Republic of Iraq
Ministry of Higher Education
and Scientific Research
Al-Nahrain University
College of Sciences
Department of Biotechnology

Macrophage Migration Inhibitory Factor Gene Polymorphism and some Immunological Aspects of Urothelial Carcinoma Patients

A Dissertation

Submitted to the Council of Science College, Al-Nahrain University, as a Partial Fulfillment of the Requirements for the Degree of Doctorate of Philosophy of Science, in Biotechnology

By

Rawaa Nazar Mohammed Ali AlChalabi

B.Sc. in Biotechnology/Al-Nahrain University / 2004

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March /2016 **Jumada Al-Awel/1437**

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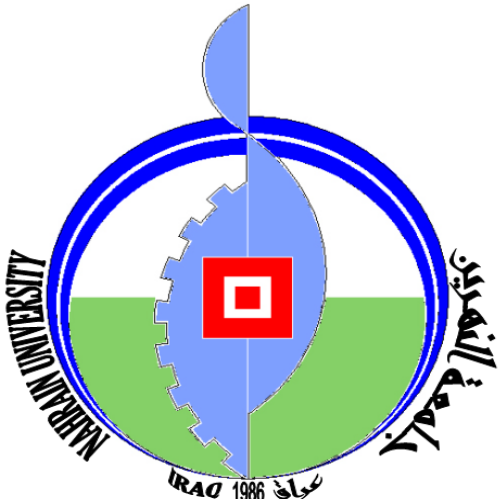

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Supervised By

Dr. Shahlaa M.Salih
(Assistant Professor)

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(Professor)

March /2016 **Jumada Al-Awel /1437**

بسم الله الرَّحْمَنِ الرَّحِيمِ

وَيَسْأَلُونَكَ عَنْ الرُّوحِ قُلْ الرُّوحُ مِنْ أَمْرِ رَبِّي وَمَا أُوتِيتُمْ مِنْ الْعِلْمِ إِلاَّ قَلِيلاً (٨٥)

صدق الله العظيم

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We, certify that this dissertation entitled "**Macrophage Migration Inhibitory Factor Gene Polymorphism and Some Immunological Aspects of Urothelial Carcinoma Patients''** was prepared by "**Rawaa Nazar AlChalabi** " under our supervision at the College of Science/Al-Nahrain University as a partial fulfillment of the requirements for the Degree of Doctorate of Philosophy in Science (Biotechnology).

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الاهداء ..

الى كل من علمني حرفه وترك بصمة في حياتي...

اساتذتي الكرام

الى ضحكة لاتنسى وملامح لاتغيب عن البال وحديث اشتقت لسماعه... الى من اراه بقلبي المشتاق واعلم انه لن يعود

والدي (رحمه الله)

الى من رآني قلبها قبل عينيها .. الى من لها في فؤادي كل حب ومع خفقات قلبي كل شوق .

والدتي

الى فخري واعتزازي...

اخي

يامن اغلى من نفسي التي بين جوانحي.... الى منبع ارتوي منه الاصرار والعزيمة ... الى من شجعني لاحقق الاكثر

حسين

الى شموع انارت طريقي ...الى فرحة ايامي وعطرها

غنى وعلي

الى عمتي الغالية ...

يسرا

الى اخت لم تلدها امي .....

غادة

الى رفيقة درب وان طال البعاد عن الخاطر لاتغيب... الى صديقة افتخر بها

حنين

الى كل من احتجته ووجدته بجانبي ... اقدم ثمرة جهدي المتواضع هذا ..

رواء

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Rawaa

Summary

This study was designed to shed light on the association between polymorphism of macrophage migration inhibitory factor (MIF) gene at -173 locus as risk factor of bladder cancer in Iraqi population by PCR-RFLP , estimation the serum level of MIF with some cytokines (IL-6, IL-10 and TNF-α) by sandwich ELISA and determine the expression of several marker (CD74, CD44, P^{53} and P^{21}) by immunohistochemical staining. Blood, urine and bladder biopsy were collected from patients and healthy volunteer at Al-Yarmook Teaching Hospital and Al-Jaibachi Private Hospital from March to November 2014.

One hundred and thirty five subjects from Baghdad (104 male and 31 female) were divided into three investigated groups: first group was urinary bladder carcinoma (UBC) (60 male and 13 female) where bladder cancer increases with age, second group was urinary bladder disorders (UBD) (22 male and 10 female) and third group was healthy control (22male and 8 female). Results showed that, after clinical laboratory diagnosis of urine samples of three groups 64 (87.7%) of UBC sample gave positive culture and 9 (12.3%) samples were negative culture; 32(100%) urine samples of UBD were positive culture while all urine sample of healthy control were negative. Gram staining and microscopic examination of UBC and UBD urine showed that 9 (9.4 %) isolates were identified as Gram positive bacteria, 85 (88.5 %) isolates as Gram negative bacteria and only 2 (2.1%) isolates as fungi. In addition , These isolates were distributed as, 2 (2.1%) isolates were identified as *Candida albicans,* 1(1%) *Citrobacter spp,* 1(1%) *Streptococcus fecalis,* 7 (7.3 %) *Pseudomonas sp.,* 9(9.4%) *Staphylococcus aureus* , 14(14.6%) *Klebsiella sp* , 19(19.8%) *Proteus sp.*, and 43(44.8%) isolates identified as *E.coli* .

According to histopathology's diagnosis, all tumors of this study have

been classified as urothelial cell carcinoma (UCC). Stage and grade for each tumor has been identified according to WHO (2004). Forty (54.8%) tumors were characterized as high grade and 33(45.2%) as low grade. While according to stage of tumors, 12(16.4%) Ta, 28(38.4%) T1 invaded subepithelial connective tissue, 22(30.1%) T2 invaded muscle, 7(9.6) T3 invaded perivesical tissue and 4(5.5%) T4 invade other organ. Forty five (61.6%) out of the 73 patients had the bladder tumor for first time while 28(38.6%) patients were suffering from recurrent bladder tumor after chemotherapy.

Results revealed serum IL-6 levels were significantly higher in patients with UBC (90.14 pg/ml) than in both of UBD (61.5 pm/ml) and healthy controls (8.4) pg/ml. Serum level of IL-10 was significantly higher in both patients with UBC (35.84pg/ml) and UBD patients (27.95pg/ml) when compared with healthy subjects (19.26pg/ml). Serum level of TNF-α was significantly higher for UBC and UBD patients when compared with mean level of control (36.19, 15.31 and 10.15 pg/ml) respectively. The mean level of serum MIF in UBC patients was significantly higher than that observed in UBD patients and healthy control (55.57 and 40.39 *vs*. 18.83) pg/ml, respectively. Positive significant correlation was found between four cytokines, and all of these were increased proportionally with advanced tumors stages and high grade.

Genomic DNA were extracted from peripheral blood of all 135 subjects and amplified by PCR with using specific primer for -173 locus that giving the PCR product (366) bp that containing both the polymorphic and a non polymorphic AluI site. Results showed that G/G genotype gave two fragments (268- and 98-bp), C/C genotype gave (62-, 206- and 98-bp) and four fragments as (62-, 98-, 206- and 268-bp) of G/C genotype after digestion by Alu I. The frequency distributions of genotypes and alleles of the groups

showed that the GG and G allele were (61.9 and 78.77) % among the UBC cases and (56.3 and 75)% among the UBD cases, while represent (53.3and 70)% respectively among healthy cases. In addition, and no significant variation was recorded between UBC and control with no significant departure from Hardy- Weinberg equilibrium. Results also showed that MIF level in GG was (62.69) pg/ml which significantly higher in UBC and level was significantly lower in UBD and healthy (37.98 and 16. 61 pg/ml), respectively.

