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To study the relationship between cadmium, zinc and mtDNA copy number in North Indian patients suffering from prostate cancer: A case control study

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Gleason score

Abstract
Objective: To examine the variation of cadmium (Cd), zinc (Zn) and mitochondrial DNA (mtDNA) copy number in prostate cancer (PCa) patients and their age match controls and correlations with clinicopathological parameters.
Subjects and methods: This study was conducted between January 2012 and January 2015. Blood Cd and Zn level was measured by inductively coupled plasma spectrophotometer. The variation of mitochondrial copy number in blood was measured by real-time PCR. We scrutinized the correlation of Cd, Zn and mtDNA with clinicopathological parameters like prostate specific antigen (PSA) and [Gleason] score.
Results: Mean Cd level (μg/L ± SD) was significantly higher (3.89 ± 1.49) while Zn level was low (85 ± 10.4) in PCa patients comparison to control patients (2.92 ± 1.23; 116 ± 19.1). A wide

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distribution of variance and higher mtDNA copy numbers were found in PCa patients [Range (fold difference) 1968–39,245 (17.4)] as compared to controls [10,178–332,243.9], (p value: 0.036). Clinicopathological analysis showed that Cd, Zn and mtDNA DNA copy numbers were significantly associated with increased [Gleason] score but not with serum PSA level (Zn: $r = -0.68$; Cd: $r = 0.64$; mtDNA: $r = 0.84$) ($p < 0.05$).

Conclusion: Cd, Zn and mtDNA copy number significantly correlate with [Gleason] score which suggests that the former may serve as marker for therapeutic guidance.

Introduction

Prostate cancer (PCa) is frequently being diagnosed in elderly men, with multi-factorial etiology. Cadmium (Cd) is characterized by various degrees of toxicity to non-target species, including human beings. It is also classified as human carcinogen, based on epidemiological and rodent experiments [1,2]. Cd enters the cells by utilizing transport pathways evolved for essential metals. Cd follows a Trojan horse strategy by molecular mimicry and interferes with essential metals homeostasis. In prostatic cells, cadmium toxicity has been closely related to zinc homeostasis. Zinc (Zn) is a key regulatory factor in the intermediary metabolism of prostate cells and any changes in Zn homeostasis contributes to the development and progression of PCa [3].

Several mechanisms of metal carcinogenesis have been proposed, the most important appears to be oxidative stress which responsible for DNA damage [4]. Mitochondria implemented intracellular targets for various metals including Cd are central to the formation of reactive oxygen species (ROS). Mitochondrial dysfunction is closely related to mitochondrial DNA (mtDNA) copy number which is expressed as increase or decrease of mtDNA copy number. In addition, human mtDNA lacks protective histones, introns, mtDNA proofreading and has limited DNA repair capacity [5]. These features make it highly susceptible to ROS and other types of damage that could lead to sequence mutations or copy number alterations in mtDNA. Normally the amount of mtDNA remains relatively stable in order to maintain the energy demand and protect its standard physiological condition [6]. Fluctuation in mtDNA copy number may affect mitochondrial dysfunction, and has been observed in various types of malignancy including PCa [7].

Since Cd and Zn play a vital role in normal and cancerous tissues, we hypothesize that Cd and Zn influence distribution pattern of mtDNA copy number. Hence, present study focuses on investigation of Cd, Zn and mtDNA from blood of PCa patients and their respective controls. We also examined the association of Cd, Zn and mtDNA copy number with other markers of prostate cancer such as Gleason score and serum PSA.

Subjects and methods

Ethical consideration and sample collection

This study was approved by the Research Ethics Committee (775/R-Cell-12) of King George's Medical University (Lucknow, India).

Samples were collected after obtaining a written informed consent from each subject (cases and controls) prior to their inclusion in this study. A total of 102 patients with histopathologically confirmed prostate cancer cases were recruited between January 2012 to January 2015 at Department of Urology, King George's Medical University, Lucknow. Benign prostatic hyperplasia (BPH) ($n = 107$) patients were recruited as control during the same time frame in this study and were defined as men with no evidence of PCa or any other malignancy. All were interviewed to collect information including age, alcohol consumption, smoking/tobacco chewing, and family history of cancer. After proper consent, 5 ml venous blood was collected and stored as per protocol.

