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Prostate Tissue Metal Levels and Prostate Cancer Recurrence in Smokers

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Abstract

Although smoking is not associated with prostate cancer risk overall, smoking is associated with prostate cancer recurrence and mortality. Increased cadmium (Cd) exposure from smoking may play a role in progression of the disease. In this study, inductively coupled plasma mass spectrometry was used to determine Cd, arsenic (As), lead (Pb), and zinc (Zn) levels in formalin-fixed paraffin embedded tumor and tumor-adjacent non-neoplastic tissue of never- and ever-

smokers with prostate cancer. In smokers, metal levels were also evaluated with regard to biochemical and distant recurrence of disease. Smokers ($N=25$) had significantly higher Cd (median ppb, $p=0.03$) and lower Zn ($p=0.002$) in non-neoplastic tissue than never-smokers ($N=21$). Metal levels were not significantly different in tumor tissue of smokers and non-smokers. Among smokers, Cd level did not differ by recurrence status. However, the ratio of Cd ppb to Pb ppb was significantly higher in both tumor and adjacent tissue of cases with distant recurrence when compared with cases without distant recurrence (tumor tissue Cd/Pb, 6.36 vs. 1.19, $p=0.009$, adjacent nonneoplastic tissue Cd/Pb, 6.36 vs. 1.02, $p=0.038$). Tissue Zn levels were also higher in smokers with distant recurrence (tumor, $p=0.039$ and adjacent non-neoplastic, $p=0.028$). These initial findings suggest that prostate tissue metal levels may differ in smokers with and without recurrence. If these findings are confirmed in larger studies, additional work will be needed to determine whether variations in metal levels are drivers of disease progression or are simply passengers of the disease process.

Keywords

Prostate cancer; Recurrence; Smoking; Heavy metal; Cadmium

Introduction

Although existing evidence suggests that smoking is not related to overall risk of prostate cancer [1, 2], recent reports implicate smoking in aggressive [3, 4] and recurrent [5] prostate cancer and in mortality [6, 7] due to the disease. Several mechanisms [3] have been proposed to explain these associations including increased exposure of smokers to the heavy metal cadmium (Cd). Cd is known to inhibit DNA repair, induce oxidative stress, promote aberrant gene expression, and induce, as well as inhibit, apoptosis [8, 9].

Smoking is considered to be one of the predominant sources of non-occupational Cd exposure in the USA. Cd is naturally present in the earth's crust and is readily taken up from soil by tobacco plants. In addition, fertilizers applied to crops such as tobacco, frequently contain Cd [10]. Cd is recognized as a carcinogen by the International Agency for Research on Cancer (IARC), and early animal and human studies, used by the IARC to classify Cd as a level IA carcinogen, suggest a link between the heavy metal and risk of prostate and lung cancer [11]. More recent epidemiological studies of Cd exposure and prostate cancer risk and progression have been mixed and inconclusive, and have relied on indirect measures of exposure such as estimated lifetime occupational exposure [12, 13] or peripheral measures of Cd in blood [14] or toenails [15, 16]. Only a few studies have assessed metals directly in prostate tissue specimens, and even fewer have accounted for smoking status or disease recurrence. In general, these studies have found higher Cd and lower zinc (Zn) levels in prostate cancer cases than in controls [17, 18] and higher Cd levels in smokers compared with non-smokers [19], leading some to suggest that the ratio of Cd to Zn may be more important than considering Cd alone as Cd can replace Zn or may have other indirect effects on Zn proteins. Zn is required for normal prostate development. A study by Sarafanov et al. [20], which assessed metal levels in 40 prostate cancer cases with known biochemical recurrence and 40 matched cases without biochemical recurrence, found significantly lower

Zn levels in adjacent non-neoplastic tissue of recurrent cases. There were no differences noted in Cd levels by recurrence status; however, the study did not account for individuals' smoking history.

In this study, we examine prostate tissue Cd and Zn levels in prostate cancer cases by smoking status. Data on two additional heavy metals associated with smoking [21], arsenic (As) and lead (Pb), are also provided, and we evaluate metal levels by biochemical and distant recurrence status within ever-smokers.

