

Detection of Mycobacterium tuberculosis In Urinary Bladder Cancer Tissues By Using Transmission Electron Microscope.

الكشف عن وجود عصيات السل الرئوية باستخدام المجهر الإلكتروني الانتقالي

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الخلاصة

خلفية البحث: سرطان المثانة هو أحد الأمراض السرطانية الإنسانية الأكثر شيوعاً في العراق والعالم. ويعتبر التدخين، الجنس، العمر، والتعرض المهني إلى الأمين العطري من بين عوامل الخطورة الأبرز لهذا المرض، يضاف إلى هذه العوامل عاملاً الالتهاب لفترة طويلة و الإصابات المرضية المزمنة والذات قد يُعبان دور كبير في نشوء سرطان المثانة. في الدراسة الحالية عملنا على عينات نسيج المثانة المحفوظة في الفورمالين لمرضى عراقيين وقد شملت العينات عينات سرطان المثانة وعينات لإصابات مرضية غير سرطانية، أجريته الدراسة الحالية للكشف عن عصيات السل في الإنسان باستعمال صبغة زيهيل- نيلسون وتصوير الشرائح باستخدام مجهر الإلكتروني الانتقالي.

الهدف: الكشف المجهرية عن وجود عصيات السل في عينات سرطان المثانة ومقارنتها بعينات لإصابات مرضية غير سرطانية .
المنهجية: الدراسة الحالية عملت على 75 نموذج من الأنسجة المثبتة بالفورمالين للمثانة خمسون عينة من العينات كانت لمرضى سرطان المثانة وخمس وعشرون عينة مثانة لإصابات غير سرطانية مختلفة جمعت العينات من عدة مختبرات خاصة في محافظة النجف. 84 % من عينات سرطان المثانة كانت المجموعة السائدة فيها من الذكور والفئة العمرية كانت 61-80 سنوات (60 %) لكلا الجنس. حالات انتشار من مستوى الدرجة واطي كانت الأكثر شيوعاً وقد سجلت (64 %). جزء كبير من الدراسة تم إجراؤه في مركز البحوث الطبية في كلية الطب / جامعة أيوا/ الولايات المتحدة الأمريكية. تم التقطيع النسيجي للعينات وتحضير العينات للتصوير الإلكتروني باستخدام تقنية خاصة.

النتائج: أظهرت نتائج فحص شرائح أنسجة المثانة والتي أُطخت بصبغة زيهيل-نيلسون، وجود العديد من عصيات السل الحمضية (أي إف بي) في الحقل الواحد النسبة المئوية الأعلى سجلت بمرضى سرطان المثانة مقارنة بمرضى الحالات الغير سرطانية (76.6 % و 23.4 %) على التوالي. لدراسة نتائج صبغة زيهيل- نيلسون والتأكد منها تم فحص اللطخات باستخدام المجهر الإلكتروني، التصوير اظهر وجود أشكال مختلفة من خلايا العصيات ظهرت في حقول المجهر الإلكتروني وغالبية الخلايا كانت خارج غشاء الخلايا البولية الظهارية.

الاستنتاج: تم الكشف عن وجود نسبة عالية من عصيات السل في سرطان المثانة مقارنة بحالات إصابات المثانة الغير سرطانية.
التوصيات: اعتماد التقنيات الحديثة والبسيطة في التشخيص المجهرية للكشف عن علاقة عصيات السل وتحديد العلاقة بين الإصابة المزمنة بعصيات السل وتأثيرها في استحداث سرطان المثانة قد يساهم في تقليل الإصابة بهذا المرض.

Abstract:

Background: Bladder cancer is one of the most common human cancers in Iraq and the world. While smoking, age sex, and occupational exposure to aromatic amines are the most prominent among the risk factors identified, long term inflammation and chronic infection may largely play some role in urinary bladder cancer (UBC) development. In the present study we worked on urinary bladder cancer and noncancerous Formalin-Fixed Paraffin Embedded (FFPE) Tissue specimens of Iraqi patients, the current study used Acid Fast Stain and Transmission Electron Microscope for detection of *Mycobacterium tuberculosis* in Urinary Bladder tissues.

