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Original Research Article

Diagnosis using Cytogenetic DNA Damage (Comet Assay) and IL-17 Serum in Patients with Prostate Cancer

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Abstract: ***Background***: The roles of chronic inflammation and genomic instability have recently emerged as risk factors in prostate carcinogenesis. In this research, single cell gel electrophoresis (comet assay) parameters such as DNA damage, serum interleukin-17 (IL-17), and immature granulocyte (IG) levels were studied to identify the difference between prostate cancer patients and healthy control groups. ***Method***: DNA damage was determined by alkaline comet assay in peripheral blood lymphocytes of prostate cancer patients and controls. The following variables were assessed as DNA damage parameters: Tail DNA (%), Tail Length, Tail Moment, Olive Tail Moment, and Head DNA (%). Outliers were defined and removed by 1.5× IQR rule. The concentration of IL-17 was determined by ELISA method in patients (n=40) and controls (n=40), ROC analysis was performed to determine the usefulness of the variable. IG counts were determined from the complete blood count analysis. Since comet assay variables had non-normal distribution (Shapiro-Wilk p <0.05), Mann-Whitney U test was used to compare the two groups. ***Results***: In analyses where outliers had been excluded, Tail Length and Tail Moment were found to be significantly increased in patients compared to control subjects (p<0.001 & p=0.008, respectively), as expected due to greater DNA movement. The amount of Tail DNA (%) was found to be significantly decreased in patients (median 1.97%, IQR 0.21-3.59) compared to control subjects (median 3.97%, IQR 2.63-5.35; p<0.001), whereas Head DNA (%) was significantly increased correspondingly. Olive Tail Moment did not show a significant difference (p=0.377). Serum IL-17 concentration was significantly higher in control subjects (411.38 ng/mL) than in patients (204.58 ng/mL, p<0.0001), showing AUC of 0.8532 (95%CI: 0.7585-0.9480), 84% sensitivity and 80% specificity with a cut-off value of 237.6 ng/mL. IG levels were significantly increased in patients (mean 0.4431) compared to control subjects (mean 0.19). ***Conclusion***: Parameters assessed using comet assay and serum IL-17 can be used to distinguish prostate cancer patients from controls. However, in some cases, these differences do not occur in the direction usually expected, indicating the complexity of the relationship between genomic instability, systemic inflammation, and prostate cancer.

Keywords: Prostate Carcinoma, Comet Test, DNA Fragmentation, IL-17, Immature Granulocytes.

INTRODUCTION

One of the most common diagnoses, and also one of the most common causes of cancer-related deaths among men worldwide, is prostate cancer (PCa) [1, 2], however, chronic inflammation is considered as one of the key factors contributing both to the development and progression of prostate cancers [3, 4]. The cytokine, like IL-17, released by the Th17 cells, triggers prostatic inflammation and promotes transition from premalignant lesions into adenocarcinoma [5]. In addition, the signaling pathway mediated by IL-17A causes increased oxidative stress and genotoxicity in epithelial cells [6], thus potentially explaining how inflammation leads to genomic instability. At the same time, the alterations in counts of granulocytes in the peripheral blood, and particularly in immature granulocytes (IG), are the markers of myeloid inflammation [7].

The cytogenetic instability, which can be assessed at the level of a single cell using the comet assay, is a kind of indicator of DNA breaks and their repair, which is critical to the development of cancer [8]. The goals of this study include (1) evaluation of DNA damage in the peripheral lymphocytes of prostate cancer patients versus controls using the comet assay, (2) assessment of the potential of serum IL-17 as a biomarker of the disease, and (3) estimation of immature granulocytes as an alternative inflammatory biomarker.

MATERIALS AND METHODS

Population for the Study

Cases of prostate cancer and disease-free age-matched individuals were chosen for this study. In the comet assay, 38 comets of the patients' group and 172 comets of the control group were tested. In the serum IL-17 and hematological assays, 20 patients and 10 controls were enrolled.