Materials and Methods

Subjects and Tissue Samples

Formalin-fixed paraffin embedded (FFPE) prostate tissue of a subset of men who participated in the Gene-Environment Interaction in Prostate Cancer Study (GECAP) [22] and underwent prostatectomy for treatment of their cancer were selected for inclusion in this study. As part of the GECAP study, men completed a face-to-face interview to determine lifetime smoking history and occupational exposure to metals (Cd, Pb, and Zn), and completed a food frequency questionnaire. Ever-smokers included cases that had a history of past or current smoking. Detailed clinical information included PSA at diagnosis, Gleason score, stage, margin status, and information on biochemical recurrence. Biochemical recurrence was defined as two consecutive PSA tests greater than 0.02 ng/mL after prostatectomy. In addition, distant recurrence was captured from the certified tumor registry of the Henry Ford Health System, Detroit, MI where the GECAP study took place.

Prostate tissue samples were selected and prepared according to the methods reported by Sarafanov et al. [23]. Areas of interest (tumor and adjacent non-neoplastic) were marked on an H&E slide by the study pathologist (O.N.K.) who was blinded to the smoking and recurrence status of subjects. Designated tissue areas of tumor and non-neoplastic were macro dissected with a titanium knife, using metal-free methods, and placed in tubes that had been pre-washed with Optima grade nitric acid and MilliQ water and dried. Tissue was deparaffinized using hexane (Optima grade, Thermo Fisher) at 20 °C for 1 week with frequent changes of solvent.

Metal Measurement by Inductively Coupled Plasma Mass Spectrometry

Tumor and adjacent non-neoplastic prostate tissue levels were measured by inductively coupled plasma mass spectrometry (ICP-MS) in the laboratory of (B.M.). In short, samples were dried until a constant tissue weight was achieved. Samples were then digested with concentrated nitric acid (high purity Optima Grade from Thermo Fisher) in pre-washed and dried polypropylene tubes overnight at room temperature. Approximately 10 mg of each sample was digested with nitric acid at 90 °C for 30 min. The samples were then diluted with MilliQ pure water (Sigma) to the appropriate range of metal concentrations. Metal measurements were carried out in a PE Sciex Elan 9000 ICP-MS with a cross flow nebulizer and Scott type spray chamber. The RF power was set at 1000 W and the argon flow optimized at 0.92 l/min. The optimum lens voltage was centered on rhodium sensitivity. Standard external calibrations curves were generated from stock solutions purchased from

VWR (Radnor, PA). All samples included internal standards, Yttrium and Indium, also from VWR. The ICP-MS measurements were repeated on duplicate samples. Data for subjects that had both tumor and matched adjacent non-neoplastic tissue are presented.

Statistical Analysis

Chi-sq and *t* tests were used to determine differences in subject characteristics across smoking status. The average level of As, Cd, Pb, and Zn was determined for each subject using two replicate measurements of two separately digested samples from each tissue sample. To accommodate the data, which was not normally distributed, the median metal level and interquartile range (25th to 75th percentile) are reported for never- and ever-smokers in tumor and adjacent nonneoplastic tissue. The differences across smokers and non-smokers and across smokers with recurrence and without recurrence were evaluated by the Mann–Whitney *U* test, and statistical significance was set at a $p < 0.05$. Metal levels were also log transformed and ANCOVA was used to account for potential confounders.

Results

The prostate cancer cases in this study included 21 never-smokers and 25 ever-smokers. Smokers and never-smokers (Table 1) were similar in age, PSA at diagnosis, and Gleason grade. In addition, dietary zinc intake and estimated occupational exposure to metals did not differ between ever- and never-smokers. Ever-smokers had a median follow-up period of 47.4 months compared with 48.7 months for never-smokers.

As Table 2 shows, metal levels were evaluated by smoking status in prostate tumor tissue and adjacent normal tissue. Overall, smokers had significantly higher Cd and lower Zn levels in tumor-adjacent non-neoplastic tissue. Cd/Zn and Cd/As ratios in adjacent non-neoplastic tissue were significantly higher in smokers as well. Metal levels in tumor tissue did not differ significantly between ever- and never-smokers.

