Diagnostic Study Of Bladder Cancer Using Urine Methylation Biomarkers Of Iraqi Patients

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KEYWORDS
Bladder Tumors, Epigenetics, DNA Hypermethylation

ABSTRACT
Background: Bladder cancer (BC) is the 4th most frequently occurring malignancy and the 9th most common cause of death worldwide in men. The invasive and metastatic form of the cancer is the main cause of death or unfavorable prognosis for BC patients. The presence of a bladder tumor is often discovered after episodes of painless macroscopic hematuria. At initial diagnosis, the disease is non-muscle-invasive in approximately 75% of patients. In recent years, detecting aberrantly methylated DNA in urine has emerged as a promising and noninvasive approach to aid BC diagnosis. Objective: To detect bladder tumors in Iraqi patients. Methods: In this cross-sectional study, sixty cases were suspected of having bladder tumors according to the opinion of the physician and some signs and symptoms, the best sign was hematuria in urine (per diagnosis). An additional 30 healthy controls with similar age and sex were recruited as control groups, with an average age of 37_85 years. from Baghdad Medical City / Ghazi al–Hariri Teaching Hospital and AlKindi Specialized Hospital during the period from September / 2023 to April / 2024. Using molecular polymerase chain reaction (high-resolution melting-PCR). Results: Out of 60 bladder inflammations, the result of hypermethylation genes ZNF154, EOMES, and POU4F2, Sensitivity were (71.7, 85, and 75) %, and Specificity (60, 33.3, and 50) % respectively with high significant differences ($p < 0.01$). In addition. Conclusions: Detecting DNA hypermethylation is considered a good diagnosis method for bladder tumors.

1. Introduction

Bladder cancer (BC) is the 4th most frequently occurring malignancy and the 9th most common cause of death worldwide in men (Sung et al., 2021). The invasive and metastatic form of cancer is the main cause of death or unfavorable prognosis for BC patients (Chen et al., 2015). The presence of a bladder tumor is often discovered after episodes of painless macroscopic hematuria. At initial diagnosis, the disease is non-muscle-invasive in approximately 75% of patients. Since non-muscle invasive bladder cancer (NMIBC) tends to recur or progress to muscle-invasive disease, regular and long-term cystoscope evaluations are mandatory (Degerge et al., 2017). Yet, cystoscopy has important disadvantages as it is an invasive procedure that is associated with high costs (Svatek et al., 2014). In recent years, detection of aberrantly methylated DNA in urine has emerged as a promising and noninvasive approach to aid BC diagnosis. Hypermethylation in specific genomic regions has been well characterized for bladder tumors versus normal epithelia (Xylinas et al., 2014).

2. Methodology

Study design and setting

This is a face-to-face interview-based cross-sectional study conducted in Baghdad, Iraq, from September / 2023 to April / 2024. Sixty bladder tumor patients were recruited for the study.

Inclusion and exclusion criteria

The study included sixty cases suspected of having bladder tumors according to the opinion of the physician and some signs and symptoms, in Baghdad, Iraq. Any patient with Renal failure and cancer, also every patient who has pro–diagnosis bladder tumors and prostate cancer has been excluded from our study.

Data collection and outcome measurements

The interview was conducted by the same researcher among participants, with clarification of any question that seemed unclear to make the answer more accurate.

Ethical consideration

The Medical Research Ethics Committee at the College of Health and Medical Technology obtained ethical approval. We solely used the data for this study. We obtained written informed consent from the participants.

Statistical analysis

All data were analyzed using SPSS (Ver.28) (IBM) program and GraphPad Prism (Ver. 8). The statistical analyses were performed using analysis of variance (ANOVA) and independent t-test as appropriate by calculating least significant difference (LSD) to obtain a p-value. Data were presented as Mean ± S.E. and a p-value $p<0.05$ was considered significant.