Objectives: microscopical detection of *Mycobacterium tuberculosis* in urinary bladder cancer.

Methods: The current study used urinary bladder cancer (UBC) formalin-fixed paraffin embedded tissues (FFPET) specimens of Iraqi patients collected from several private histopathology labs in AL-Najaf province, the specimens were 50 specimens of UBC patients and 25 different noncancerous pathological bladder specimens. Slides of urinary bladder FFPE tissues were stained with Ziehl-Neelsen(ZN) stain. Examining of slides were conducted using Transmission Electron Microscope (TEM) imaging was performed special procedure was used for TEM slides preparation.

Results: FFPE tissues were stained with Ziehl-Neelsen(ZN) stain. Examining of slides showed presence of high number of acid fast bacilli (AFB) in one microscopical field. UBC tissue slides showed the highest percentage comparing to noncancerous patients (76.6% and 23.4%) respectively. For further confirmation and study of ZN stain results, Transmission Electron Microscope (TEM) imaging was performed. The results showed presence of different morphological shapes of acid fast bacilli cells appeared in TEM fields and most cells were observed attaching to the urothelial cell membrane.

Conclusions: high percentage of MTBC and MTB were detected among patients with UBC compared to noncancerous cases.

Recommendations: modern techniques should be used for MTBC and MTB detection in laboratories, and further study for the relationship between chronic MTB infection and UBC are required.

Keywords: urinary bladder cancer, *Mycobacterium tuberculosis*, Transmission Electron Microscope.

INTRODUCTION

Urinary Bladder Cancer (UBC) can be divided into different types, including transitional cell carcinoma, squamous cell carcinoma and adenocarcinoma. Bladder cancer was the 9th most common cancer globally in 2008; it was the 7th most common cancer among males and 17th most common malignancy in females ⁽¹⁾.

UBC is the third most common cancer among Iraq patients ⁽²⁾. Risk factors including smoking, male sex and old age are the most common risk factors and accounts for approximately half of all UBCs, Occupational exposure to aromatic amines and polycyclic aromatic hydrocarbons are other important risk factors ⁽³⁾.

However, chronic infections of the human urinary bladder as well as, is an important risk factor in the pathogenesis of bladder cancer ⁽⁴⁾. It has been noticed that preneoplastic lesions of humans and rodents bladder included a variety of types of proliferative cystitis, which are caused by infection ⁽⁵⁾. Studies found that presence of endogenous bacterial infections (cystitis) and some intestinal opportunistic bacterial infection would metabolically activate the bladder procarcinogens ^(6,7,8).

Although, the connection between bacterial infection and UB carcinogenesis is still with some controversy this might be due to absence of clear agreement on the molecular mechanisms by which bacteria might induce carcinogenesis. However, some studies hypothesized that, Bacterial toxins and secondary metabolites formed by chronic bacterial infection might predispose to carcinogenesis in addition, Chronic inflammation resulted from persistent bacterial infections may lead to tissue neoplasia ⁽⁹⁾. The oldest and well known cause of chronic infection is *Mycobacterium tuberculosis* (MTB), during the course of infection within the host, Nitric Oxide Species NOSs is produced by both macrophages and Mycobacterial production of nitrate reductase that can lead to DNA damage and mutations in urothelial cells ⁽¹⁰⁾. The theory by which tuberculosis promote cancer consider that Inflammation and fibrosis are an important factors in carcinogenesis ⁽¹¹⁾.

AIM: Investigation for the potential association of MTBC as risk factors for urinary bladder cancer among Iraqi patients.

MATERIALS AND METHODS

Specimens used in this study were Urinary Bladder (UB) Formalin Fixed Paraffin Embedded (FFPE) tissues. They were totally 75 specimens, 50 specimens were from patients with different stages of Urinary Bladder Carcinoma (UBC) and 25 specimens were for non-cancerous patients (cystitis). The FFPE tissues were mostly transurethral resection tissues embedded in paraffin from 2009-2013. They were collected from AL-Najaf Governorate private Histopathological Laboratories and Al-Sader Teaching Hospital Neoplasm Unit. Patient's Histopathological reports were collected for further information such as patient's history (age, gender, etc. ...), grading, and staging.

