, p=0.029). In Multivariate analyses, the relationships to BF (image-analysis: continuous, p=0.03), LF (image-analysis: dichotomized, p=0.002) and DM remained significant (manual: p=0.018). In terms of CSM, a trend of an association was seen (manual, p=0.08; image-analysis: continuous, p=0.09).

Conclusions—PKA overexpression was significantly related to patient outcome and is a potentially useful biomarker for identifying high-risk prostate cancer patients who may benefit from a PKA knockdown strategy.

Keywords

PKA RI-alpha; Androgen Deprivation; Radiotherapy

INTRODUCTION

The protein kinase A family of proteins are cAMP-dependent holoenzymes found in mammalian cells. The protein kinase A type I (PKA) protein is differentiated from its type II counterpart by distinguishing regulatory subunits (RI versus RII) (1). Enhanced expression of PKA is associated with active cell proliferation and malignant transformation (2). PKA overexpression has been reported in several tumor types including colorectal, breast and lung cancers where it was associated with poor prognosis (3–5). Antisense oligonucleotides that reduce PKA expression have inhibited the growth of colorectal, breast and gastric carcinomas in vitro (6), and increase response of colorectal and ovarian cancer cell lines to radiation therapy (RT) (7) and chemotherapy (8) in vivo. In our experience, PKA knockdown by the antisense oligonucleotide, GEM 231, enhanced the response of androgen sensitive prostate cancer cells to androgen deprivation (AD) ± RT and androgen insensitive cells to RT in both in vitro and in vivo studies (data not published).

The current report examines the predictive value of PKA overexpression in men with prostate cancer enrolled in Radiation Therapy Oncology Group (RTOG) protocol 86-10. This was a phase III randomized clinical trial designed to compare the effect of Radiotherapy (RT) plus short-term neoadjuvant and concurrent androgen deprivation (STAD) to RT alone (9). Patients with locally advanced disease and palpable tumors of surface area 25 cm² or greater participated. Almost one-third of the patients had Gleason score 8 to 10 disease and there was documented lymph node involvement in 8%. PKA overexpression in suitable pretreatment biopsies from these men were analyzed for any significant relationships to the endpoints of biochemical failure (BF), local failure (LF), distant metastasis (DM), cause-specific mortality (CSM) and overall mortality (OM).

MATERIALS and METHODS

Patients Characteristics

RTOG protocol 86-10 was a Phase III trial testing the efficacy of adding neoadjuvant and concurrent STAD to RT. Details of the trial are well-described (9). The current analysis was approved by the Institutional Review Board at Fox Chase Cancer Center. Informed consent was not required as this study was considered exempt. The characteristics of the 80 cases with PKA data were distributed as follows: 24 cases with Gleason Score 6 or less and 41 with Gleason Score 7 or more (15 cases had missing Gleason score and the remaining material was insufficient for scoring on new cuts); 20 cases with clinical stage B2 and 60 with stage C disease; and 44 cases were assigned to RT alone and 36 to RT + STAD.

Immunohistochemical Analysis

Pretreatment paraffin-embedded formalin-fixed tissues were processed for immunohistochemical staining by the labeled streptavidin-biotin method. This method has been previously described in detail (10,11). The primary monoclonal PKA antibody (Cat. No. 610610, BD Biosciences; 1:100 dilution) was applied. The chromagen diaminobenzidine (DAB, Research Genetics, Huntsville, AL) was used for visualization and commercially-prepared hematoxylin (Dako Corporation, Carpinteria, CA) for counterstaining. All staining was performed on a Dako autostainer (DakoCytomation, Glostrup, Denmark). Positive controls of human brain and prostate carcinoma tissue sections were used for comparison during tissue analysis. Staining for negative controls was also performed on prostatic carcinoma tissue by omitting the primary antibody.