3. Results And Discussion

Sixty cases were suspected of having bladder tumors according to the opinion of the physician and some signs and symptoms, the best sign was hematuria in urine (per-diagnosis). An additional 30 healthy controls with similar age and sex were recruited as control groups. **Table and Figure (1)** study the comparison between the patients & control health according to age, sex, and smoking. Show results of both parameters (gender & age) were non-significant (P> 0.05), which agrees with the plain of our paper which studies the age and gender similarities between control and patients to get high accuracy in the results, but in the smoking table & figure give also non-significant (P = 0.729) that come back for most Iraqi peoples are smokers, this agrees with (Jasim Mohammed *et al.*, 2016). But when compare between the age & gender of patients shows there differ between age groups the most affected (30 – 50) were (38.3%), while (51 – 70) were (36.7%) & (71 – 90) were (25%), the results clear most patients (75%) were between the age of (30 – 70), and the disease is four times more common in men than women the percentage were (81.7%) & (18.3%) respectively, this significant gender difference could point to a variety of underlying factors, including genetic predispositions, lifestyle differences, occupational exposures, or differences in health behaviors between men and women. Also, smoker status where the strongest risk factor for bladder cancer is tobacco smoking, which accounts for (76.7%) of all cases, this high occurrence recommends a strong correlation between smoking and the condition being studied. Smoking is a significant risk factor, contributing to both the development and progression of the disease. That agrees with many world studies (van Hoogstraten *et al.*, 2023, Saginala *et al.*, 2020, Lenis *et al.*, 2020, Catto *et al.*, 2021).

Table (1): Distribution of demographics & smoking according to studied groups.

Parameters			Studied groups		P – Value
			Control N = 30	Patient N = 60	
Age groups / Year	30 – 50	N	13	23	P = 0.441 NS
		%	43.3%	38.3%	
	51 – 70	N	13	22	
		%	43.3%	36.7%	
	71 – 90	N	4	15	
		%	13.3%	25%	
Sex	Male	N	24	49	P = 0.849 NS
		%	80%	81.7%	
	Female	N	6	11	
		%	20%	18.3%	
Smoking	Smoker	N	22	46	P = 0.729 NS
		%	73.3%	76.7%	
	Non-smoker	N	8	14	
		%	26.7%	23.3%	