In order to study the expression of CD74 molecules, staining was done by using anti-CD74 clone, then it was compared between the CD74 expressions. Results exhibited positive staining 50(68.49 %) with different scores and 23(31.51%) gave negative staining with highly significant differences. while all tissues free from bladder tumors were expressed negative staining.

Results showed highly positive CD44 immunohistochemical expressions in bladder tumor tissues, 64 (87.7%) gave positive result of staining with different score and 9(12.3%) gave negative results, while 9(90%) out of the ten normal urothelium gave positive expression with highly significant differences.

Analysis of the 73 bladder tumors revealed that nuclear p53 protein of 42(57.5%) gave positive staining which were exclusively nuclear and 31(42.5%) gave negatively staining result while expression of p53 was nil in all normal transitional epithelium specimens. Results showed that 51(69.9%) out of 73 urinary bladder tumors gave positive nuclear staining for p21 with different scores while 22 (31.1%) and all ten normal urothlium consistently demonstrated no p21 immunoreactivity, providing a negative staining pattern. Also, results showed a significant positive expressions of CD74, CD44, p53 and p21 in stages T2 and T1 with high grade.

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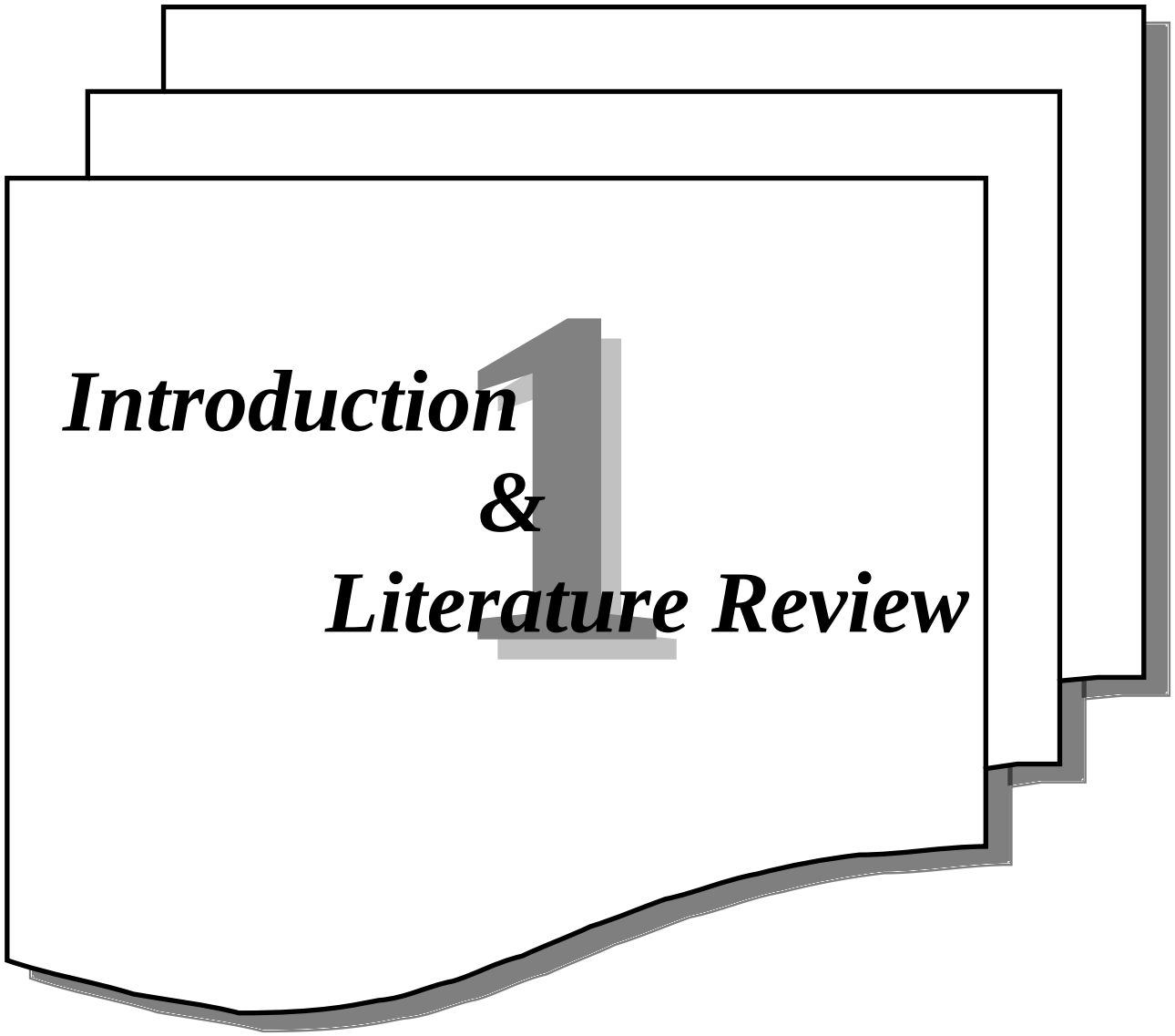
Abbreviations	Words
API	Analytical Profile Index
ASCO	American Society of Clinical Oncology
BC	Bladder Cancer
CAM	Cell Adhesion Molecule
CD	Cluster of Differentiation
CT	Computed Tomography
DC	Dendritic cell
IHC	Imuuno Histo Chemistry
IL	Interleukin
MHC	Major Histocompatibility complex
MIF	Macrophage Migration Inhibitory Factor
MRI	Magnetic resonance Imaging Scan
NMIBC	Non Muscle Invasive Bladder Cancer
PCR	Polymerase chain reaction
RT	Room Temperature
SCC	Squamous Cell Carcinoma
SNP	Single Nucleotide Polymorphism
UCC	Urothelial Cell Carcinoma
TNF	Tumor Necrosis Factor
TNM	Tumor Node Metastasis staging system

TSI	Triple Sugar Iron agar
TURBT	Trans Urethral Resection Bladder Tumor
UBC	Urinary Bladder Carcinoma
AJCC	American Joint Committee on Cancer
UBD	Urinary Bladder Disorder
UTI	Urinary tract infection
WHO	World Health Organization

Chapter One



Introduction & Literature Review

Introduction & Literature Review

1.1 Introduction

Bladder cancer is the 2nd most frequent malignancy of the genitourinary tract and the fourth most common cancer in men (Li *et al.*, 2014). The most common presenting pathology of bladder tumors is the urothelial cell carcinoma (90% of cases), while squamous cell carcinomas, adenocarcinomas and other rare subtypes comprise a minority of cases(Bartsch *et al*., 2010) .

Tumors of the bladder rarely occur before the age of 40 years arising most commonly in the seventh decade of life(Volkmer *et al*.,2012).Smoking and occupational exposure to environmental carcinogens like aromatic amines, radiation and chemotherapy are the main factors that are strongly associated with bladder cancer (Letašiová *et al.*, 2012).

Approximately 70% of bladder tumors are non muscle invasive tumors (stage Ta, Tis, or T1), 25% are muscle invasive (stage T2 or T3), and 5% are metastatic. Metastasis is the leading cause of death. Several events are required for metastasis to occur, including neovascularization, cell attachment, invasion, and cell proliferation (Sjodah *et al*., 2015).