Metal analysis

Blood Cd and Zn were measured using inductively coupled plasma spectrometer (Thermo Electron; Model IRIS Intrepid II XDL, USA) in the acid digested samples (nitric acid:perchloric acid, v/v). One ml blood samples were digested with digestion mixture (nitric acid:perchloric acid, v/v) according to the method of Siddiqui et al. [8].

DNA extraction and analyses of mtDNA copy number using Real-time quantitative PCR

The genomic DNA is isolated by conventional phenol chloroform extraction method followed by ethanol precipitation and re-suspended in Tris-EDTA buffer and stored at −20 °C for further use. mtDNA copy number was determined as described previously [9]. In brief, *ND1* region (108 bp) of mtDNA was amplified by using forward primer: 5′-TGACCCTTGGCCATAATATGATTT-3′; reverse primer: 5′-TTCGATGTTGAAGCCTGAGACTAG-3′ (Invitrogen, Cat#4304970) and Dual-labeled probe (6FAM-5′-AGACCAACCGAACCCCCTTCGACC-3′-Taq-Man TAMRA) (Invitrogen Cat#450025). Primers for β-globin gene (106 bp) was used as described previously and the sequences are as follows: forward primer: 5′-GTGAAGGCTCATGGCAAGAAAG-3′ and reverse primer: 5′-TGTCACAGTGCAGCTCACTCAGT-3′. Reactions were run in duplicate manner on ABIPRISM 7700 Sequence Detector (Applied [Biosystem], Forest City, USA). The reaction consisted of: 1x Premix Ex Taq (TaKaRa), 2.5 μl DNA sample, 200 nM each primers, 250 nM dual-labeled probe. The cycling condition was 95 °C for 10 s, followed by 40 cycles at 95 °C for 5 s and 60 °C for 30 s. Recombinant plasmid containing mtDNA fragment in *NDI* gene coding region was used as standard DNA.

Table 1 Principal clinicopathological characteristics in PCa and BPH controls patients.

Variables	Prostate cancer ($n = 102$)	Control ($n = 107$)	OR (95% CI)	p value
Age (%)				
≤70	72	76	0.86 (0.46–1.6)	0.63
>70	30	31		
Smoking History/tobacco chewing				
No	11	33	3.68 (1.7–7.7)	
Yes	91	74		0.001*
Alcohol consumption				
No	16	26	1.72 (0.86–3.44)	
Yes	86	81		0.12
Family history (%)				
No	97	–		
Yes	5	–		
Dietary habit				
Vegetarian	67	79	0.67 (0.37–1.22)	
Non-vegetarian	35	28		0.198
Occupation				
Farmer	82	60	3.21 (1.7–5.9)	
Others	20	47		0.002*
PSA (ng/ml)				
<4	5	82		
>4–10	20	15		
>10–20	39	7		
>20	38	2		0.001*

OR: odds ratio; CI: confidence interval; p: probability.
* $p < 0.05$.

Statistical analysis

Odds ratio (OR), confidence interval 95% (CI 95%) and Chi square test was calculated for demographic variable. Spearman rank correlation analysis was applied in studying the relationship between metals, mtDNA and clinicopathological parameters. One way ANOVA was perform for Cd and Zn for multiple comparisons with different Gleason score in PCa patients. One way analysis was performed for mtDNA copy number in the different groups of PSA and Gleason score. All statistical calculations were carried out using the statistical package for the social science, version 16.0 (SPSS Inc. Chicago, IL, USA) and 3D curve were plotted between the mtDNA copy number against Cd and Zn using software Statistica (Statistica AGE, version 12.0, USA). Difference were considered significant when $p < 0.05$.

Results

The patient's demographic information is demonstrated in Table 1. There were no statistically significant differences between the PCa patients and BPH controls in terms of age (OR, 95% CI: 0.86 (0.46–1.6), p: 0.63); alcohol consumption (OR, 95% CI: 1.72 (0.86–3.44), p: 0.12) and dietary habit (OR, 95% CI: 0.67 (0.37–1.22), p: 0.19). A significant difference was observed in addiction habits such as smoking/tobacco chewing (p: 0.001) and clinical parameter of serum PSA (p: 0.001) in PCa compare to BPH control.