Processing of specimens was carried out at the Department of Microbiology, Faculty of Medicine, University of Kufa and laboratories of Al-Sader Teaching Hospital in AL-Najaf Governorate. The processing included melting and recasting of tissue blocks, renumbering and slicing of tissues for slides preparation.

Two slides were prepared, one slide was used for Acid Fast (Ziehl-Neelsen) staining and the other one for Gram staining, Ziehl-Neelsen (ZN) staining results were used to confirm the results of

Mycobacterium molecular detection. Gram staining results were used as a comparative results to ZN staining results. Staining procedures were carried out according to staining kits manufacturer instruction (SYRBIO, Syria, and Syrian Arab Republic).

Slides were stained and examined under light microscope (Olympus Co.) using 100X power lens, slide examination was performed in the Department of Microbiology, Faculty of Medicine, University of Kufa. Examining of slides included recording types of infectious agents depending on bacterial cell morphology.

In the Central Microscopy Research Facility (CMRF), University of Iowa, USA, the FFPE tissues were further processed and sectioned into 4µm thickness using Leica Microtome RM2135. These tissues were laid over a Fisherbrand™ Superfrost™ Plus Microscope Slides, to confirm Histopathological diagnosis slides were stained with Hematoxylin and Eosin stain using DAKO Autostainer Universal Staining System.

Transmission Electron Microscope Imaging.

Two MTB positive cases were chosen to be imaged with Transmission Electron Microscope (TEM). They were named slide 1 and slide 2. Acid Fast positive slides were checked and specific tissue sites were chosen and pointed with water proof pin. A specific procedure for paraffin embedded tissues was used for preparation of our specimens. The procedure was adapted from the Central Microscopy research facility/ Carver College/ University of Iowa, and the procedure is as follows:

Paraffin section with stain for General TEM (Epon)

- 1) Wash off glass coverslip with Xylene
 - 2) Xylene wash 3x5 min
 - 3) Rehydrate slides to water (100%, 95%, 75% and 50%, then water 5min each step)
 - 4) 0.1M Na Cacodylate buffer* 5 min
 - 5) 1% Osmium fixation 30 min
 - 6) 0.1M Na Cacodylate buffer * 3X5 min
 - 7) Distilled water 1 min.
 - 8) 50% Ethanol* 15 min.
 - 9) 75% Ethanol 15 min.
 - 10) 95% Ethanol 30 min.
 - 11) 100% Ethanol (2 changes)** 1 hour
 - 12) Ethanol and Epon (2:1)** 1 hour
 - 13) Ethanol and Epon (1:2)** 1 hour
 - 14) 100% Epon** 2 hours
 - 15) 100% Epon** overnight
 - 16) Embed in fresh Epon with beam capsule and place in 60-70° oven 24-48h
 - 17) Microtomy using Leica EM UC6 Ultramicrotome MZ6.
 - 18) Uranyl and Lead staining.
 - 19) Examination by JEOL 1230
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*=Step in which overnight storage at 4°C is permissible

**=Step in which overnight storage at room temperature is permissible.

Training on using TEM and Slides examination were done by the laboratory principal Jian. Four slides were prepared from four different sites of the tissue. Tissue cutting was performed using Microtomy using Leica EM UC6 Ultramicrotome MZ6 Image (1-A). Sections were cut and stained

using Uranyl and Lead staining and it was performed using lab. Specific equipments including very fine and specific forceps.

RESULTS:

Seventy five FFPE Urinary Bladder tissue specimens were included in the current retrospective study, fifty specimens were from malignant UB tissues (UBC) and twenty five were for noncancerous (NCA) pathological conditions (Cystitis). The majority of cases (77.33%) were male in both UBC and NCA patients; 42 (84%) out of 50 case of UBC and 16 (64%) out of 25 cases of NCA patients, while females formed 22.77 % (8 Malignant and 9 NCA) and the male: female ratio recoded 5.25:1.0.

Table (1): Patient's age grouping and data.