Cell adhesion molecules (CAMs) play important roles in cell -cell and cell-matrix interactions. They are associated with invasion and metastasis in a wide variety of human malignancies including urothelial cancers. Superficial, low grade tumors have higher recurrence rates and higher grade tumors have a higher invasive potential and tendency to metastatic potential. Generally, standard histopathological characteristics of urothelial cancers, including tumor grade and stage determine the tumor's behavior but description of new tumor characteristics may be helpful for the patients treatment (Stivarou and Patsavoudi *et al.*, 2015).

Cytokines are low molecular weight soluble proteins that have a fundamental role in communication within the immune system and involved in a wide array of biological activities specially that regulate growth, differentiation and activation of immune cells(Dranoff, 2004 and Chokkalingam *et al*., 2013).

Each cytokine binds to a specific surface receptor followed by subsequent cascades of intracellular signaling that altered cell function and include the up regulation of several genes and their transcription factors resulting in the production of other cytokines (Horacek *et al*.,2014). Disregulation of cytokine production is thought to play an important role in the development of diseases such as autoimmune disorder and cancer. Various cytokines are involved in interactions between malignant cells of tumors and immune cells, which may influence tumor progression directly by acting on tumors cells as growth promoting or inhibiting factors or indirectly by attracting inflammatory cell types and affecting angiogenesis (Brumatti *et al*., 2010).

Gene expression of cytokines and cytokine receptors is tightly regulated and aberrant expression has been implicated in the susceptibility to a range of infectious diseases and some cytokine Single-Nucleotide polymorphisms have been demonstrated to be important in altering expression or function of the cytokine gene(Sjödah *et al*., 2013). Genetic alterations in cytokine genes may lead to a high or low production of certain cytokines that may influence native antitumor immune responses or tumor progression by acting on pathways of tumor angiogenesis (Chen *et al*., 2013 and Karra *et al.,* 2015).

Approximately 20% of all cancers arise in association with infection and chronic inflammation and even those cancers that do not develop as a consequence of chronic inflammation, exhibit extensive inflammatory infiltrates with high levels of cytokine expression in the tumor microenvironment.

investigations have shown that inflammation and proinflammatory cytokine production are correlated with advanced cases of cancer and may be indicators of a poor prognosis (Jasim and Khalil,2014 and McBeth *et al*., 2015).

MIF enhancement of macrophage transcription, activation and viability, coupled with its inhibitory effects on anti-tumor cell cytotoxic lymphocytes, suggests that MIF overexpression in developing malignancies may act in concert to facilitate increased tumor growth which present an important link between inflammation and cancer due to its pro-inflammatory role. Its molecular mechanisms involve, among others, the inhibition of p53 which promote tumor cell proliferation, cell survival and tumor-associated neoangiogenesis (Meyer-Siegler *et al*., 2010 and Souza *et al*.,2014).

MIF binds to extracellular domain of CD74with a high affinity and initiates a signaling cascade. CD74 forms a complex with CD44which is essential for the MIF-induced signaling cascade. Rare (SNPs) in the CD74 gene have been reported, but SNPs in molecules that interact with CD74, such as MIF, CD44and MHC class II are more frequent and are associated with the development of cancer(Liu and Lin,2014). The imbalance in the regulation of inflammation that occurs in many cancers can induce cellular damage. This stimulates interaction between immune cells and the damaged cells, which then proliferate, invade, and subsequently develop into tumors (Morris *et al*., 2014).

Cell cycle is a strictly controlled process regulated by protein complexes composed of cyclins and cyclin-dependent kinases (cdks) and also by several tumor suppressor gene protein products acting at the Go/G1 checkpoint of the cell-cycle. Some of these protein products are p53, and p21. Their role is the regulation of normal cell growth, proliferation and consecutively normal cell death (apoptosis). Inactivation of one or more tumor-suppressor genes and/or

loss of cell cycle control represents the first step of carcinogenesis. It is believed that cancer cells defective in p53 have lost the ability to undergo cell growth by normal cell cycle progression and apoptosis. Therefore, these cells acquire the transformed phenotype of cancer cells (Syrigos *et al*.,2004; Stadler *et al*., 2011 and Puzio-Kuter *et al*., 2015).

p21 mediates its various biological activities primarily by binding to and inhibiting the kinase activity of the cyclin-dependent kinases leading to growth arrest at specific stages in the cell cycle (Tang *et al*., 2015). The ability of p21 to promote cell cycle inhibition may also depend on its ability to mediate p53-dependent gene repression. Interactions between p53 and p21 are very interesting. Patients with p53-altered/p21-negative tumors showed a higher rate of recurrence and worse survival compared to those with p53-altered/ p21-positive tumors (Syrigos *et al*., 2004; Abbas and Dutta,2010 and).

Aims of the Study:

1- Evaluation the possible role of genetic sequence variants in *MIF*$_{-173}$ gene as a risk factor in Iraqi population with urinary bladder carcinoma .

2- Investigation the role of serum levels of IL-6, IL-10, TNF-α and MIF in the initiation and progression of bladder carcinoma .

3- Detecting the immunohistochemical expression of CD74, CD44, p53and p21 in tissue samples collected after surgery .

1.2 Literature Review

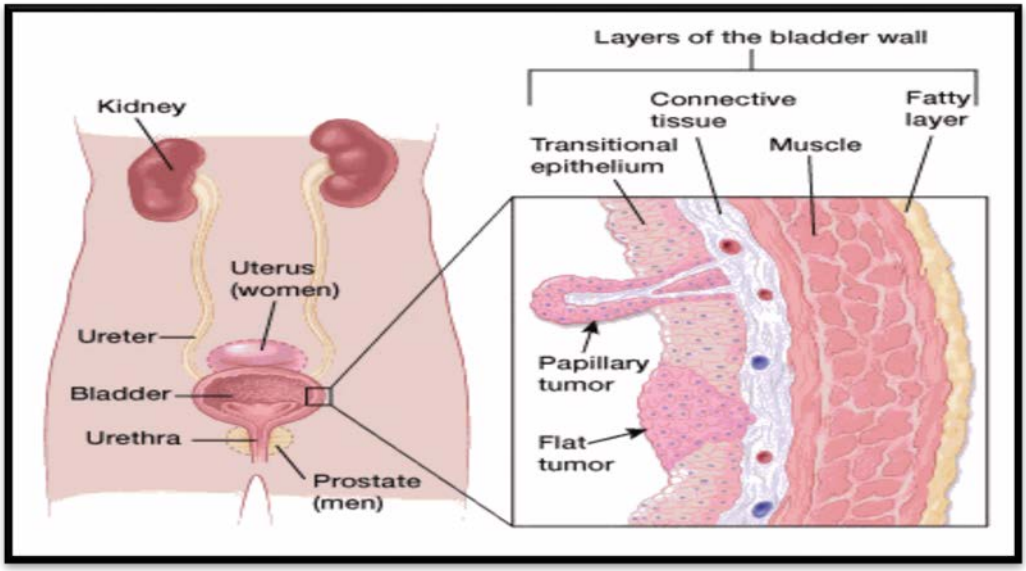
1.2.1 Cancer

Cancer is the uncontrolled proliferation of the cells. Cancer progression is a complex multi-step process that consists of transformation, tumor growth, invasion and metastasis(Rhijn *et al*., 2009 and Siegel *et al*., 2012). Tumor invasion and metastasis are the critical steps in determining the aggressive phenotype of human cancers, the obstacles to the successful treatment and major causes of cancer deaths. The spread of tumor cells from a primary tumor to secondary sites within the body is a complicated process involving the degradation of basement membrane, invasion of stroma, adhesion, angiogenesis, cell proliferation, migration, and anti-apoptosis (Shih *et al*., 2006 and Zehnder *et al*., 2014).