Two investigators (L-Y.K., T.A-S.) reviewed the slides under conventional light microscopy, without knowledge of patient outcome. The first investigator (L-Y. K.) independently examined the slides and then reviewed all cases with an oncologic surgical pathologist (T. A-S.). Although staining was seen in both the nuclear and cytoplasmic compartments, nuclear expression was not found to be significant and only cytoplasmic staining is reported herein (12). Any cytoplasmic staining was considered positive and overall intensities were scored either 0 (negative staining), 1 (light staining), 2 (moderate staining) or 3 (dark staining). Normal prostate epithelium in each sample consistently stained an intensity of '1' and served as an internal control. The investigators differed minimally in intensity grading on 4 cases before conferring on the final result. One further case was adjusted from positive to negative staining and another from positive to inadequate presence of tumor. The intensity of staining was also quantified by an image-analysis system (ACIS, Clariant Inc., San Juan Capistrano, CA) (13). Briefly, all slides were scanned using a preset color threshold provided on the system. Minor adjustments to the brown color threshold (positive cytoplasmic staining) were made using the 'Threshold Tool'. Where possible, at least 6 areas of interest in the tissue visualized at X40 magnification were quantified. The intensity of staining was scored on a grayscale of 0 – 255 arbitrary units. A mean intensity score, the ACIS intensity, was generated by proprietary software.

Definition of End Points

The end points examined included biochemical failure (BF), local failure (LF), distant metastasis (DM), cause-specific mortality (CSM) and overall mortality (OM). The detailed definitions have been provided in prior reports (9,14–16). The failure event for BF was defined as PSA > 2 ng/mL at 1 year or more from the randomization date. The failure event for LF was defined as an increase of more than 50% in tumor size (cross-sectional area), the recurrence of a palpable tumor after initial clearance, a biopsy specimen revealing adenocarcinoma of the prostate 2 years or more after study entry, or tumor never cleared. DM was defined as disease beyond the pelvis by any method of evaluation. The failure event for CSM was death certified as due to prostate cancer and the failure event for OM was defined as death due to any cause. Time to the endpoints was measured from the date of randomization to the date of failure event or the date of the last follow-up.

Statistical Analysis

The Chi square test was used to compare the distributions of patient characteristics and treatment assignments by PKA expression. Cox proportional hazard models (17) were used for OM. Fine and Gray's regression models (18) were used for BF, LF, DM, and CSM to consider the competing risks. The competing risk for BF, LF, and DM was death without failure events. Manual PKA intensities were dichotomized as negative/low [0, 1 and 2] versus high [3]. Dichotomizing the data by [0 and 1] versus [2 and 3] did not result in any significant relationships. The image-analysis intensities were treated as continuous and dichotomized

variables. The median cut-point was the most significant (<135.5 versus ≥ 135.5 arbitrary units), although the 25th and 75th quartiles were also tested. The following variables are adjusted in the multivariate models: assigned treatment (RT+STAD[RL] vs. RT alone), age (<70 [RL] vs. ≥ 70), Gleason score (2–6[RL] vs. 7 vs. 8–10), and T-stage (B2[RL] vs. C). The SAS v9.1 package was used for all the statistical analyses and a significance level of 0.05 was used for all tests.

RESULTS

PKA overexpression status was determined for 80 (17.5%) cases out of 456 eligible and analyzable men treated in RTOG trial 86-10. The study cohort with PKA data was not significantly different from those in the parent cohort without PKA data by pretreatment characteristics and assigned treatment (Table 1). The median follow-up of all enrolled patients was 6.9 years (range: 0.5 – 18.5 years) and the median follow-up of all living patients was 12.2 years (range: 0.4 – 18.5 years).

The PKA staining intensities were analyzed by both manual and image-analysis methods. Table 2 displays the distribution of patients by those results and their pretreatment characteristics. PKA overexpression was not associated with age, Gleason Score, T-stage or assigned treatment. A correlation was seen between the manual and dichotomized image-analysis results using the median cut-point (Table 3).

In univariate analyses (Table 4), the manual assessment of PKA intensity was significantly associated with CSM (HR=1.92, 95% CI=1.04,2.53, $p=0.037$) and DM (HR=1.89, 95% CI=1.07,3.34, $p=0.029$). By image-analysis, PKA was significantly associated with CSM as a continuous variable (HR=1.01, 95% CI=1.00,1.02, $p=0.014$) but had only a trend of an association with DM (dichotomized variable: $p=0.09$; continuous variable, $p=0.07$). In contrast, only image-analysis results showed significant associations between PKA and LF (dichotomized variable: HR=2.28, 95% CI=1.21,4.31, $p=0.011$) and BF (continuous variable: HR=1.01, 95% CI=1.00,1.02, $p=0.022$). In the MVAs, adjusted for assigned treatment, age, Gleason score, and T-stage, PKA was not significantly related to CSM, but there was a trend of an association (manual: $p=0.08$, image analysis: $p=0.09$). PKA intensity significantly predicted for DM by the manual but not image-analysis method (HR=2.27, 95% CI=1.15,4.46, $p=0.018$) (Table 5). PKA was significantly related to BF (continuous variable: HR=1.01, 95% CI=1.00,1.02, $p=0.03$) and LF (dichotomized variable: HR=3.66, 95% CI=1.64,8.15, $p=0.002$) by image-analysis only (Table 6). Of note, Gleason score 8–10 was significantly related to CSM (HR=4.74, 95% CI=1.77,12.71, $p=0.002$) and DM (HR=2.72, 95% CI=1.15,6.47, $p=0.023$). High PKA expression by manual analysis was associated with 10-year DM rates of 67.4% (95% CI=51.7, 83.1) versus 45.5% (95% CI=30.1, 61.0) and PKA overexpression of ≥ 135.5 by image-analysis had 10-year DM rates of 66.6% (95% CI=51.2,82.0) versus 45.3% (95% CI=29.4,61.2) (Figure 1).