The Cd level (mean ± SD μg/L) in BPH control was 2.92 ± 1.23 whereas in PCa group was 3.89 ± 1.49 (Fig. 1). Meanwhile Zn level was low (85 ± 10.4) in PCa as compared to BPH (116 ± 19.1) and the differences was found statistically significant (p: 0.001) (Fig. 2). No correlation was found between metals (Cd and Zn) and serum PSA in case of PCa and BPH (Table 2). Low mean value of Cd was found in low and intermediate risk patients (Gleason score: 5,

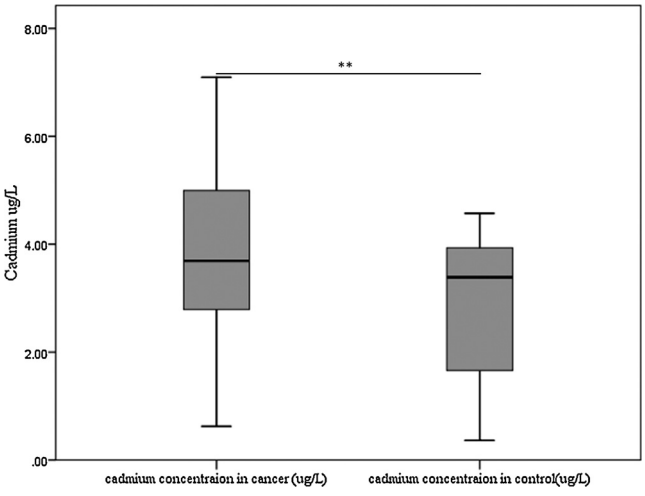

Figure 1 Bar diagram indicating the mean ± SD values of cadmium in PCa and BPH controls. Line and asterisks indicate statistically significant difference (**$p < 0.05$).

Table 2 Correlation of cadmium and zinc with serum PSA and Gleason score in PCa and BPH Control.

	PCa		Control	
	R	p	R	p
Cd with serum PSA	0.00	0.969	−0.16	0.105
Zn with serum PSA	−0.10	0.292	0.12	0.232
Cd with Gleason score	0.64	0.001*		
Zn with Gleason score	−0.68	0.001*		

R: correlation; p: probability.
* $p < 0.05$.

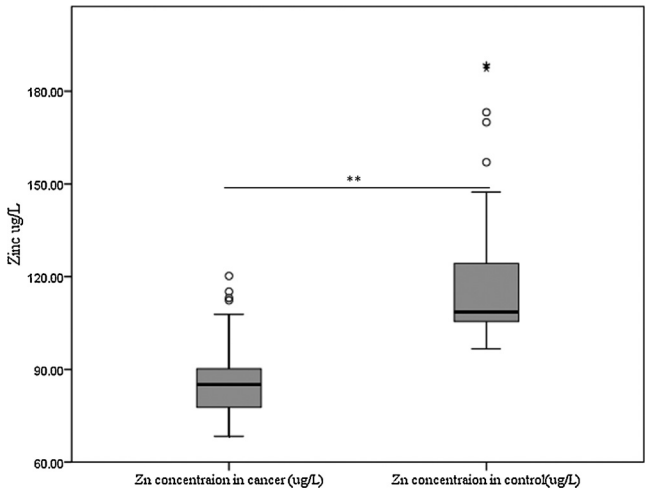

Figure 2 Bar diagram indicating the mean ± SD values of zinc in PCa and BPH controls. Line and asterisks indicate statistically significant difference (**$p < 0.05$).

Table 3 Comparison of zinc and cadmium level in PCa between different levels of Gleason score.