Numerous genetic changes and a variety of positive and negative factors may be involved in the molecular basis of metastasis. During cancer progression, several rounds of mutation and selection result in highly invasive ability of some cancer cells(Davies,2003). In particular, increased expression of metastasis promoting genes or decreased expression of metastasis suppressor genes can provide cancer cells with a selective invasive advantage and lead to the clonal outgrowth of a tumor .Mutations accumulate in the human body which hence the cancer progresses to different stages(Siegel *et al*., 2013). Main mutations involve conversion of proto-oncogenes to oncogenes and mutations in the tumor suppressor genes. A cure or a therapy for cancer has not been successful because each cancer has a different combination of gene mutations which make it difficult to make a common drug for cancer (Sen and Hopwood ,2011and Grapsa *et al*., 2014).

1.2.2 Bladder Cancer

The bladder is a hollow organ in the lower part of the abdomen that stores urine until it is passed out of the body which is made up of a number of layers. A thin surface layer called the urothelium lines the inside of the bladder, loose connective tissue called the lamina propria, bladder muscle and the layer of fat as shown Figure (1-1). Bladder cancer means that the abnormal cells multiply without control in the bladder arising from the epithelial lining (the urothelium) of the urinary bladder that has the highest recurrence rate of any malignancy. (Dejong *et al* .,2014).



(1-1): Location of Bladder and It's Layers (Walsh *et al*., 2012)

Approximately 95% of malignant bladder tumors are urothelial cell carcinomas (UCC) which can be classified as papillary (most common type, tend to grow slowly towards the lumen), solid (less frequent, infiltrate the bladder wall and are more aggressive) or Carcinoma In Situ (CIS, a very aggressive kind of cancer that involves the inner lining of the bladder (Chan *et al*., 2009;Akli *et al*., 2012).

Solid tumors comprise not only malignant cells, but also extracellular matrix and many other non-malignant cell types, including fibroblast, endothelial cells and inflammatory cells such as macrophages, neutrophils, mast cells and lymphocytes(Cha *et al.*, 2012). Recurrent or persistent inflammation has long been considered to contribute to cancer development. The presence of inflammatory cells in tumors was first described in 1863 and inflammatory microenvironment plays a key role to promote tumor development and progression(Miremami and Kyprianou, 2014).

The macrophage is the pivotal member of inflammatory cells within the tumor stroma. It has now been well understood that the majority of malignant tumors contain numerous macrophages as a major component of the host leukocytic infiltrate(Bucala and Donnelly, 2007). These macrophages are referred to as tumor associated macrophages (TAMs) and most are derived from peripheral blood monocytes recruited into the tumor mass which can release a vast diversity of growth factors, cytokines, inflammatory mediators, and proteolytic enzymes. Many of these factors are key agents in tumor progression (Sergei *et al.*,2010 and Zhu *et al*., 2012).

The main symptoms of bladder cancer causes are painless gross hematuria (blood in the urine), frequent urination, dysuria, urgency and flank pain. The blood in the urine may be visible to the naked eye (gross/macroscopic hematuria) or detectable only by microscope (microscopic hematuria) (Jacobs *et al*., 2010 and Bryan,2011). Hematuria is the most common symptom in bladder cancer that occurs in approximately 80-90% of the patients but these signs and symptoms are not specific to bladder cancer, and are also caused by non-cancerous conditions, including prostate infections, over-active bladder and cystitis. There are many other causes of hematuria, such as bladder

or ureteric stones, infection, kidney disease. All above symptoms that are present in UTI more common in women thus may be misdiagnosed and leads to delaying the treatment and more advanced diseases (Bangma *et al.*,2013and Sanford *et al*., 2015).

BC is the most common malignancy of the urinary tract. Approximately 70-75% of patients are diagnosed with a superficial, low-grade, and noninvasive disease (NMIBC). Standard primary treatment for NIMBC is transurethral resection (TUR) followed by chemotherapy or immunotherapy of nearly 60% to 70% of these tumors will recur(Seydlitz *et al*., 2014). Surgery for superficial lesions is often followed by intravascular therapy and 25% will progress into a higher stage or grade and 30% of patients present with high-grade or invasive or metastatic disease. The risk of distant metastasis, the lethal phenotype of bladder cancer, increases dramatically with invasion of the deep muscle layer of the bladder wall (Lacarrière *et al*., 2013and Yang *et al*., 2015).

1.2.3 Types of Bladder Cancer

BC is classified on the basis of the appearance of its cells under the microscope (histological type). The type of bladder cancer has implications in selecting the appropriate treatment for the disease. There are three types of bladder cancer:

1.2.3.1 Urothelial cell Carcinoma

Urothelial cell carcinoma occurs in the cells that line the inside of bladder. Depending on the genetic alterations that occur, these cells may follow different pathways in the expression of their phenotype(Nuno *et al*., 2007). Approximately 90-95% of malignant bladder tumors is (UCC) and can be divided into 2 subtypes based on how they grow as:papillary (most common type

grow in finger-like projections from the inner surface of the bladder toward the hollow center and Papillary tumors often grow toward the center of the bladder without growing into the deeper bladder layers) or Carcinoma In Situ (CIS, a very aggressive kind of cancer that involves only the inner lining of the bladder) (Pasin *et al*., 2008; Kamat *et al*., 2012).

Also, UCC can be divided based on how far they have invaded into the wall of the bladder: non-invasive bladder cancers are still in the inner layer of cells (the transitional epithelium) but have not grown into the deeper layers and Invasive cancers grow into the lamina propria or even deeper into the muscle layer (James *et al*.,2012). Invasive cancers are more likely to spread and are harder to treat. A bladder cancer can also be described as superficial or non-muscle invasive. These terms include both non-invasive tumors as well as any invasive tumors that have not grown into the main muscle layer of the bladder (Ploeg *et al.,* 2009; and Darwiche *et al.,* 2015).

The most common pathway for UCCs involves the development of a papillary tumor that projects into the bladder lumen and, if untreated, eventually penetrates the basement membrane, invades the lamina propria, and then continues into the bladder muscle, where it can metastasize(Smith and Guzzo, 2013).

Nearly90% of urothelial cell bladder tumors exhibit this type of behavior while the remaining 10% of UCCs follows a different molecular pathway and are called CIS. Many urothelial tumors are primarily composed of UCC but contain small areas of squamous differentiation, squamous cell carcinoma (SCC), or adenocarcinoma (Cheung *et al.,* 2013).

1.2.3.2 Squamous Cell Carcinoma

It is a malignant neoplasm derived from bladder urothelium and comprises 5% of all urinary bladder carcinomas, with pure squamous phenotype in response to infection and irritation. A diagnosis of squamous cell carcinoma of the bladder should be rendered only when the tumor is solely composed of a squamous cell component in the absence of a conventional urothelial carcinoma component(Vikram *et al*., 2009 and Haung *et al*., 2013). It is more common in parts of the world where a certain parasitic infection (Schistosomiasis) is a prevalent cause of bladder infection especially in African countries. In the bladder, the deposition of *Schistosoma* eggs commonly provokes a severe inflammatory response and fibrosis which associated with the development of cancer at a younger age and with a predominance of SCC relative to egg-negative cases(Lagwinski *et al*., 2009).