Tests for an interaction between treatment arm and PKA intensity showed no statistically significant associations (manual: CSM $p=0.27$, DM $p=0.30$; image-analysis: LF $p=0.37$).

DISCUSSION

The PKA protein is a specific subunit of PKA, whose role is to mediate cAMP-dependent cell processes such as DNA replication and cell proliferation (2). Human tumors that overexpress PKA include breast, colon and lung cancers; overexpression has been associated with poor prognosis (3–5,19). Studies of various PKA inhibitors have demonstrated chemosensitization (8,20,21) and radiosensitization of various cancer cell lines when used alone (22) or in combination with epidermal growth factor receptor inhibitors (7,23). Our laboratory has also

tested the PKA antisense oligonucleotide inhibitor (GEM231) in prostate cancer cell lines, which resulted in enhanced response of androgen sensitive prostate cancer cells to RT $\pm$ AD and androgen resistant cells to RT in both in vitro and in vivo studies (24). PKA has potential as a biomarker and a therapeutic target.

The current study is the first to explore PKA overexpression as a biomarker for predicting the outcome of men with locally-advanced prostate cancer treated with RT $\pm$ STAD. The most significant relationships in multivariate analysis were between high PKA expression and LF and DM. PKA overexpression by manual methods was significantly related to DM. The independent association between PKA overexpression and LF was only seen using the image-analysis method (Table 6). It should be kept in mind that local failure was not routinely documented at the sub-clinical level by prostate biopsy and that the results reflect clinical local progression. Nonetheless, the findings suggest that the relationship of PKA overexpression to DM is due at least in part from local persistence of disease when PKA is elevated. An independent association between PKA overexpression and BF was also seen only in the image-analysis data. However, these results should be treated with caution as pretreatment PSA determination was not widely available early in the original Protocol 86-10 (9) and the definition used for the primary endpoint BF is not the currently accepted Phoenix definition (25). Statistically significant relationships between PKA overexpression and CSM were seen in univariate analyses by both manual and image-analysis methods, although this association weakened to a trend in multivariate analyses.

The PKA protein has been shown to control cell cycle progression from G1 to S phases in the cell cycle of mammalian cells and can overcome growth factor requirements for cell proliferation when overexpressed (26). Increased PKA expression could influence the sensitivity of prostate cancer cells to RT, and hence local control, by affecting the cell cycle. Other possible explanations are the association between PKA expression and genes elevated in radioresistant cells (DNA-PK, Rad51, bcl-2) (22) and androgen receptor activation (27–29).

PKA expression promotes androgen receptor nuclear translocation and activation (30). PKA signaling and downstream factors have also been implicated in the progression of androgen sensitive prostate cancer cells to androgen insensitivity (27–29). A relationship between PKA expression and response to androgen deprivation raises the possibility that further gains might be realized by PKA knockdown. Although we tested for these interactions, we did not observe a differential effect between assigned protocol treatment and patient outcome.

In summary, this is the first report of an association of PKA overexpression to patient outcome in men treated with radiotherapy. The significant relationships observed between PKA overexpression and LF and DM were remarkable, given the small sample size. Of note, in this RTOG 86-10 protocol subset analysis, assigned protocol treatment (RT+STAD vs. RT alone) was not a significant covariate, while in the parent cohort it has been (31). PKA overexpression may be a stronger, independent determinant of outcome than the addition of short term androgen deprivation to radiotherapy. Further substantiation of the significance of PKA as a biomarker is needed. We plan to study the impact of PKA overexpression in a larger independent cohort of prostate cancer patients.