	Cadmium (μg/L) *N*, Mean, median ± SD	Zinc (μg/L) Mean, median ± SD
Gleason score 5	4, 1.78, 2.04 ± 0.78	92.21, 91.09 ± 8.72
Gleason score 6	15, 1.86, 1.89 ± 0.61	96.82, 92.22 ± 10.51
Gleason score 7	45, 2.97, 2.89 ± 0.95	87.61, 86.66 ± 7.42
Gleason score 8	15, 4.28, 4.55± 0.96	79.8, 78.56 ± 6.25
Gleason score 9	23, 4.98, 5.22 ± 0.91	74.68, 73 ± 6
	F: 38.86; *p*: 0.001*	*F*: 22.83; *p*: 0.001*

* $p < 0.05$.

6 and 7) compare to high risk patients (Gleason score: 8 and 9) while high mean value of Zn was seen in low risk patients and vice versa (Table 3). Cd level positively correlated with Gleason score (*r*: 0.64) and Zn level negatively correlated with Gleason score (*r*: −0.68) ($p < 0.05$). Comparison amongst metals (Cd and Zn) and different Gleason scores are shown in Table 3.

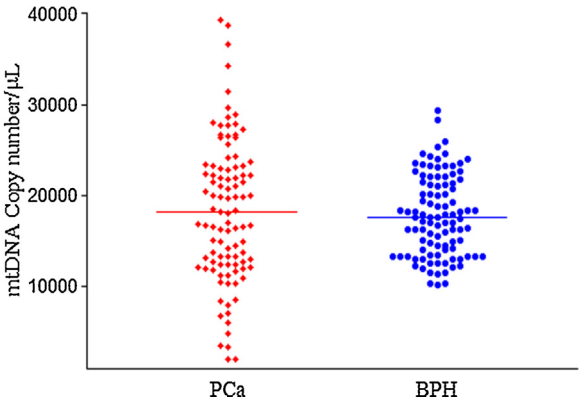

Figure 3 Distribution of mtDNA copy number in PCa and BPH patients.

For the study of distribution pattern of mtDNA, we [compared] the PCa group with their age matched BPH control. mtDNA copy number/μL in BPH group [range (fold difference)] was 10,178–33,224 (3.9) whereas in PCa group mtDNA was 968–39,245 (17.4); this difference was statistically significant (*p*: 0.036) (Fig. 3). There was no correlation between mtDNA copy number and serum PSA in PCa patients and their age match BPH control (*F*: 1.874; *r*: 0.14; *p*: 0.139) (Table 4). PCa Patients with higher [Gleason] score (8–9) were more likely to have higher mtDNA copy number than those with lower [Gleason] score (5–7) (*F*: 81.87; *r*: 0.849; *p*: 0.0001) (Table 3). Patients with a Gleason score of 4 + 3 (7) had higher mtDNA copy number compared to those with lower score (3 + 4: 7) (Fig. 4). Finally, correlation between mtDNA, Cd, and Zn levels are documented in Fig. 5.

Discussion

In this study, 88% PCa and 59% BPH control patients were farmers. Cadmium was detected in almost all samples and their concentration was significantly higher in PCa compare to BPH control group. Cd

Table 4 Comparison of mtDNA copy number with PSA and Gleason score.

Variables	PCa Number (%), mtDNA copy number; median (range)	BPH Control Number (%), mtDNA copy number; median (range)	
Serum PSA			
<4	5; 14,208 (3331–24,214)	78; 17,596 (10,178–28,237)	
>4–10	20; 13,250 (2015–34,247)	12; 17,658 (10,354–29,342)	
>10–20	39; 20,487 (1968–31,353)	7; 19,925 (14,736–23,237)	
>20	36; 16,781 (3471–29,712)	2; 14,883 (12,244–17,522)	*p*: 0.13
Gleason score			
5	4; 3471 (1968–7895)		
6	15; 11,247 (3331–14,987)		
7	45; 15,370 (9436–24,681)		
8	15; 25,658 (21,747–31,353)		
9	21; 26,733 (20,487–34,247)		*p*: 0.0001*

p: probability.
* $p < 0.05$.