Comet Assay

Single cell gel electrophoresis (alkaline comet) assay was used for the measurement of DNA damage in the peripheral blood lymphocytes. The pictures of comets were captured using fluorescence microscope and the analysis was done with the help of automated comet-scoring system providing six comet's parameters: Comet Length, Head DNA (%), Tail Length, Tail DNA (%), Tail Moment, and Olive Tail Moment following the comet assay nomenclature for human biomonitoring [8].

IL-17 Serum Level and Immature Granulocytes

The level of IL-17 in serum was measured using ELISA method. The IG level was measured by means of the routine CBC test using automated hematology analyzer.

Statistical Analysis

For the comet assay parameters, outlier values for each group were identified and excluded from statistical analysis according to the rule of 1.5× interquartile range (IQR) (values below Q1 – 1.5×IQR and above Q3 + 1.5×IQR). Normal distribution of the remaining data for comet assay parameters was determined by the Shapiro-Wilk test and because the data did not follow a normal distribution, the Mann-Whitney U test was used, and data were presented as mean ± SD and median (interquartile range, IQR). For serum IL-17 parameter, both comparison of means of groups and ROC curve analysis for calculation of the area under the curve (AUC), the cut-off value, the sensitivity, and the specificity was carried out; youden's index calculation (J = Sensitivity + Specificity – 1) was conducted in order to confirm the cut-off value. P-value < 0.05 was regarded as statistically significant.

RESULTS

Comet Assay – DNA Damage Parameters

Apart from the Tail DNA (%) parameter, all other DNA damage parameters analyzed in comet assays were statistically different between patients and control groups after the exclusion of the outliers (1.5×IQR criteria; Table 1). The Tail Length parameter was significantly higher in patients (n = 37; median 13.0 px, IQR 3.0-31.0) than in control subjects (n = 152; median 2.0 px, IQR 0.0-5.0; p < 0.001), and the Tail Moment parameter was significantly higher in patients (n = 32; median 0.193 vs. n = 144; median 0.052 in controls; p = 0.008).

The Tail DNA (%) parameter, considered to be the most sensitive comet assay parameter, was significantly lower in patients (n = 33; median 1.97%, IQR 0.21-3.59) than in control subjects (n = 149; median 3.97%, IQR 2.63-5.35; p < 0.001), and the Head DNA (%) was correspondingly significantly higher in patients. The Olive Tail Moment parameter, being an integrated comet assay parameter based on the Tail Length and %DNA parameters, was not statistically different between the two groups (p = 0.377).

Table 1: Parameters used in comet assay (Tail DNA %, Tail Length, Tail Moment, Olive Tail Moment, and Head DNA %) for prostate cancer patients and controls excluding outliers using 1.5×IQR criteria

Parameter	Control (n≈149–152)	Patients (n≈132–137)	MWU p-value
Tail DNA (%), mean±SD (median, IQR)	4.13±2.16 (3.97, 2.63–5.35)	2.34±2.20 (1.97, 0.21–3.59)	<0.001
Tail Length px, mean±SD (median, IQR)	3.43±4.18 (2.00, 0.00–5.00)	17.51±17.09 (13.00, 3.00–31.00)	<0.001
Tail Moment, mean±SD (median, IQR)	0.128±0.170 (0.052, 0.000–0.195)	0.406±0.486 (0.193, 0.000–0.713)	0.008
Olive Tail Moment, mean±SD (median, IQR)	1.222±0.626 (1.143, 0.835–1.534)	1.798±1.723 (1.325, 0.091–3.108)	0.377

Parameter	Control (n≈149–152)	Patients (n≈132–137)	MWU p-value
Head DNA (%), mean±SD (median, IQR)	95.87±2.16 (96.03, 94.65–97.37)	97.66±2.20 (98.03, 96.41–99.79)	<0.001

Note: n is slightly different for each parameter due to outlier removal using the 1.5×IQR criterion.