Age Groups/y	Malignant (50 cases)						NCA (25 cases)					
	M	%	F	%	Total	%	M	%	F	%	Total	%
< 20 /y	1	100		0	1	2	2	100	0	0	2	8
21-40 /y	1	100		0	1	2	1	50	1	50	2	8
41-60/y	15	88.23		11.76	17	34	4	40	6	60	10	40
61-80/y	24	80		20	30	60	9	90	1	10	10	40
81-100/y	1	100		0	1	2	0	0	1	100	1	4
Total	42	84		16	50	100	16	64	9	36	25	100

y= years, NCA=noncancerous, M = Male & F=Female

The age of patients was (17 – 99) years; the mean age was 60.43 and standard deviation was 14.44 (table-1). The predominant age group for both malignant and an NCA pathological condition for males and females were between 61 and 80 years old. UBC patient’s average age was 63.52 it was between (20-99) years.

Ziehl-Neelsen staining results:

ZN staining was performed for 66 slides prepared from urinary bladder FFPE tissues, 45 from malignant and 21 from NCA UB tissues, slides were examined under 100X lens table(2). There was a significant difference between positive and negative results P = 0.021 (1 DF). UBC tissue showed the highest percentage of AFB positive cases in comparison to a lower percentage recorded by NCA patients (76.6% and 23.4%) respectively out of 47 total positive slides.

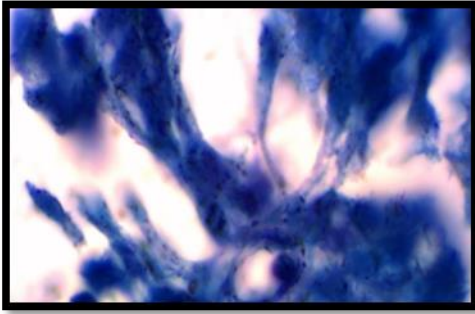
Table(2): AFB stained slides of urinary bladder cancer and NCA patients.

Slides results	UBC	NCA	Total
Positive	36(72%)	11(44%)	47(62.67%)
Negative	9(18%)	10(40%)	19(25.33%)
No slides	5(10%)	4(16%)	9(12%)
Total	50(100%)	25(100%)	75(100)
* X ² = 5.328		P = 0.02099 (1 df)	

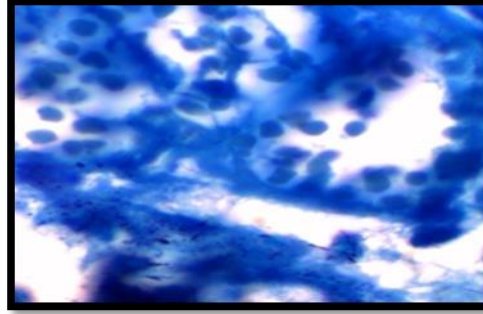
*chi-square included only positive and negative results.

UBC tissue slides showed, the presence of few number of AFB positive bacterial cells (*Mycobacterium tuberculosis*). In addition, there were variations in size between cells infecting the same tissue section Figure (1), a large number of AFB positive results recorded for UBC patients comparing

to NCA, Figure (2). Moreover, NCA-AFB stained slides showed a larger bacterial cells and spreading widely in tissues.



Figure(1): AFB positive slides for patients with UBC infected with high number of bacterial cells.



Figure(2): AFB positive slides for patients with UBC infected with low number of bacterial cells.

High number of AFB bacterial cells were detected in the acid fast stained slide in addition to other types of bacteria which stained negatively with this stain Figures (3 and 4). AFB positive stained bacteria were pleomorphic varied from cocci to bacilli in addition to diplococci cells presented intra- and extracellular.

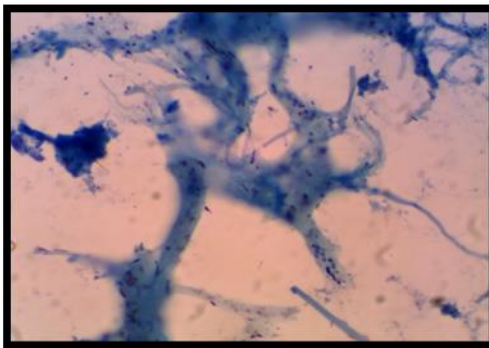


Figure (3) AFB positive slide for NCA patient with unbounded cells.