We also assessed metal levels by biochemical recurrence and distant recurrence status within ever-smokers. Eight smokers had biochemical recurrence of disease and four of these had evidence of distant recurrence after prostatectomy. All subjects were pathologically staged as M0 at diagnosis, and there were no significant differences in tumor stage, margin status, or Gleason score among those that recurred or did not recur (e.g., Table 1, smokers within Gleason scores 8 and 9, one subject had recurrence and two subjects did not have recurrence). Compared with cases without biochemical recurrence, those with recurrence had significantly lower median Pb levels in tumor tissue and a significantly higher Cd/Pb ratio in tumor tissue (Table 3). Tumor Pb level was even lower in the smaller group of distant recurrent cases and resulted again in significantly higher Cd/Pb ratios in both tumor and adjacent non-neoplastic tissue of recurrent versus non-recurrent cases (tumor tissue Cd/Pb, 6.36 vs. 1.19, $p = 0.009$, tumor-adjacent normal tissue Cd/Pb, 6.36 vs. 1.02, $p = 0.038$) (Table 4). The median Zn level, on the other hand, was nearly fourfold higher in tumor and adjacent tissue of cases with distant recurrence than levels of Zn found in non-recurrent cases. Results from multivariable analyses of log transformed metal levels adjusting for age, race, zinc intake, packyears of smoking, or occupational metal exposure supported the non-

parametric univariate results. Results remained the same when we limited the analyses to current smokers and those who quit most recently (within the past 20 years).

Discussion

In this study, we observed significantly higher Cd to Zn and Cd to As ratios in non-neoplastic tissue of ever-smokers compared with never-smokers. However, with regard to recurrence in ever-smokers, we observed significantly higher Cd to Pb ratios and no difference in Cd to Zn ratio in cases that had evidence of distant recurrence. Specifically, we observed lower Pb levels in tumor tissue of ever-smokers who had biochemical recurrence or distant recurrence and higher Zn levels in both tumor and adjacent non-neoplastic tissue of ever-smokers with distant recurrence. These initial findings, although identified in a small set of prostate cancer cases with a wide range of smoking history, suggest a potential role for metals in both biochemical and distant recurrence of prostate cancer within ever-smokers.

Several mechanisms through which smoking may lead to progression of prostate cancer have been proposed [3] and include (1) increased levels of total and free testosterone, (2) epigenetic changes, (3) genetic variation, which may modify xenobiotic and other pathways, (4) carcinogenic effects of cadmium and/or N-nitroso compounds, and (5) other non-mutagenic effects of carcinogens such as increased neovascularization of tumors. Only a handful of previous studies have measured Cd and other metals directly in prostate tissue, and none to our knowledge have measured metals in recurrent and non-recurrent tumors of smokers. An autopsy study conducted by Anetor et al. [19] in Germany assessed prostate tissue metal levels in 68 smokers and 61 non-smokers without regard for prostate cancer and showed higher Cd to selenium ratios in smokers compared with non-smokers. This is similar to our finding of a higher Cd to Zn ratio in prostate cancer cases who were classified as ever-smokers. Sarafanov et al. [20] found prostate cancer cases with biochemical recurrence to have significantly lower prostate tissue Zn (279 vs. 346 ug/g, $p=0.04$) and iron (95 vs. 111 ug/g, $p=0.04$) in tumor-adjacent non-neoplastic tissue compared with cases without recurrence. They observed no significant difference in Cd level overall ($p=0.40$) but did observe a significant difference at the third quartile with recurrent cases having higher prostate tissue Cd than non-recurrent cases. Sarafanov et al. [20] did not report subjects smoking history.

In our smoking-stratified analyses, prostate cancer cases with a history of smoking that progressed to biochemical or distant recurrence had lower levels of Pb in tumor tissue and higher Cd/Pb ratio in tumor and adjacent non-neoplastic tissue when compared with cases that did not recur. The combined effects of Cd and Pb, which can be additive, synergistic, or antagonistic, may differ from that of either metal individually [24]. Our finding that non-recurrent cases had a nearly 1:1 ratio of Cd to Pb while recurrent cases had more than six times the ppb of Cd than ppb of Pb may indicate that Cd and Pb are antagonists in the prostate. Those with high Cd to Pb ratios may be at greater risk of the toxic effects associated with Cd and at greater risk for recurrence of disease. Lower Pb levels in tumor as well as near adjacent non-tumor tissue could be the result of lower Pb exposure in these cases or perhaps a protein or proteins that bind Pb are lost or reduced in tumor and the tumor microenvironment of prostate cancer cases that will progress. Although no predominant Pb

binding protein has been reported for the prostate at least three proteins, thymosin β_4 , acyl-CoA binding inhibitor [25, 26] and delta-aminolevulinic acid dehydratase [27] have been shown to bind a high proportion of Pb in other tissues, and all could theoretically play a role in cancer progression [28–30]. Other proteins could be responsible for binding Pb in the prostate as well. Further work is needed to determine whether our observation of lower Pb levels and higher Cd/Pb in smokers with prostate cancer recurrence is due to differences in Pb exposure, differences in Pb binding proteins, or due to other factors.