Risk factors led to increase chance of SCC are: infection with schistosomiasis **,** chronic irritation, e.g. indwelling catheter, bladder calculi, chronic infection and intravesical BCG(Abdulamir *et al*., 2009and Makboul *et al*., 2015). SCC is often present at an advanced stage; however, radical cystectomy with lymph node dissection appears to offer a significant benefit in survival in a subset of patients (Yurdakul *et al*., 2005and Rambau *et al*., 2013).

1.2.3.3 Adenocarcinoma

Adenocarcinoma of the urinary bladder begins in cells that make up-mucous –secreting glands in the bladder which accounting for 0.5 - 2.0 % of all malignant vesicle tumors (Somak *et al*., 2011).The histologic variants show a predominant colonic (enteric) type glandular morphology with varied histologic

patterns It can arise anywhere in the urinary bladder. However, they involve the trigone and posterior bladder wall in most cases. About two-thirds of the adenocarcinomaspresent as solitary, discrete lesions, unlike the "usual" urothelial carcinomas, which tend to be multifocal. These tumors arise in patients who have a long history of cystitis; glandular cystitis frequently is associated with this lesion. Histologically, these tumors resemble colonic carcinoma, and most of them have invaded into muscle at the time of initial diagnosis (Kapur *et al*., 2011).

1.2.4 Epidemiology and Etiology of Bladder Cancer

Bladder cancer is a common malignancy worldwide that is associated with significant morbidity and mortality. Unfortunately, the incidence rate of bladder cancer continues to increase over the last 60 to 70 years specially in the developed countries where industrialization has led to carcinogenic exposure and represents the fourth most common cancer in men and the ninth most common cancer in women (Jemal *et al*, 2008).

Males are 3 to 4 times more likely to develop bladder cancer than females, presumably because of an increased prevalence of smoking and exposure to environmental toxins(Griffiths, 2013). Although the majority of patients with bladder cancer are diagnosed with a superficial, low-grade noninvasive disease can often be treated effectively but invasive cancers not only require invasive surgery, but are also refractory to aggressive chemotherapy and radiotherapy Rosenberg and Hahn, 2015).

The American Cancer Society predicted that 72,570 new cases of bladder cancer will be diagnosed in the USA in 2013 and that 15,210 people will die of the disease. The incidence of BC increases with age which rarely diagnosed before age 40 years (Ferlay *et al*., 2010).

A white male has a 3.7% chance of developing urothelial cancer in his lifetime, which is roughly 3 times the probability for white females or African-American males and more than 4 times the probability for African-American females (Siegel *et al*.,2013). Also, the risk of developing invasive bladder cancer is age dependent For men from birth to age 39 years, the incidence rate of invasive bladder cancer is 0.02% ages 40 to 59 years, 0.41%; ages 60 to 69 years, 0.96%; ages 70 years and older, 3.5%; and from birth to death, 3.7%.The bladder cancer incidence for women from birth to age 39 years is 0.1%; ages 40 to 59 years, 0.13%; ages 60 to 69 years, 0.26%; ages 70 years and older, 0.99%; and from birth to death, 1.17% while based on the report of the International Agency for Research on Cancer, there are about 386,000 new cases of urothelial bladder cancer and 150,000 deaths annually worldwide (Hatina and Schulz, 2012 and Keymoosi *et al*.,2014).

The incidence rate of bladder cancer has been rising in Asia and Russia because of an increased prevalence of smoking. Sixty-three percent of all bladder cancer cases occurs in developed countries, with 55% from North America and Europe. The histologic cell type of bladder cancer is very geographically dependent, but urothelial cancer is the most common (Malats and Real, 2015). In North America and Europe, 95% to 97% of cases are urothelial carcinoma, in Africa 60% to 90% are urothelial and 10% to 40% are squamous cell and Egypt has the highest rate of squamous cell carcinoma because of the endemic infections with *Schistosoma* species .The mortality rate from bladder cancer in Egypt is 3 times higher than in Europe and 8 times greater than in North America because of the aggressive nature of squamous cell carcinoma that is highly prevalent in Egypt (Salem *et al*.,2010).

In Iran, bladder cancer is the third most common cancer among males and the ninth most common among females with an incidence of 13.03 and 3.32 per 100000 population among men and women, respectively (Salehi *et al*., 2011).

Bladder cancer in Iraq was studied by AL-Shwani,(2013) who found that the incidence rate is on constant rise, with 80% were males and 20% were female and average age for males were (66,07 years) and for the females were (67.82years) as well, Al-Biaty,(2015) who reported that 87.7% were male and 12.3% were female with a mean age of 63 year.

Bladder cancer is often described as a polyclonal field change defect with frequent recurrences due to a heightened potential for malignant transformation. However, bladder cancer has also been described as resulting from implantation of malignant cells that have migrated from a previously affected site. Bladder cancer is caused by genetic abnormalities and external risk factors(Chan *et al*., 2009). Molecular pathways are likely responsible for the development of noninvasive and invasive bladder tumors for example somatic mutations in fibroblast growth receptor3 (FGFR-3) and tumor suppressor protein p53 (Tp53) in tumor cells that appear to be important and early molecular events in the noninvasive and invasive pathways, respectively (Madka *et al.*,2013). Alterations in the *TP53* gene are noted in approximately 60% of invasive bladder cancers. Progression-free survival is significantly shorter in patients with *TP53*mutations and is an independent predictor of death among patients with muscle-invasive bladder cancer. Also, there are several polymorphisms that seem to be related to the formation of bladder cancer, in particular the susceptibility to environmental carcinogens (Salinas-Sánchez *et al*.,2008 and Ghafouri-Fard *et al* .,2014) .

In addition to the skin and lungs, the bladder is the main internal organ affected by occupational carcinogens. Environmental carcinogens can enter the system and cause bladder cancer from inhalation or through skin absorption. Up to 80% of bladder cancer cases are associated with environmental exposure. There are many risk factors that are strongly associated with bladder cancer such as :

- **Smoking:** Tobacco use is by far the most common cause of bladder cancer. Smoking duration and intensity are directly related to increased risk. Developing bladder Carcinoma is 2-6 times greater in smokers than in nonsmokers. If a person smokes 1 to 9 cigarettes versus more than 21 cigarettes per day, the relative risk of bladder cancer is 1.5 versus 5.4, respectively(Figueroa *et al*., 2014). If a person smokes 1 to 10 years versus more than 40 years, the relative risk of bladder cancer is 1.2 versus 3.0, respectively. If a person smokes more than 60 years, they have a six fold increased risk of developing urothelial cancer compared to a nonsmoker (Walsh *et al*., 2012).The type of tobacco smoked appears to be associated with bladder cancer formation because of different carcinogens present within the tobacco. Black tobacco appears to be worse than blonde tobacco because of the greater amount of aromatic amines.Nitrosamine,2-naphthylamine and4-aminobiphenyl are possible carcinogenic agents found in cigarette smoke (Freedman *et al*., 2011).
- **Occupational exposure** to aromatic amines or aniline dyes is presumed to be the cause of bladder cancer in up to 25% of cases. Numerous occupations associated with diesel exhaust, petroleum products, and solvents (Ex: auto work, truck driving, plumbing, leather and apparel

work, rubber and metal work) have also been associated with an increased risk of bladder cancer(Brown *et al.*, 2012).

People living in urban areas are also more likely to develop bladder cancer. The etiology in these cases is thought to be multifactorial, potentially involving exposure to numerous carcinogens(Burger *et al*., 2013).