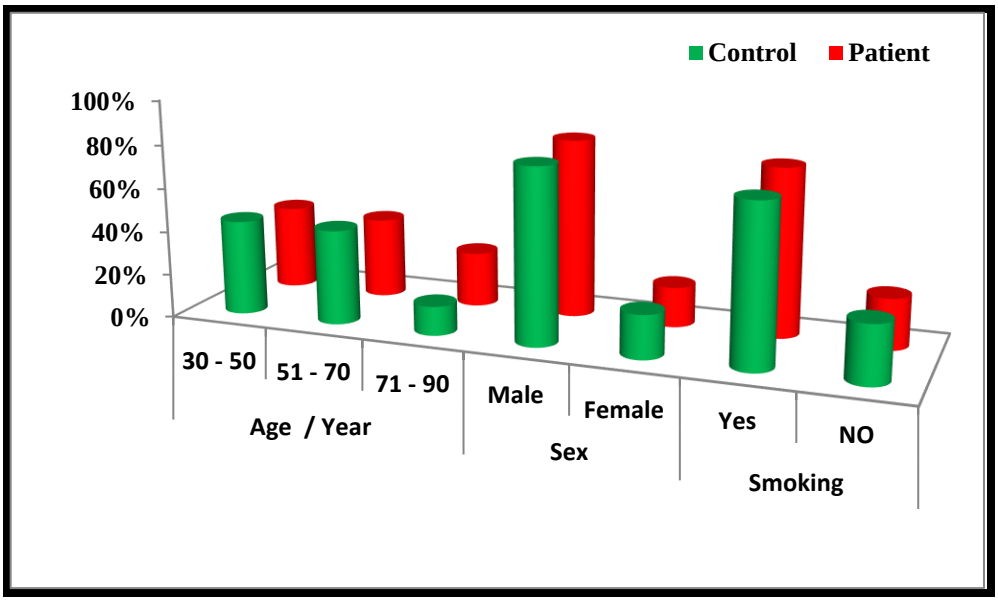

Table (2) revealed significant and highly significant differences between the control and patient groups for several parameters, including chronic bladder inflammation, pioglitazone use in diabetes mellitus, Lynch syndrome, and other genetic syndromes. Chemical substances may affect the infection or alter genetics expression when direct exposure, Certain industries, such as dye, rubber, leather, textiles, and paint, involve exposure to carcinogenic chemicals like aromatic amines, which are linked to an increased risk of bladder cancer, but our study revealed non- significant differ between controls and patients. While the percentage of individuals exposed to chemicals in the patient group (13.3%) is higher compared to the control group (6.7%), the p-value (0.343) shows that the difference is not statistically significant. This suggests that most of the selected samples were not exposed to chemicals, or the sample size was too small to show a significant change. Chronic bladder problems include a wide range of conditions that affect bladder function & health of the urinary system, Interstitial Cystitis, Overactive Bladder, Chronic Bacterial Cystitis, Radiation Cystitis, Urinary Incontinence, Chemical Cystitis & Neurogenic Bladder. The percentage between patients and controls shows a significant difference (P = 0.017), with prevalence in control groups at 33.3% & patient groups at 60% indicating that chronic bladder problems are a risk factor or a consequence of the condition being studied in the patient group. This agrees with other studies (Tan *et al.*, 2020, Lobo *et al.*, 2022, Jubber *et al.*, 2023). Pioglitazone (Actos) is used D.M., Actos is an oral diabetes medicine that helps control blood sugar levels. The use of Pioglitazone has been associated with an increased risk of bladder cancer in some studies, leading to regulatory warnings and recommendations for careful consideration of the drug's risks and benefits. The findings in the current study, showing a highly significant difference in Pioglitazone use in the control was 13.3% while in the patient group 65% with P-Value 0.0006, align with these concerns and suggest that further investigation is needed to fully understand the relationship between Pioglitazone and bladder cancer. Patients prescribed Pioglitazone should be monitored for signs of bladder cancer, such as blood in the urine or urinary symptoms, and should discuss the potential risks and benefits with their healthcare provider. These results agree with studies in other countries (Gangopadhyay and Singh, 2023, Xu *et al.*, 2022, Grunberger, 2013, Yki-Järvinen, 2005). Table & figure describe the Lynch syndrome and other genetic syndromes between controls & patients (P = 0.048) with a statistically significant difference, results show the Lynch syndrome was more common among the patient population considered a risk factor for the bladder cancer, Lynch Syndrome, also known as hereditary nonpolyposis colorectal cancer (HNPCC), is a genetic disorder that increases the risk of several types of cancer, particularly colorectal cancer, endometrial cancer, and cancers of the urinary tract, stomach, small intestine, liver, brain, and skin. It is caused by inherited mutations in mismatch repair (MMR) genes, which normally help repair DNA replication errors.

The results of this study are consistent with the findings of other researchers (Dominguez-Valentin *et al.*, 2020) this extensive study involved over 6,000 Lynch Syndrome carriers and confirmed an increased risk of various cancers, including bladder cancer. The study reported that carriers of MSH2 mutations had a higher risk of developing bladder cancer compared to the general population, which aligns with the findings of the current study.

Table (2): Distribution assays among studied groups.

Parameters			Studied groups		P – Value
			Control N = 30	Patient N = 60	
Chemicals	Yes	N	2	8	P = 0.343 NS
		%	6.7%	13.3%	
	NO	N	28	52	
		%	93.3%	86.7%	
Chronic Bladder Problems	Yes	N	10	36	P = 0.017 S
		%	33.3%	60%	
	NO	N	20	24	
		%	66.7%	40%	
Pioglitazone (Actos) use D.M	Yes	N	4	39	P = 0.0006 HS
		%	13.3%	65%	
	NO	N	26	21	
		%	86.7%	35%	
Lynch syndrome and other genetic syndromes	Yes	N	1	11	P = 0.048 S
		%	3.3%	18.3%	
	NO	N	29	49	
		%	96.7%	81.7%	

Table (3) provides various diagnostic tests PCR (ZNF154, EOMES, and POU4F2), using several metrics:

PCR / ZNF154, the sensitivity of 71.7% indicates that PCR / ZNF154 can correctly identify 71.7% of true positive bladder cancer cases. This makes it a reasonably reliable method for detecting the presence of the disease with a specificity of 60%, making it identify 60% of true negative cases. This suggests that there is a considerable rate of false positives, but it is better than some other methods, Positive Predictive Value (78.2%) means that when PCR / ZNF154 gives a positive result, there is a 78.2% chance that the patient actually has bladder cancer. This is a strong predictive value, showing reliability in positive cases, the accuracy 67.78% reflects the overall effectiveness of PCR / ZNF154 in correctly diagnosing both positive and negative cases and an AUC of 0.763 suggests that PCR / ZNF154 has good overall test performance, with a higher AUC indicating better diagnostic ability.