We also observed high levels of Zn in ever-smoker smoking cases with recurrent disease. Prostate cancer is considered unusual in that risk of the disease is generally associated with low Zn levels [31]. At least two previous studies, however, found increased risk of advanced or fatal prostate cancer in men using high levels of Zn supplements [32] or multivitamin supplements containing Zn [33], although Zn is generally considered to have protective effects for prostate cancer, overall. Work by Prasad et al. [34] using the transgenic adenocarcinoma of the mouse prostate (TRAMP) model suggests that both high and low Zn levels may be associated with greater tumor weight compared with tumors in mice with normal Zn levels. Zn imbalance, therefore, may play a role in prostate recurrence among smokers.

This study had many strengths including long-term follow-up of recurrence, direct measurement of metals in the tissue of interest, use of ICP-MS for low level detection of metals, and detailed clinical, occupational metal exposure and dietary information. Still, there were several limitations. Our sample size was small, and there was a wide variation in smoking behavior (duration, intensity) within our ever-smokers. As expected, we did observe a higher rate of distant recurrence among our ever-smokers compared with our never-smokers even though PSA and Gleason score at diagnosis did not differ between the two groups. However, with only one recurrence among our never-smokers, metal levels in recurrent cases could only be assessed for ever-smokers. Therefore, we cannot be sure that our observations of low Pb, high Zn, and high Cd/Pb ratio within recurrent cases are unique to smokers. As has been previously reported [23], there is also some loss of Zn due to the paraffin embedding process. Our samples were all processed in the same pathology department during a limited number of years and it is unlikely that the difference we observed can be explained by differences in the embedding process. Further, in order to obtain an adequate amount of tissue for analysis, macro dissection is used, which could introduce variations in the amount of stromal versus epithelial tissue contained in each specimen. Still with these limitations, this is the only study to our knowledge to have assessed metal levels in recurrent and non-recurrent prostate cancer cases taking smoking status into account.

Larger studies of smoking, metals and prostate cancer recurrence with a longer period of follow-up are warranted and should measure metals directly in prostate tissue. If confirmed, these findings may shed new light on the biological mechanisms connecting smoking and prostate cancer progression.

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Abbreviations

ACBP/	acyl-CoA binding inhibitor/diazepam binding
DBI	inhibitor
ALAD	Delta-aminolevulinic acid dehydratase
As	Arsenic
Cd	Cadmium
FFPE	Formalin-fixed paraffin embedded tissue
ICP-MS	Inductively coupled plasma mass spectrometry
Pb	Lead
ppb	Parts per billion
PSA	Prostate specific antigen

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Table 1

Prostate cancer case characteristics

Characteristic	Never-smokers N=21	Ever-smokers N=25
Age	58.7 (sd 8.2)	59.9 (sd 5.8)
Occupation with metal exposure	13 (61.9 %)	15 (60.0 %)
Dietary zinc intake (mg/day)	13.6 (sd 6.9)	15.0 (sd 6.5)
Mean PSA at dx (ng/dl)	6.8 (sd 7.5)	6.6 (sd 4.3)
Gleason		
6	6 (28.6 %)	7 (28.0 %)
7	13 (61.9 %)	15 (60.0 %)
8-9	2 (9.5 %)	3 (12.0 %)

Table 2

Metals in tumor and matched adjacent non-neoplastic prostate tissue of smokers and non-smokers