- **Chemotherapy**: Chemotherapy destroys malignant cells by causing significant DNA and cellular damage, but can also have a profound effect on rapidly dividing normal epithelium such as in the bladder((Parkin, 2011). The only chemotherapeutic agent that has been proven to cause bladder cancer is cyclophosphamide. The risk of bladder cancer formation is linearly related to the duration and intensity of cyclophosphamide treatment, supporting a causative role(Siamak *et al*., 2015).
- **Radiation:**. The issue of second cancers following therapeutic radiation for a wide variety of malignancies is currently receiving increased attention as it is well recognized that patients who receive radiation therapy have an increased long-term risk for developing second primary cancers compared with patients who do not receive radiation therapy (Romanenko *et al*., 2003). Radiation therapy has been linked to occurrences of secondary malignancies, including leukemia, sarcomas, thyroid carcinoma, lung carcinoma, and bladder carcinoma. UBC cases diagnosed following prostatic radiation therapy differ in histology and biological behavior from bladder cancers diagnosed in patients with prostate cancer who did not receive radiation therapy. Histology in these cases shows an undifferentiated malignant tumor which does not resemble

prostate adenocarcinoma. Radiation has been shown to be associated with the progression of low-grade urothelial tumors to high-grade tumors and a higher rate of p53 mutations (Sountoulides *et al*., 2010;Suriano *et al*., 2013). Those secondary radiation-induced bladder tumors are usually aggressive and sometimes lethal. The release of radioactivity after the accident at the Chernobyl nuclear facility show an increase in DNA damage, DNA damage-repair mechanisms, and urinary bladder lesions and the incidence of bladder cancer increased from 26.2 to 43.3 per 100,000 between 1986 and 2001 (after the Chernobyl accident) (Ruben *et al*., 2008and Kukreja *et al*., 2014).

- **Family History** : First-degree relatives of patients with bladder cancer have a two fold increased risk of developing urothelial cancer themselves, but high-risk of urothelial cancer families are relatively rare(Rachakonda *et al*., 2013). The inherited risk of bladder cancer formation appears to affect all stages of urothelial carcinoma and is not associated with bladder cancer formation at an earlier age. Unfortunately, there are no clear mendelian inheritance patterns, making classic linkage studies impossible (Wang *et al*., 2009). Sometimes family members with bladder cancer have all been exposed to the same carcinogen, other times, they may all have certain genetic abnormalities associated with BC such as telomerase reverse transcriptase (TERT). Promoter mutations were associated with recurrence, mutation in the gene retinoblastoma (RB1), can increase the bladder cancer risk as well mutation of p53 (Santos *et al*., 2014) .
- **Urinary Bilharziasis (Schistosomiasis)**:It is a parasitic disease caused by flatworms that live in snail-infested fresh water. It is endemic to 74 countries and affects some 200 million people worldwide, causing an

estimated 200 000 deaths annually (Chistulo *et al,*2004*)*. Chronic infection with *Schistosoma hematobium* leads to squamous cell formation of the bladder. The *Schistosoma* ova are deposited in the wall of the bladder and produce chronic inflammation that converts the urothelium to a squamous cell epithelium. Squamous cell epithelium has a much greater proliferation rate,and with the presence of chronic inflammation. Over time, this greater proliferation rate leads to cancer formation (Botelho *et al*., 2011 and Honeycutt *et al.,* 2014). The exact mechanism by which *Schistosoma* ova can cause squamous cell carcinoma is unclear, but two factors are suspected. One is the increased proliferation rate, and the second is the chronic inflammation and exposure to environmental agents. The increased proliferation of the squamous epithelium leads to a higher risk of spontaneous genetic alterations that can cause cancer. Bilharzial ova deposited in the bladder provokes an intense inflammatory reaction in addition to environmental agents that combine and associated with production of oxygen-derived free radicals may induce genetic mutations by generating genotoxic substances in the urine, such as *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine is known bladder cancer carcinogen. This substance is generated in very high levels in the urine of patients chronically infected with *Schistosoma* organisms (Abol-Enein, 2008, Salem *et al*., 2010). Chronic schistosomiasis leads predominantly to squamous cell carcinoma rather than urothelial carcinoma, with 70% of infected patients who develop bladder cancer having squamous cell carcinoma, although many will have both urothelial and squamous cell cancer (Botelho *et al*., 2013). Spinal cord–injured patients are also at risk

for developing squamous cell carcinoma, most likely because of chronic catheter irritation and infection. This supports the concept that chronic infection and foreign bodies can lead to bladder cancer formation (Fliex *et al*., 2008; Levinson ,2010).

- **Urinary Tracts Infections (UTIs):** Urinary tract infection (UTI): is an infection involving part or all of the urinary tract which remains a common and troublesome health problem in many different countries all round the world. In most hospitals, very large numbers of UTI were recorded daily among different ages and sexes. These infection may involve the urethra, bladder, ureters, the kidneys and prostates, the effect of infection depends on the interaction between the bacteria and host's defense mechanisms (Josip,2006; Wagner *et al*., 2010 and Jasmina *et al*., 2011). The most important causes of UTI represented by Gram negative bacteria belong to Enterobacteriacae family specially *Escherichia coli*. Although *E.coli* is a major component of the normal intestinal flora, but it is recognized as very active opportunistic pathogen associated with UTI, because *E.coli* has several virulence factors which contribute to *E.coli* pathogenicity. The distribution of virulence factors can also vary depending on host characteristic and type of infection. UTIs usually occur as a consequence of colonization of the periurethral area by a virulent organism that subsequently gains access to the bladder (Jiang *et al*., 2009; Kalpana *et al.*, 2010 and Fadhil *et al*., 2013). UTIs are predominant in females, Almost 95% of cases of UTIs are caused by bacteria that multiply at the opening of the urethra and travel up to the bladder (known as the ascending route). Much less often, bacteria spread to the kidney from the

blood stream. A history of urinary tract infection significantly elevated the risk of bladder cancer, particularly in individuals who reported three or more infections. Significantly increased bladder cancer risk was also found for bladder stones (Sabate *et al*., 2009).

UTI may predict a higher stage bladder cancer and greater mortality among older patients which appears to delay the diagnosis of bladder cancer in both sexes, but more notably in women (Arlene *et al.*, 2013).

1.2.5 Diagnosis of Bladder Cancer

A complete medical history is used to identify potential risk factors and the tests that doctor may order for diagnosing according to American Cancer Society, National Cancer Institute and American Society of Clinical Oncology (ASCO) (Todenhoffer *et al*., 2013;Hong and Zu , 2013 and Tadin *et al*., 2014)which include:

- **Urine lysis** for the detection of microscopic hematuria.
- **Urine culture** may be done to see if an infection (rather than cancer)is the cause because infections and bladder cancers can cause similar symptoms.
- **Urine tumor marker test** :different urine tests look for specific substances released by bladder cancer cells. These tests may be used along with urine cytology to help determine if a person has a bladder cancer.
- **Urine cytology:** a sample of urine flushed from bladder during urination is looked at under a microscope to see if it contains any cancer or pre-cancer cells, but this test is not perfect. Not finding cancer on this test does not always mean you are cancer free.
- **Ultrasound (ultrasonography)** : it uses sound waves to create pictures of internal organs. It can be useful in determining the size of a bladder cancer

and whether it has spread beyond the bladder to nearby organs or tissues. It can also be used to look at the kidneys.