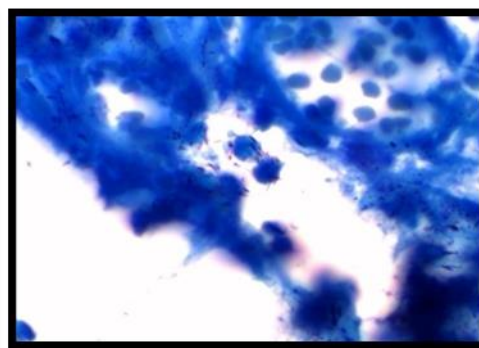
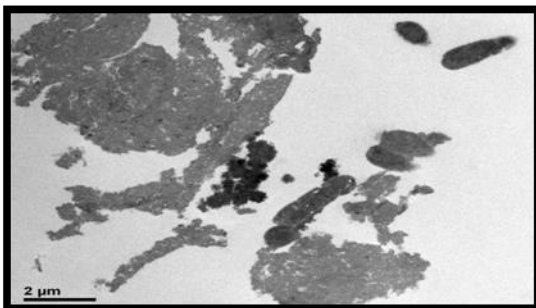


Figure (4): AFB positive slides for NCA patient .

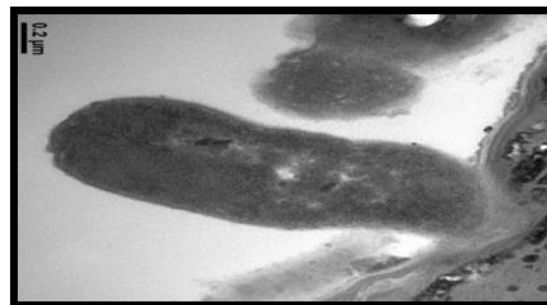
Images of UBC and NCA *Mycobacterium* infected tissues showed that AFB positive cells were unbounded and no fibrotic or immune cells surrounding *Mycobacterium* cells.

Transmission Electron Microscope imaging :

Two MTB positive slides for UBC patients were chosen to be examined and imaged by JEOL 1230 TEM microscope.



Figure(5): TEM image for AFB bacterial cells.



Figure(6): TEM image for AFB positive bacterial cell in contact to the urothelium.

The results showed a direct contact between the bacilli cell and the urothelial cell membrane with variable size cells Figure(5). The second image Figure(6) showed presences of bacterial cells that vary in morphology from bacilli to coccobacilli. Most cells were found either in contact to cells or extracellular.

DISCUSSION

Urinary Bladder cancer is the 9th most common cancer worldwide ⁽¹¹⁾. Data collected in the current study showed that the majority of cases were of male group for both UBC and noncancerous patients, in which 77.33% specimens out of 75 specimens were from males, and 84% out of 50 case of UBC.

The male: female ratio recoded 5.25:1.0. These findings are higher than that published by other studies ⁽¹²⁾, which estimated that male: female ratio is of 3.8 1.0 and higher than that reported by the American Bladder Cancer Society ⁽¹³⁾ which reported that in the general population a man has a 1 in 27 and a woman a 1 in 84 chance of getting bladder cancer in their lifetime, this equates to a 3% chance in men and a 1% chance in women. This high percentage of UBC recorded among our study group can be attributed to many reasons including socioeconomic reasons (e.g. smoking), environmental reasons and due to exposure to a wide range of potentially carcinogenic substances such as weapons residue at war time.

Ziehl-Neelsen staining results:

The diagnosis of tuberculosis is based mainly on conventional approaches, which depend on clinical features and the result of culture and microscopy. Culture methods are sensitive and specific but they are slow and take 2-6 wks ⁽¹⁴⁾. There are frequent occasions when tissue obtained by biopsy is not sent for culture because the diagnosis was not a clinical consideration before the report of findings on microscopic examination of the tissues ⁽¹⁵⁾. In such cases ZN staining can be the most rapid and inexpensive method for the detection of *Mycobacterial* infection in tissue sections ⁽¹⁶⁾.