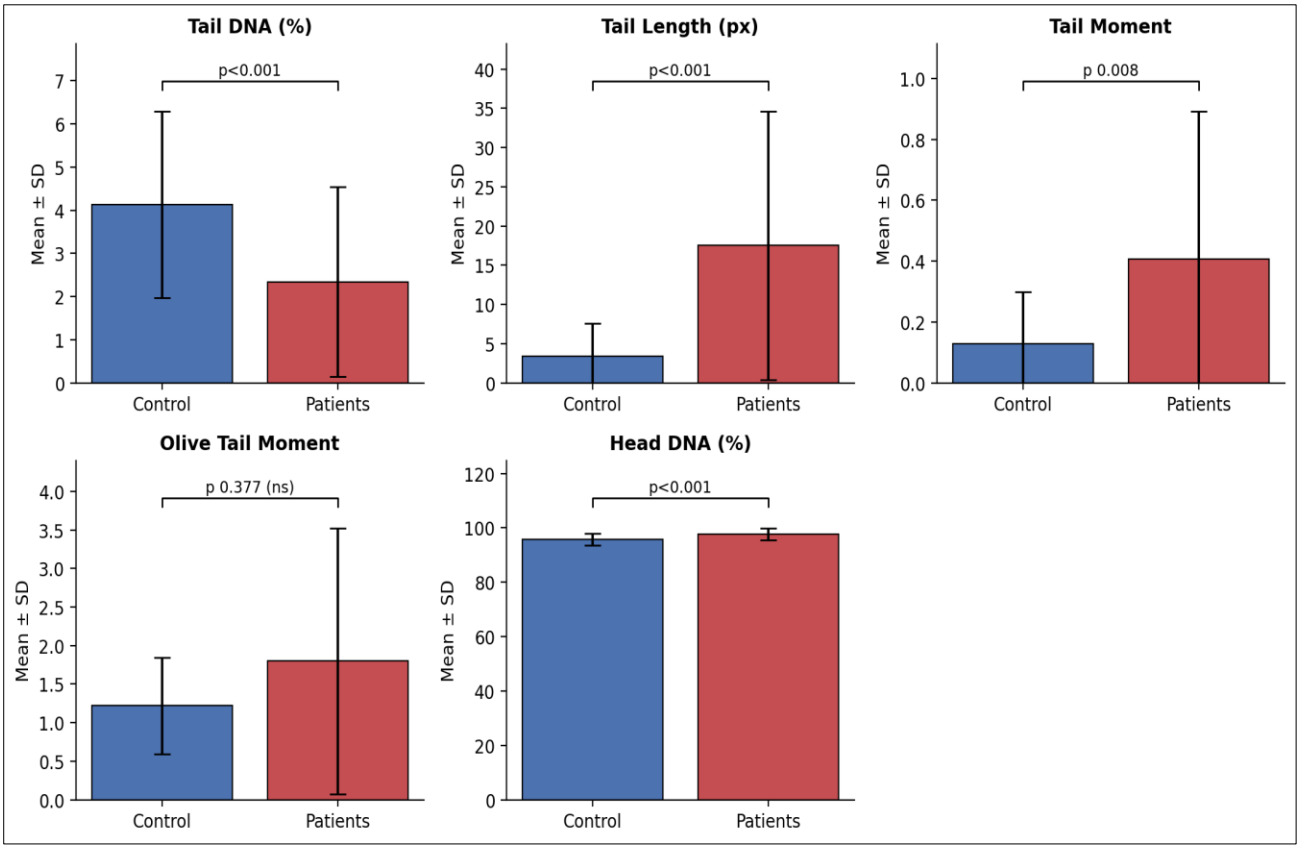

Figure 1: Parameters of comet assay in controls and prostate cancer patients (mean ± SD) following exclusion of outliers (1.5×IQR). P values derived from Mann Whitney U test. (ns=not significant)

Serum IL-17

There were significant differences in serum IL-17 concentrations in the two study groups, where the control group showed a mean concentration ± SD of 411.38 ng/mL and the patient group of 204.58 ng/mL ($p < 0.0001$). Using the ROC curve, the AUC was calculated to be 0.8532 (95% CI: 0.7585 – 0.9480), and the sensitivity and specificity values were 84% and 80%, respectively, using the optimal cut-off point of 237.6 ng/mL ($p < 0.0001$). The corresponding Youden's index ($J = 0.64$) confirms this cut-off as the optimal balance point on the ROC curve.