PCR / EOMES, the sensitivity of 85% shows that PCR / EOMES can correctly identify 85% of true positive cases, making it one of the most sensitive methods for detecting bladder cancer, while; specificity is 33.3% means that the test correctly identifies only 33.3% of true negative cases, indicating a high rate of false positives, the PPV of 71.8% means that when the test is positive, there is a 71.8% chance that the patient has bladder cancer. This value is relatively high, showing good reliability for positive diagnoses. other shows accuracy 66.67% reflects the overall effectiveness of PCR / EOMES in correctly diagnosing bladder cancer. This is relatively high, indicating its utility as a diagnostic tool, and an AUC of 0.716 suggests good overall test performance, with a higher AUC indicating better diagnostic ability.

The last test PCR / POU4F2 shows a sensitivity 75% indicating that the test can correctly identify 75% of true positive bladder cancer cases. This makes it a fairly reliable method for detecting the presence of the disease, with a specificity 50%, showing half of the true negative cases. This indicates a moderate rate of false positives, A positive Predictive Value (75%) means that when PCR / POU4F2 gives a positive result, there is a 75% chance that the patient actually has bladder cancer. This is a good predictive value, showing reliability in positive cases, the Accuracy of the test was 67.1% reflecting the overall effectiveness of PCR / POU4F2 in correctly diagnosing both positive and negative cases. It is relatively high, making it a useful diagnostic tool, and an AUC of 0.754 suggests that PCR / POU4F2 has good overall test performance, with a higher AUC indicating better diagnostic ability. P-values of the cystoscopy, ELISA / BTA, PCR / ZNF154, PCR / EOMES, and PCR / POU4F2 have highly significant p-values (HS), indicating statistically significant results, while cytology and ELISA / NMP-22 have non-significant p-values (NS), indicating their results are not statistically significant.

PCR-based methods targeting ZNF154, EOMES, and POU4F2 genes show promise in bladder cancer diagnostics. These tests offer good sensitivity and overall performance, as indicated by their AUC values and statistically significant p-values. However, they often need to be used in conjunction with other diagnostic methods, such as cystoscopy and cytology, to improve specificity and reduce false positives. Recent studies support the integration of these molecular diagnostics into comprehensive bladder cancer detection and monitoring strategies. that align with the findings for the PCR-based methods targeting ZNF154, EOMES, and POU4F2 in bladder cancer diagnostics (Silva-Ferreira et al., 2024, Koukourikis et al., 2023, Harsanyi et al., 2022), These studies found a significant correlation between ZNF154, EOMES, and POU4F2 methylation levels and the presence of bladder cancer, supporting its use as a diagnostic marker. and agree with other studies (Papavasiliou et al., 2023), This paper discussed the high sensitivity of EOMES in bladder cancer detection and its potential integration into diagnostic panels, and the study by (Chin et al., 2023) discussed the sensitivity and specificity of POU4F2 as a diagnostic tool for bladder cancer.

Table (3): Validity tests of assays by using ROC test according to clinical diagnosis (patients and controls).

Validity test	ELISA / BTA	ELISA / NMP-22	PCR /ZNF154	PCR /EOMES	PCR /POU4F2
Sensitivity	78.3%	41.7%	71.7%	85%	75%
Specificity	56.7%	53.3%	60%	33.3%	50%
Positive predictive value (PPV)	78.3%	64.1%	78.2%	71.8%	75%
Negative predictive value (NPV)	56.7%	31.4%	51.4%	52.6%	50%
Accuracy	71.12%	45.56%	67.78%	66.67%	67.1%
Area under the curve (UAC)	0.792	0.442	0.763	0.716	0.754
Cut-off value	0.129	0.183	85	84	83.9
P - value	0.0031 HS	0.373 NS	0.004 HS	0.001 HS	0.006 HS

4. Conclusion

DNA Methylation detection of some genes is highly specific to the diagnosis of bladder tumors if compared with cystoscopy and cytology methods.

Conflict of interests

No conflict of interest was declared by the authors.

Funding source

The authors did not receive any source of funds.

Data sharing statement

Supplementary data can be shared with the corresponding author upon reasonable request.

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