Present study used ZN stain to detect presence of *Mycobacterium* in UB tissues. There was a significant difference between positive and negative results $P < 0.05$. 62.67% of slides showed AFB positive result the UBC tissue showed the highest percentage 76.6% of them compared to a lower percentage recorded by noncancerous patients 23.4%. The ZN stain results confirmed UB tissue infection with MTBC species, with high percentage among UBC patients using of FFPE tissues may affect ZN stain results, in which some studies showed that the number of bacilli in tissue section stained with ZN stain seems to be much lower than that expected and the number can be higher if different type of specimens were used ^(17, 18).

Presence of high percentage of MTBC among UBC tissue can give a sign for the association of those bacteria with cancer and this can be attributed to either the fact that *Mycobacteria* inhibit phagosome maturation by blocking the fusion of phagosomes with early endosomes and lysosomes, and by causing alterations in membrane proteins that normally promote the formation of an acidic phagolysosome leading to impaired immune response to infection ⁽¹⁹⁾.

Or due to the presence of MTB intracellularly this may give a chance for gene-gene interaction or for less extent DNA damaging through production of DNA damaging materials (e.g. Reactive Oxygen Intermediates) ^{(20) (21)}. Gene -gene or gene-environment interaction may better predict the risk of bladder cancer in the presence of a high percentage of insertional sequence *IS6110* positive MTBC in UBC ⁽²²⁾. Our results are in agreement with BURIN *et al.*, (1995) ⁽²³⁾, who mentioned that urinary tract infections predispose UBC and its mechanism differ between both sexes and most of these infections are bacterial in origin. They mentioned several case-control studies that found a significant association between acute

bacterial UTI and UBC. On the other hand, our results disagree with the results of Kjaer *et al.*, (1989)⁽²⁴⁾ and Piper *et al.*, (1986)⁽²⁵⁾ who found no association between UTI and UBC.

Transmission Electron Microscope imaging

The association between cancer and *Mycobacterium* was investigated in our study through imaging of two MTBC positive cases for UBC patients using JEOL 1230 TEM microscope. The images showed a direct contact between the bacilli cell and the urothelial cell membrane. The second image showed presence of bacterial cells that vary in morphology from bacilli, *coccobacilli* and *cocci* cells with no boundaries around infected cells. In addition, it shows a direct contact between the cell membranes of bacterial cell and urothelial cell without internalization with the presence of two morphologically different cells.

There were no previous study which used UBC FFPE tissues slides stained with ZN stain to compare with, but our overall results were in agreement with the results of Markova *et al.*, (2012)⁽²⁶⁾ who reported that tubercle bacilli has the ability to use L-form conversion as an adaptive strategy to survive and multiply under unfavorable conditions in which urinary bladder tissue is not the primary site for tuberculosis infection. *Mycobacterial* L-forms lose their acid-fastness, change their morphology and have wide morphological variability such as coccoids and small granular forms, as well as the appearance of unusual modes of irregular cell division. Variable sizes of tubercle bacilli were also isolated in different studies including Farnia *et al.*, (2010)⁽²⁷⁾ and Dahl, .and (2004) studies⁽²⁸⁾.

Cancer microbes were described by some researchers as being primarily in cell-wall-deficient form. As a result of the loss of cell wall bacteria appear as round, coccus-like, granular forms that are found both within the cell and outside the cell. Different types of bacteria may look similar when they are in this form and in the body and culture those bacteria have the high capacity to enlarge in size⁽²⁹⁾.

CONCLUSIONS:

The majority of the UB specimens were for male patients and they were mostly of old age group and for less extent for females. ZN staining is very important in addition to other tissue staining procedures to determine pathogens associated with UBC cases.

RECOMMENDATIONS:

Combination of Anti-Mycobacterial drugs with routine UBC therapy in MTBC positive cases might help in good prognosis for such cases. Further investigation for the role of MTBC in UBC and other type of cancers using tissue cultures and animal experimental studies.

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