Table 2: IL-17 concentrations in serum and performance indices in diagnosing prostate cancer patients vs healthy subjects. Results of ROC curve analysis for AUC, optimum cutoff, sensitivity, specificity, and Youden index

Parameter	Control (n=40)	Patients (n=40)	p-value
Serum IL-17 (Mean ± SD, ng/mL)	411.38	204.58	<0.0001
AUC (95% CI)	—	0.8532 (0.7585–0.9480)	<0.0001
Sensitivity (%)	—	84	—
Specificity (%)	—	80	—
Cut-off value (ng/mL)	—	237.6	—
Youden's Index (J)	—	0.64	—

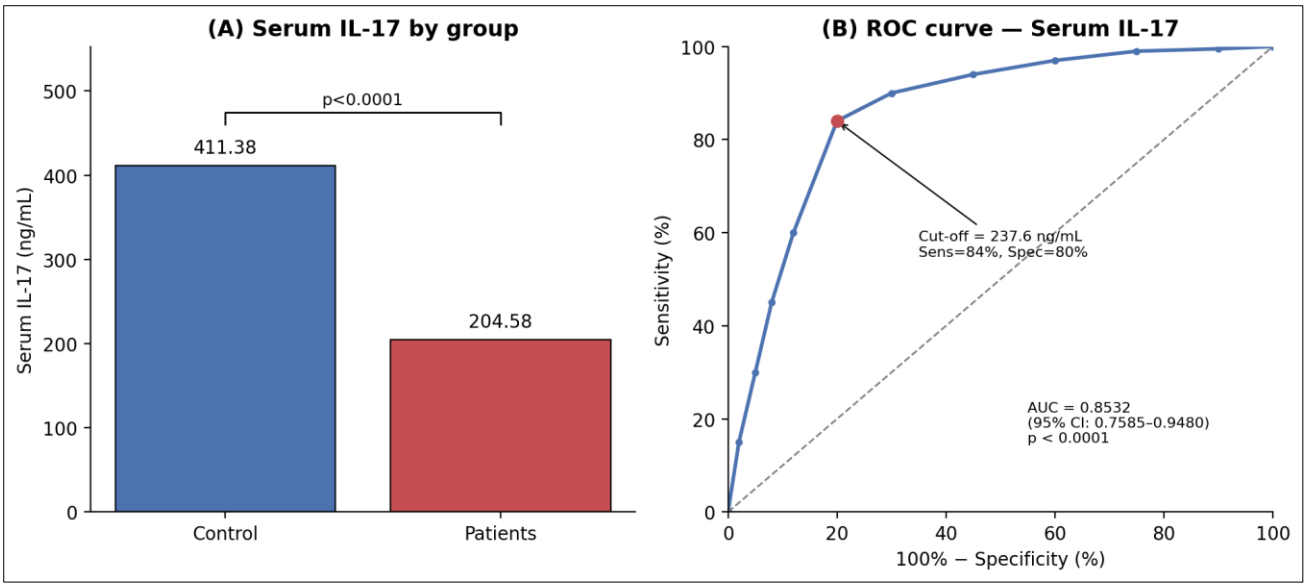

Figure 2: (A) Mean serum IL-17 levels (ng/mL) in controls versus patients. (B) Receiver Operator Characteristics (ROC) curve for serum IL-17 to distinguish between patients and controls (AUC = 0.8532). Shape of the curve is for illustration purposes only since the exact numbers for each subject are not provided.

Immature Granulocytes (IG)

The immature granulocytes showed significantly higher mean values among patients (mean = 0.4431, SD = 0.08258, Range = 0.