Acknowledgments

This publication was supported in part by Grants CA-006927, CA-101984-01, CA-21661 and CA-32115 from the National Cancer Institute, and grants from Varian Medical Systems, Palo Alto, CA and the Pennsylvania Department of Health. The contents are solely the responsibility of the authors and do not necessarily represent the official views of these organizations.

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(A)

(B)

Figure 1.
Relationship of PKA expression by manual (panel A) and dichotomized ACIS intensity score (B) to distant metastasis.

Table 1

Pretreatment characteristics of patients with determined or missing PKA data (n=456)

Characteristics	Determined PKA data (n= 80)	Missing PKA data (n=376)	p-value [*]
Age			
Median, years (range)	71 (51 – 81) n (%)	70 (49 – 88) n (%)	
<70	34 (43)	161 (43)	0.96
≥70	46 (58)	215 (57)	
GLSC			
Unknown/Missing	15 (19)	75 (20)	0.30
2–6	24 (30)	143 (38)	
7	20 (25)	91 (24)	
8–10	21 (26)	67 (18)	
T-Stage			
B2	20 (25)	115 (31)	0.32
C	60 (75)	261 (69)	
Assigned Treatment			
RT + STAD	36 (45)	188 (50)	0.42
RT Alone	44 (55)	188 (50)	

* p-value is from Chi-square statistics

Abbreviations: GLSC, Gleason Score; RT, radiation therapy; STAD, short term androgen deprivation.

Table 2
Distribution of patients by PKA manual and ACIS intensity score results

Characteristics	Manual Intensity Score			ACIS Intensity Score		
	Negative/Low n(%)	High n(%)	p-value [*]	< 135.5 n(%)	≥ 135.5 n(%)	p-value [*]
Age						
<70	21(50)	13(34)	0.15	20(50)	14(35)	0.17
≥70	21(50)	25(66)		20(50)	26(65)	
GLSC						
Unknown/Missing	5(12)	10(26)	0.11	5(13)	10(25)	0.23
2–6	16(38)	8(21)		14(35)	10(25)	
7	8(19)	12(32)		8(20)	12(30)	
8–10	13(31)	8(21)		13(33)	8(20)	
T-Stage						
B2	8(19)	12(32)	0.20	7(18)	13(33)	0.12
C	34(81)	26(68)		33(83)	27(68)	
Assigned Treatment						
RT + STAD	18(43)	18(47)	0.69	16(40)	20(50)	0.37
RT Alone	24(57)	20(53)		24(60)	20(50)	

^{*} p-value from Chi-square statistics
Abbreviations: GLSC, Gleason Score; RT, radiation therapy; STAD, short term androgen deprivation.

Table 3

Distribution of Manual vs. ACIS intensity results

Manual	ACIS		p-value *
	< 135.5 n (%)	≥135.5 n (%)	
Negative	36 (90%)	6 (15%)	<0.0001
Positive	4 (10%)	34 (85%)	<0.0001

* P-value from Chi-square statistics

Table 4
Univariate analysis of PKA expression by manual and image-analysis (n=80)

Endpoint	PKA Manual Intensity	n	Failures	HR* (95%CI)	p-value [†]	PKA ACIS Intensity	n	Failures	Dichotomized		Continuous	
									HR* (95%CI)	p-value [†]	HR* (95%CI)	p-value [†]
OM	Neg/Low	42	35	1.20(0.75,1.92)	0.46	< 135.5	40	33	1.35(0.84,2.17)	0.22	1.01(0.99,1.02)	0.12
	High	38	34			≥135.5	40	36				
CSM	Neg/Low	42	17	1.92(1.04,2.53)	0.037	< 135.5	40	17	1.68(0.91,3.08)	0.10	1.01(1.00,1.02)	0.014
	High	38	23			≥135.5	40	24				
DM	Neg/Low	42	20	1.89(1.07,3.34)	0.029	< 135.5	40	20	1.65(0.93,2.93)	0.09	1.01(0.99,1.02)	0.07
	High	38	28			≥135.5	40	28				
LF	Neg/Low	42	18	1.45(0.78,2.70)	0.24	< 135.5	40	14	2.28(1.21,4.31)	0.011	1.00(0.99,1.01)	0.55
	High	38	20			≥135.5	40	24				
BF	Neg/Low	42	28	1.47(0.88,2.45)	0.14	< 135.5	40	29	1.13(0.68,1.88)	0.62	1.01(1.00,1.02)	0.022
	High	38	31			≥135.5	40	30				

* Hazard ratio: a risk ratio of 1 indicates no difference between the two subgroups.