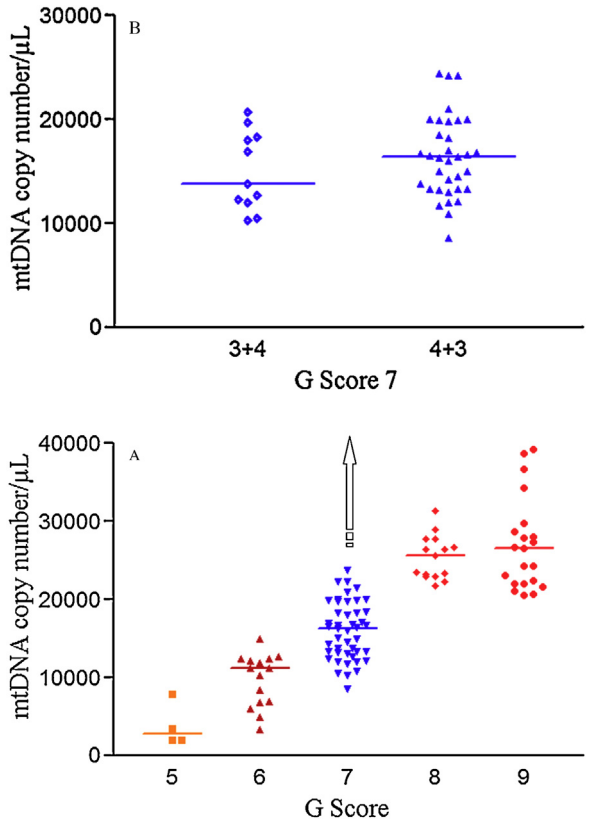

Figure 4 Distribution pattern of mtDNA at different Gleason score in PCa patients (A); Distribution of mtDNA in Gleason score 7 (B).

positively and Zn negatively correlated with [Gleason] score. The high level of Cd might be due to the habit of chewing betel quid and smoking homemade tobacco leaf (Biri) which is a major source of Cd exposure [10]. This unfiltered tobacco chewing is an endemic habit especially in northern part of India.

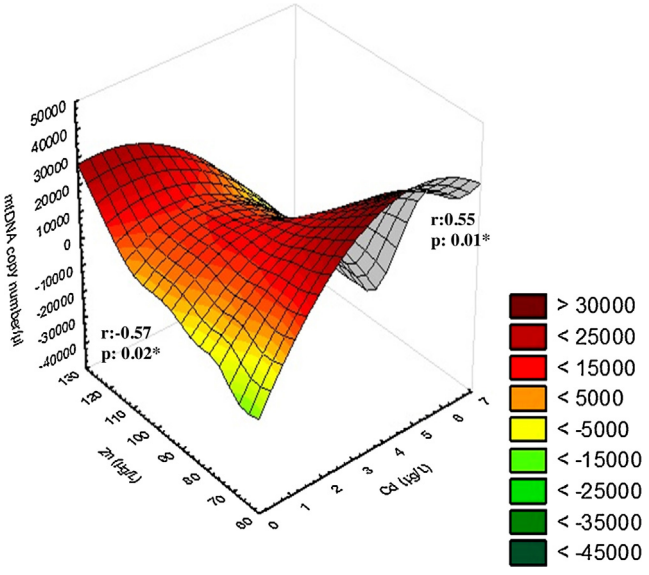

Figure 5 Correlation of mtDNA against cadmium and zinc. *r*: correlation; *p*: probability.

Previously, it was though that in India occurrence of PCa is far less compared to the western world. As a result of rural to urban migration, changing life style, increasing awareness regarding PCa, better access to medical facility; more and more cases of PCa are being discovered. Jain et al. [11] reported that PCa is the second most frequently diagnosed cancer in major cities of India. Almost all regions of India are equally affected by PCa. The incidence rate (after age adjustment) of PCa in big cities of India is 10.66/100,000 [12] which is higher when compared to northern Africa (8.1) and south-east Asia (8.3) but is still lower than United State (85.6) and other countries of Europe (Eastern zone: 29.1 and Southern zone: 50.0) [13,14]. The incidence rate of PCa in India is constantly and rapidly increasing. It has been projected that the number of patients suffering from PCa will double by 2020 [11].