Table 3: IG counts in patients with prostate cancer compared to those of healthy individuals. Numbers given as mean ± SD, median, minimum, maximum, and standard error of mean

Parameter	Control (n=40)	Patients (n=40)
IG, Mean ± SD	0.19	0.4431
Median	0.15	0.10
Minimum	0.000	0.000
Maximum	0.600	4.500
Std. Error of Mean	0.05667	0.2230

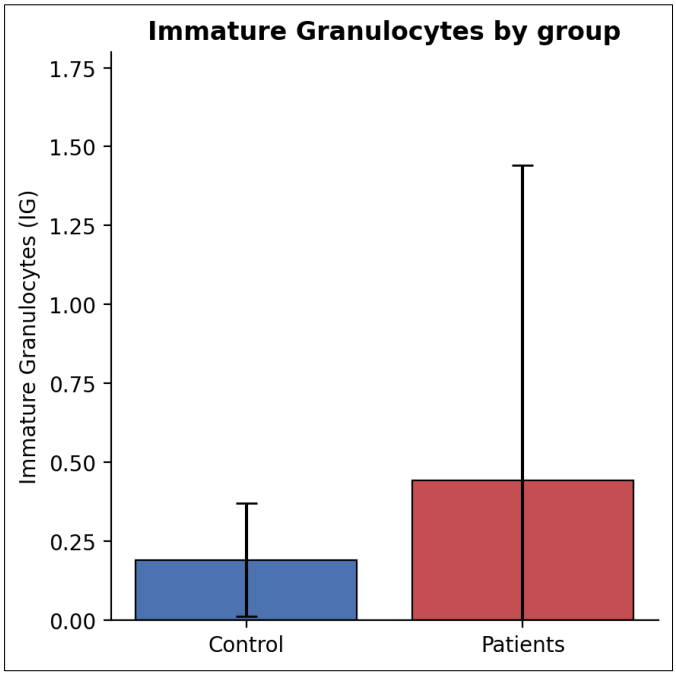

Figure 3: Levels of immature granulocytes (IG) (Mean ± SD) for Controls and Prostate Cancer Patients.

DISCUSSION

Findings by Comet Assay

The results obtained through the use of comet assay technique concerning the genomic instability of patients suffering from prostate cancer are contradictory. The higher values of Tail Length and Tail Moment in cases of patients are consistent with the expectations due to the greater damage and migration of DNA along with the increased level of oxidative stress and diminished DNA repair process in those with tumors [9, 10]. Conversely, the lower values of Tail DNA (%) and increased values of Head DNA (%) found among patients are contradictory to the expected values of Tail DNA (%) due to the presence of the disease [8].

Discrepancies observed between tail length/moment and %DNA in the tail can be explained by the following factors, none of which is mutually exclusive. Firstly, comet parameters can differ in their sensitivity to certain DNA damage: whereas the parameter of tail length is sensitive to low molecular weight DNA fragments and high mobility of the fragments, %DNA in the tail refers to the percentage of the genome that is displaced, and its value is likely to be lower because of repair of DNA damage or the preference for less damaged cells [11]. Secondly, technical factors such as the number of scored cells per group (38 in the group of patients vs. 172 in the control group), electrophoresis conditions or cell selection criteria can have an effect on tail parameters only. Finally, it is possible that patients enrolled in the research were undergoing some treatment at the moment of blood collection [12].

The absence of a statistically meaningful difference in Olive Tail Moment, because mathematically it combines both tail length and % DNA, may suggest that in this case these two factors showed opposite trends and hence neutralized each other. Overall, the results obtained from the comet assay should be regarded with caution and further study needs to be done using an equal number of cells in each group and stratification by treatment, with an additional genotoxicity assay like the micronucleus test used for confirmation.