Metal	Adjacent non-neoplastic tissue				Tumor tissue			
	Never-smokers N=21	Ever-smokers N=25	<i>p</i>	<i>p</i> ^{Cd ratio}	Never-smokers N=21	Ever-smokers N=25	<i>p</i>	<i>p</i> ^{Cd ratio}
	Median (IQR) ppb	Median (IQR) ppb			Median (IQR) ppb	Median (IQR) ppb		
Cd	202.9 (163.8)	308.4 (189.6)	0.03	–	219.9 (260.7)	266.4 (271.4)	0.31	–
As	5.38 (3.96)	5.12 (6.29)	0.80	0.014*	6.41 (4.97)	4.33 (4.58)	0.10	0.06
Pb	178.7 (213.1)	268.8 (199.1)	0.36	0.20	155.8 (337.1)	176.1 (339.9)	0.31	0.98
Zn	441,005.4 (338,391.4)	263,792.1 (528,864.7)	0.002	<0.001*	484,799.4 (204694.2)	350,304.1 (380057.9)	0.20	0.21

As Arsenic, Cd cadmium, Pb lead, Zn zinc, IQR interquartile range, ppb parts per billion, *p* difference in metal levels, *p*^{Cd ratio} difference in Cd/As, Cd/Pb, or Cd/Zn ratio,

*, Mann–Whitney *U*, *p* <0.05 considered significant

Table 3

Metal levels in prostate tissue of ever-smokers by biochemical recurrence status

Metal	Adjacent non-neoplastic tissue of ever-smokers				Tumor tissue of ever-smokers			
	No recurrence N=17	Biochemical recurrence N=8	<i>p</i>	<i>p</i> ^{Cd ratio}	No recurrence N=17	Biochemical recurrence N=8	<i>p</i>	<i>p</i> ^{Cd ratio}
	Median (IQR) ppb	Median (IQR) ppb			Median (IQR) ppb	Median (IQR) ppb		
Cd	302.6 (170.0)	374.1 (612.4)	0.72	–	261.0 (210.0)	380.3 (402.2)	0.40	–
As	4.46 (5.72)	6.70 (4.25)	0.53	0.73	3.71 (5.24)	5.75 (3.12)	0.31	0.85
Pb	268.8 (234.8)	227.5 (567.6)	0.72	0.24	292.7 (328.8)	104.4 (92.7)	0.005*	0.017*
Zn	273,903.6 (163580.9)	216,729.6 (800875.4)	0.98	0.95	336,287.4 (300386.9)	430,700.7 (1,108,278)	0.34	0.73

Biochemical recurrence defined as two consecutive PSA >0.02 ng/mL. *As*, Arsenic, *Cd* cadmium, *Pb* lead, *Zn* zinc, *IQR* interquartile range, *ppb* parts per billion, *p* difference in metal levels, *p*^{Cd} ratio difference in Cd/As, Cd/Pb, or Cd/Zn ratio in those with and those without biochemical recurrence,

* Mann–Whitney U, *p* <0.05 considered significant

Table 4

Metal levels in prostate tissue of ever-smoker prostate cancer cases by distant recurrence status

Metal	Adjacent non-neoplastic tissue of ever-smokers				Tumor tissue of ever-smokers			
	No distant recurrence N=21	Distant recurrence N=4	<i>p</i>	<i>p</i> ^{Cd ratio}	No distant recurrence N=21	Distant recurrence N=4	<i>p</i>	<i>p</i> ^{Cd ratio}
	Median (IQR) ppb	Median (IQR) ppb			Median (IQR) ppb	Median (IQR) ppb		
Cd	302.6 (190.3)	547.9 (1084.9)	0.23	–	261.0 (245.6)	380.2 (798.1)	0.18	–
As	4.46 (4.61)	7.61 (20.2)	0.08	0.55	3.97 (4.72)	6.08 (4.27)	0.18	0.64
Pb	296.3 (293.7)	86.1 (846.4)	0.29	0.038*	219.2 (330.8)	76.4 (62.2)	0.011*	0.009*
Zn	246,235.4 (147,867.7)	850,717.4 (1640271.2)	0.028	0.30	336,287.4 (275,103.9)	1,264,243.7 (1,249,075.0)	0.039*	0.34

As Arsenic, Cd cadmium, Pb lead, Zn zinc, IQR interquartile range, ppb parts per billion, *p* difference in metal levels, *p*^{Cd ratio} difference in Cd/As, Cd/Pb, or Cd/Zn ratio in those with and those without distant recurrence,

* Mann-Whitney U, *p* <0.05 considered significant