- **Bladder biopsies** :bladder biopsy samples are most often obtained during cystoscopy. A biopsy can show whether cancer is present and what type of bladder cancer it is. If bladder cancer is found, two important features are its invasiveness and grade (Cystoscopy**:** A medical procedure in which your urologist inserts a narrow tube through urethra. This tube has a lens and fiber-optic lighting system that allows urologist to see the inside of urethra and bladder. This procedure is generally performed using a local anesthetic to make patient more comfortable. It is the only procedure that will allow to locate a tumor, and then perform a possible transurethral resection of bladder tumor (TURBT) by cystoscopy as shown in figure (1-2).

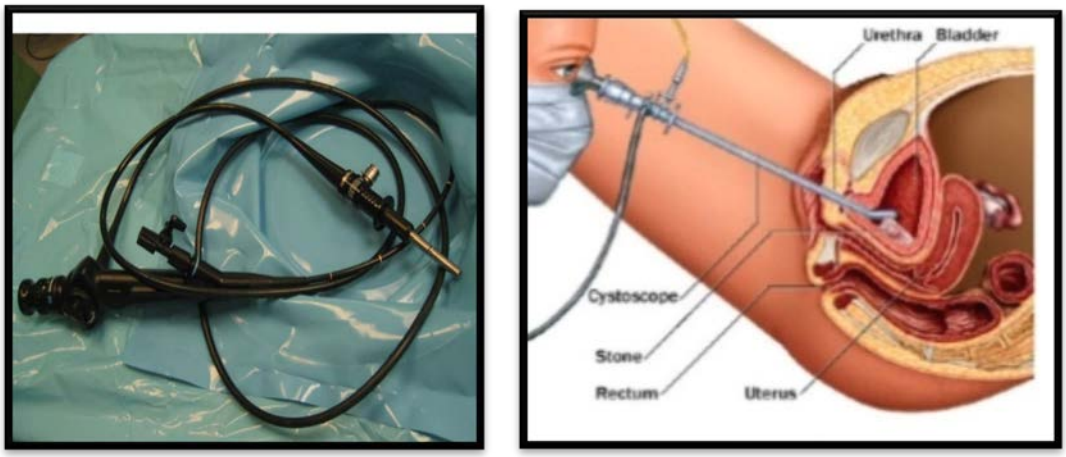


Figure (1-2):Transurethral resection of bladder tumor by Cystoscopy (Todenhoffer *et al*., 2013)

The biopsy can show how deeply the cancer has invaded (grown into) the bladder wall, which is very important in deciding treatment. Urologist passes the cystoscope into the bladder and collects a sample of

cells, which will be biopsied to determine if cancer is present. TURBT can also be used to treat bladder cancer.

- **Intravenous pyelogram** (IVU): is an x-ray of the urinary system taken after injecting a special dye into a vein. This dye is removed from the bloodstream by the kidneys and then passes into the ureters and bladder. The dye outlines these organs on x-rays and helps show urinary tract tumors.
- **Retrograde pyelogram** :For this test, a catheter (thin tube) is placed through the urethra and up into the bladder or into a ureter. Then a dye is injected through the catheter to make the lining of the bladder, ureters, and kidneys easier to see on x-rays. This test is not used as often as IVP, but it may be done (along with ultrasound of the kidneys) to look for tumors in the urinary tract in people who can not have an IVP because they are allergic to x-ray dyes.
- **Computed Tomography (CT) scan** is known as a CT urogram. It can provide detailed information about the size, shape, and position of any tumors in the urinary tract, including the bladder. It may be used instead of an IVP to look at the upper part of the urinary system. It can also help show enlarged lymph nodes that might contain cancer, as well as other organs in the abdomen and pelvis.
- **Magnetic resonance imaging (MRI) scan :**MRI scans provide detailed images of soft tissues in the body, but MRI scans use radio waves and strong magnets instead of x-rays. The energy from the radio waves is absorbed and then released in a pattern formed by the type of body tissue and by certain diseases. A computer translates the pattern into very detailed images of parts of the body. MRI images are particularly

useful in finding signs that the cancer has spread outside of the bladder into nearby tissues or lymph nodes. A special MRI of the kidneys, ureters, and bladder known as MRI urogram, can be used instead of an IVP.

1.2.6 Staging and Grading of Bladder Tumor

The American Joint Commission on Cancer in combination with the International Union Cancer Consortium meets on a regular basis to determine the tumor, nodes, and metastases (TNM) staging classifications (T" plus a letter and/or number (0 to 4) is used to describe the size and location of the tumor, The "N" stands for lymph nodes, the tiny, bean-shaped organs that help fight infection. Lymph nodes near where the cancer starts are called "regional lymph nodes". Lymph nodes in other parts of the body are called "distant lymph nodes" and M indicates whether the cancer has spread to other parts of the body called distant metastasis) (Sobin *et al.,* 2009 and Edge *et al*., 2010). The 2009 staging system is shown in Table (1-1).Staging is a way of describing where the cancer is located, if or where it has invaded or spread, and whether it is affecting other parts of the body. It's determination is' based on examining the sample removed during a TURBT (Descotes *et al*.,2014). Early bladder cancer is also called "superficial bladder cancer" or "non muscle invasive bladder cancer" that includes noninvasive papillary urothelial carcinoma (pTa), carcinoma *in situ* (CIS) (pTis), and tumor invading into the lamina propria (pT1) while invasive bladder cancer has spread into or through the muscle layer of the bladder. The cancer may have spread into the muscle layer of the bladder (T2), grown through the muscle layer (T3) and spread into the prostate, uterus or vagina, or into the wall of the pelvis or tummy (abdomen) (T4) or spread to a

nearby lymph node (N1) as shown in Figure (1-2) (Cheng *et al*.,2009; Goodison *etal*.,2013). However, nearly 60% to 70% of these tumors will recur, and 25% will progress into a higher stage or grade. Although many chemical agents have shown some evidence of activity against tumor recurrence, their toxicity and incomplete efficiency have limited their use as common chemotherapy agents. These factors highlight the urgency of novel adjuvant agents. Natural products, including those from plants and microorganisms, provide much potential for anticancer drug discovery (Yang *et al*., 2015).Doctors also describe the type of cancer by its grade (G), which describes how much cancer cells look like healthy cells when viewed under a microscope who compares the cancerous tissue with the healthy tissue. Healthy tissue usually contains many different types of cells grouped together. If the cancer looks similar to healthy tissue and contains different cell groupings, it is called "differentiated" or "a low-grade tumor". If the cancerous tissue looks very different from healthy tissue, it is called "poorly differentiated" or" a high-grade tumor". Classification of bladder cancer is important to determine the appropriate treatment strategy and predict outcomes. Most systems are based upon the degree of tumor cell anaplasia that is, the loss of cellular "differentiation," the distinguishing characteristics of a cell (Oosterhuis *et al*.,2002;Lightfoot *et al*.,2011;Anastasiadis and Reijke,2012).Traditionally, bladder carcinomas have been graded according to the World Health Organization (WHO) 1973 grading of urothelial papilloma: well differentiated (G1), moderately differentiated (G2), or poorly differentiated (G3). In 2004, the WHO and the International Society of Urological Pathology (ISUP) published a new grading system that employs specific cytological and architectural criteria.The new WHOclassificationdifferentiates betweenurothelial pilloma (completely benign lesion),papillary urothelial neoplasm of low

malignant potential (PUNLMP), and low-grade and high-grade cancer.