Serum IL-17

It is also important to mention that the finding of increased levels of IL-17 in the serum in the control group, compared to prostate cancer patients, is also a remarkable one, since the IL-17 level is usually associated with cancer patients, being a pro-inflammatory factor and a tumor-promoter through Th17 cells activation [5-13]. However, cytokine levels seem to differ depending on the type of tumor: in patients with colorectal cancer, IL-17A was found elevated in patients' serum, whereas IL-33 was decreased compared to controls [14]. The findings mentioned above make it reasonable to suggest that decreased IL-17 levels in the current study may be related to the specific microenvironment of prostate cancer (e.g., androgen deprivation effect).

Biologically, IL-17 is produced mainly by Th17 cells and acts in chronic inflammation pathways that have been linked to prostatitis, leading to prostate cancer formation [16]. In this regard, IL-17 activation involves NF-κB pathway with activation of IL-6, IL-8, and IL-1 cascades in the prostatic tissue [17], normally providing anti-tumorigenic immune responses. Nevertheless, IL-17 has been shown to exhibit both pro-tumor actions, such as angiogenesis and immune escape, and tumor-suppressing actions by recruiting cytotoxic immune cells [5]. Such dual effects can possibly be attributed to reduced IL-17 levels found in patients in this work, either due to IL-17 trapping into tumor microenvironment, systemic immune exhaustion, or shifting toward a more suppressive (Treg-dominated) cytokine environment [13,15]. Regardless of its nature, highly discriminatory power (AUC = 0.8532, sensitivity 84%, specificity 80%) makes IL-17 a valuable candidate as a biomarker for prostate cancer.

Immature Granulocytes

The elevated level of IG in prostate cancer patients suggests a systemic inflammatory and myeloid skewing of hematopoietic response, a phenomenon frequently reported in various cancers and inflammatory conditions as a hallmark of tumor associated inflammation, granulocyte emergencies or early MDSC mobilization [18]. IG has already proved high discriminatory ability for systemic inflammation in different clinical settings like sepsis with high sensitivity and specificity [7]. Unlike IL-17 that exhibited an inverse trend in this patient group, IG has a positive trend and supports its candidacy as an inexpensive and routinely available biomarker to complement IL-17 and other specific cytokine based biomarkers. IG is not specific for malignant conditions but can be elevated in various infections and inflammatory conditions, therefore its utility as a diagnostic marker in this study needs to be taken as supplementary rather than diagnostic.

Limitations

There are several important limitations inherent to this investigation. These include the disparate number of comets scored per group (38 patients vs. 172 controls after outliers removal), as well as insufficient sample size for IL-17 and IG measurements at subject level (20 patients, 10 controls). There was no incorporation of the clinical variables such as tumor stage, Gleason score, previous treatment, and medical history into this analysis, which are known to have an impact on both cytokines levels and DNA damage biomarkers. Moreover, the absence of such physiological and clinical risk factors as age, body mass index (BMI), blood pressure, smoking status, serum prostate specific antigen (PSA), Gleason

score, testosterone, and physical activity, which are known to contribute to the risk and progression of prostate cancer and affect the inflammation tone and DNA damage, is a significant limitation. This is due to the fact that they might be responsible for some of the differences observed between patient and control cohorts, therefore limiting our ability to attribute the difference in IL-17 levels and DNA damage to the prostate cancer status only. The opposite directionality seen for the Tail DNA (%) and IL-17 levels points to this necessity.

CONCLUSION

In this study, comet assay parameters as well as serum IL-17 level proved to be effective indicators to distinguish patients with prostate cancer from healthy subjects, the latter proving to have high diagnostic accuracy (AUC = 0.8532). But at the same time, there were some parameters that contradicted the standard "the higher the value, the worse it is for the patient" hypothesis: these include, among others, Tail DNA (%), serum IL-17, Tail Length, Tail Moment, and immature granulocytes. These results suggest a more complicated relation between genomic instability and systemic inflammation in prostate cancer development than commonly believed. Further research is needed on the topic using a larger sample size and clinically well-characterized population. A future study should use an extensive list of physiological and clinical variables, which includes body mass index, blood pressure, smoking status, prostate specific antigen, Gleason score, testosterone, and physical activity.

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