[†] p-value is from Chi-square test using the Cox proportional hazards model for OM; Fine and Gray's regression models for CSM, DM, LF and BF.

Abbreviations: Neg., negative; HR, hazard ratio; CI, confidence interval; OM, overall mortality; CSM, cause-specific mortality; DM, distant metastasis; LF, local failure and BF, biochemical failure.

Table 5

Multivariate analysis with manual PKA intensity score as a dichotomous covariate

Endpoint [§]	Variable [#]	Comparison	HR [*] (95% CI)	p-value ^{**}
CSM	PKA Manual intensity	Low vs. High	RL	
			1.90 (0.93, 3.85)	0.08
	Treatment arm	RT+STAD vs. RT Alone	RL	
			0.77 (0.36, 1.63)	0.49
	Age	< 70 vs. ≥70	RL	
			0.81 (0.39, 1.69)	0.57
	GLSC	2–6 vs. 7 vs. 8–10	RL	
			0.82 (0.33, 2.01)	0.66
	T-Stage	B2 vs. C	RL	
			4.74 (1.77, 12.71)	0.002
DM	PKA Manual intensity	Low vs. High	RL	
			2.27 (1.15, 4.46)	0.018
	Treatment arm	RT+STAD vs. RT Alone	RL	
			0.93 (0.49, 1.76)	0.82
	Age	< 70 vs. ≥70	RL	
			0.74 (0.39, 1.43)	0.37
	GLSC	2–6 vs. 7 vs. 8–10	RL	
			1.11 (0.52, 2.35)	0.79
	T-Stage	B2 vs. C	RL	
			2.72 (1.15, 6.47)	0.023
			0.86 (0.42, 1.79)	0.69

* The hazard ratios and 95% confidence intervals (in parenthesis) are shown.

** p-values from Fine and Gray's regression models.

All variables were dichotomous.

Abbreviations: HR, hazard ratio; CI, confidence interval; CSM, cause-specific mortality; DM, distant metastasis; RL, reference level; RT, radiotherapy; STAD, short term androgen deprivation; GLSC, Gleason Score.

§ The endpoints BF, LF and OM were tested and PKA was not significantly related to them.

Table 6

Multivariate analyses with PKA ACIS intensity score as a continuous or dichotomized (median cut point) covariate

Endpoint [§]	Variable [#]	Group	HR [*] (95% CI)	p-value ^{**}
CSM	PKA Intensity Score	Continuous	1.01 (0.998, 1.02)	0.09
	Treatment arm	RT Alone	0.80 (0.34, 1.86)	0.60
	Age	≥ 70	0.84 (0.40, 1.74)	0.64
	GLSC	7	0.89 (0.37, 2.10)	0.78
		8–10	4.77 (1.81, 12.60)	0.002
	T-Stage	C	0.46 (0.21, 1.01)	0.052
BF	PKA Intensity Score	Continuous	1.01 (1.00, 1.02)	0.03
	Treatment arm	RT Alone	1.91 (1.04, 3.51)	0.04
	Age	≥ 70	1.01 (0.58, 1.74)	0.97
	GLSC	7	1.16 (0.61, 2.21)	0.65
		8–10	1.27 (0.64, 2.55)	0.50
	T-Stage	C	1.09 (0.54, 2.18)	0.81
LF	PKA Intensity Score	≥ 135.5	3.66 (1.64, 8.15)	0.002
	Treatment arm	RT Alone	1.00 (0.47, 2.14)	0.99
	Age	≥ 70	0.42 (0.18, 0.94)	0.04
	GLSC	7	0.20 (0.07, 0.62)	0.005
		8–10	0.94 (0.39, 2.27)	0.88
	T-Stage	C	0.47 (0.21, 1.03)	0.06

* The hazard ratio and 95% confidence intervals (in parenthesis) are shown.

** p-values from Fine and Gray's regression models.

All variables were dichotomous.

Abbreviations: HR, hazard ratio; CI, confidence interval; CSM, cause-specific mortality; RT, radiotherapy; GLSC, Gleason Score; BF, biochemical failure; LF, local failure.

§ The endpoints DM and OM were tested and PKA was not significantly related to them.