Zinc is very important for the structural integrity of prostate and high zinc levels are characteristic of healthy prostate cell. Zinc influences various molecular repair mechanisms of mitochondria [3] and is involved in the development and normal functioning of the immune system [15]. Cd toxicity has been closely related to Zn homeostasis. Once inside the cells, cadmium displaces zinc from proteins and enzymes where zinc has a sulfur-dominated coordination sphere [16]. Higher concentration of Cd is capable of inhibiting DNA repair and leading to genomic instability, which may be associated with various types of malignancy [17]. The deleterious effects of Cd are associated with either acute exposure to high levels of the element or chronic exposure to low level of metal. In our study, no correlation was found between metals (Cd and Zn) and serum PSA in PCa as well as BPH control group. This study suggests that rise of Cd level and depletion is of zinc level correlates with increasing Gleason score and is more pronounced with aggressive disease. Our result corroborate with Cortesi et al. [18] who found significant zinc depletion with increasing Gleason grade. Evidence suggests that mitochondrial integrity is affected by Cd and zinc level.

The association between mtDNA copy number variation and malignancy is still subject of debate. Both an increase and decrease in mtDNA copy number have been associated with an increased risk for tumorigenesis. In genomic DNA extracted from blood, an elevated copy number has been observed in patients with various cancers. A prospective study of renal cancer revealed that high mtDNA copy number in blood was allied with increased future risk of renal cell carcinoma [19]. On other hand, decrease in mtDNA copy number was found in patients with stomach cancer [20].

A precise mechanism for increase in the mtDNA copy number in blood has not been fully understood. Possibly, increased mtDNA copy number indicates the increased levels of oxidative damage that has been associated with possible cause of different type of malignancies [21]; it may reflect indirect evidence of carcinogenesis rather than direct cause [9]. Moreover, low mtDNA copy number in cell can be caused by mitochondrial DNA polymerase γ (POLG1) mutation, resulting in tumor initiation and progression [22].

This study revealed that mtDNA copy number is more scattered in PCa patients compare to controls and the difference was statistically significant. This significance indicates that increase in mtDNA copy number in PCa as compare to BPH control. Our results support previous tissue-based study which showed that the average mtDNA content increased in prostate cancer tissue as compared to benign

prostatic tissue [9]. Although we compared blood mtDNA copy number with two indices of PCa (Serum PSA and Gleason score). mtDNA copy number had no correlation with Serum PSA in PCa patients. Gleason score is the most important pathological finding in PCa and main indicator of tumor burden. When we compared mtDNA copy number with different Gleason scores (6–9), a positive correlation was found. PCa patients with higher [Gleason] score were more likely to have higher mtDNA copy number than their with lower [Gleason] score. In prostate cancer, [Gleason] score 7 was split into two category i.e. 4 + 3 and 3 + 4. The increase of [Gleason] score is directly correlated with tumor aggressiveness. Patients with a [Gleason] score of 4 + 3 (7) tended to have slightly but significantly higher mtDNA copy number compare to their lower score (3 + 4: 7) (Fig. 4B). Zhou et al. [23] found significant higher blood mtDNA in advanced stage of PCa as compare to lower stages.

There are few limitations in our study. As with any prospective case control biomarker study, our study does not allow us to determine whether mtDNA copy numbers found in PCa patients was the cause or the result of cancer onset on progression. Moreover, relatively small sample size in this study limited our statistical capacity to detect interactions between metals and mtDNA copy number and other major risk factors. Large prospective studies (with a larger sample size) may confirm our findings.

Conclusion

This result suggests that an increased variation of cadmium level correlates with increasing [Gleason] score and zinc level negatively correlates with increasing [Gleason] score. The Distribution variance pattern of mtDNA copy number also correlates with [Gleason] score in PCa patients compare to BPH control.

Authors' contributions

Protocol/Project development was done by A. Abhishek and V. Singh. Data Collection and management was done by A. Abhishek., V. Singh., R.J. Sinha., N.G. Ansari., and M. Verma. Data Analysis was done by A. Abhishek., V. Singh M. Verma, D. Verma and M. Kumar. Manuscript writing/editing was done by A. Abhishek., V. Singh., R.J. Sinha., N.G. Ansari., M.K.J. Siddiqe., and M. Verma.

Ethical committee approval

Consent from the patient: Informed written consent taken from patients.

Conflict of interest

None declared.

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