Table (1-1): TNM Classification System of Bladder Cancer (Walsh *et al*., 2012)

Primary Tumor (T)	
TX	**Primary tumor cannot be assessed**
T0	**No evidence of primary tumor**
Ta	**Noninvasive papillary carcinoma**
Tis	**Carcinoma in situ: "flat tumor"**
T1	**Tumor invades Subepithelial connective tissue**
T2	**Tumor invades Muscularis propria**
pT2a	**Tumor invades Superficial Muscularis propria (inner half)**
pT2b	**Tumor invades deep Muscularis propria (outer half)**
T3	**Tumor invades Perivesical tissue**
pT3a	**Microscopically**
pT3b	**Macroscopically (Extravesical mass)**
T4	**Tumor invades any of the following: prostatic stroma, seminal vesicles, uterus, vagina, pelvic wall, abdominal wall**
T4a	**Tumor invades prostatic stroma, uterus, vagina**
T4b	**Tumor invades pelvic wall, abdominal wall**
Regional lymph nodes (N)	
NX	**Lymph nodes cannot be assessed**
N0	**No lymph node metastasis**
N1	**Single regional lymph node metastasis in the true pelvis (hypogastric, obturator, external iliac, or presacral lymph node)**
N2	**Multiple regional lymph node metastasis in the true pelvis (hypogastric, obturator, external iliac, or presacral lymph node metastasis)**
N3	**Lymph node metastasis to the common iliac lymph nodes**
Distant Metastasis (M)	
MO	**No distant metastasis**
M1	**Distant metastasis**

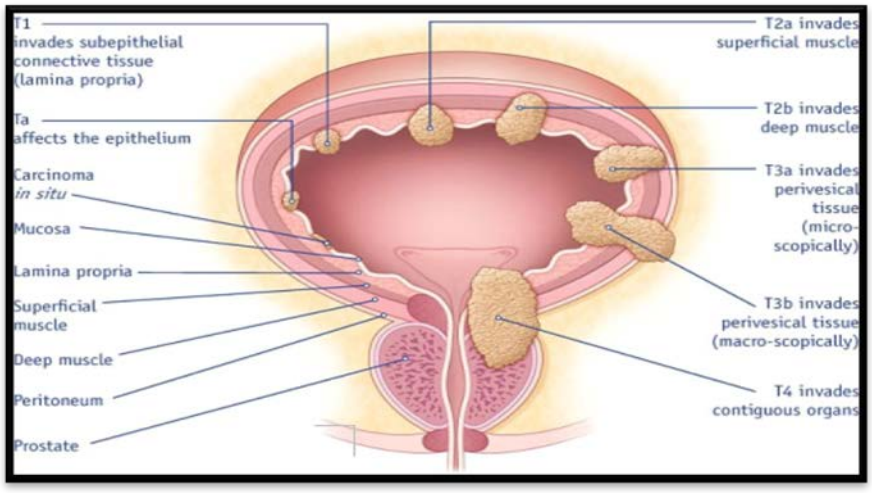


Figure (1-3): Tumor Staging in Human Bladder (Cheng *et al*., 2009)

Comparisons of the 1973 and 2004 classification systems are shown in Table (1-2). According to American Joint Committee on Cancer (AJCC), staging system and grading system which incorporate a range of histologic descriptions such as, More recently, ,bladder cancer grading to only two categories: 1) well-differentiated or low grade, and 2) poorly differentiated or high grade (Colombel *et al*., 2008; Kenneth *et al*.,2009; Cheung *et al*.,2013).

Table (1-2): World Health Organization grading of tumor in 1973 and 2004

WHO 1973	WHO 2004
Urothelial papilloma completely benign lesion	**Urothelial papilloma completely benign lesion**
Grade 1: well differentiated	**PUNLMP Low-grade papillary urothelial carcinoma**
Grade 2: moderately differentiated	**Low-grade papillary urothelial carcinoma**
Grade 3: poorly differentiated	**High-grade papillary urothelial carcinoma**

PUNLMP = papillary urothelial neoplasms of low malignant potential

1.2.7 Cytokines

Cytokines represent a large family of proteins molecules that have a broad range of functions including interleukins(ILs), interferons (IFNs) and members of tumor necrosis factors family (TNF) produced by a large variety of cells like macrophages, B-lymphocytes, T-lymphocytes and mast cells, as well as endothelial cells, fibroblasts, and various stromal cells; a given cytokine may be produced by more than one type of cell (Zhang and Jianxiong ,2009).

Cytokine is a general name; other names include lymphokine (cytokines made by lymphocytes),monokine (cytokines made by monocytes),chemokine (cytokines with chemotactic activities), and interleukin (cytokines made by one leukocyte and acting on other leukocytes) (Pollard, 2004;).All of them play an important role in the initiation and regulation of immune response. Therefore, cytokines signaling are thought to be contributing in the tumor via two mechanism: stimulation of cell growth and inhibition apoptosis of damaged cells (Gilson *et al*., 2015).

1.2.7.1Interleukin -6

Interleukin-6 is a glycoprotein of a molecular weight of 26 KDa. It is composed of 184 amino acids. The human gene for IL-6 is located on 7p15-p21 chromosome and has the structure similar to the gene for granulocyte colony-stimulating factor, which explains the functional similarity of both cytokines (Tsui *et al*., 2013). IL-6 produced by many different cell types and the main sources are stimulated monocytes, and in a smaller percentage by fibroblasts, endothelial cells, T-cells and B-lymphocytes, granulocytes, smooth muscle cells, eosinophils, osteoblasts, mast cells, glial cells, and keratinocytes also produce IL-6 after stimulation (Abdulmohymen and Ashoor, 2010).

The IL-6 receptor is expressed on T-cells, activated B-cells, peripheral monocytes and some macrophage. Physiological stimuli for the synthesis of IL-6 are IL-1, bacterial endotoxins, TNF (Lukaszewicz *et al*., 2007). It was also termed a β-cell stimulatory factor, β2-interferon, a hybridoma growth factor, or a cytotoxic T cell differentiation growth factor. IL-6 is a multifunctional cytokine with pleiotropic effects (Mastorakos and Ilias, 2007). Its importance lies in the stimulation of lymphocytes B differentiation and induction of a permanent differentiation of lymphocytes B into plasma cells which produce different classes of immunoglobulin. IL-6 stimulates lymphocytes T to the production of interleukin-2 (IL-2) and the synthesis of its receptors. Both IL-6 and IL-1 also activate lymphocytes T which recognize antigens, and stimulate the proliferation and differentiation of cytotoxic lymphocytes in the presence of IL-2. Thus it plays a major role in immunologic response (Chen *et al*., 2013).

IL-6 plays a major role in pathogenesis and development of malignancies. It helps tumor to grow through inhibiting apoptosis and the induction of tumor angiogenesis (Lee and Margel,2011).IL-6 may be involved in the regulation of solid tumor growth in paracrine and autocrine ways. IL-6 contributes to the proliferation of bladder cancer cells and other cancer especially those at the advanced stage of development and It's concentrations depends on the tumor stage and histological grade (Salgado *et al*., 2003+ and McBeth *et al*., 2015).

1.2.7.2 Interleukin-10

IL-10 is also known as cytokine synthesis inhibitory factor (CSIF) that functions as a positive or negative mediator in innate and adaptive immunity under different circumstances(Saraiva and O-Garra, 2010). IL-10 is coded by a

gene located on chromosome 1 produced by numerous cell types including T cells (Th1, Th2 and Treg), B cells, monocytes/macrophages, keratinocytes epithelial cells and binds to its receptor (IL-10R) expressed on the cell surface, which consists of R1 and R2 subunits (Chan *et al*; 2013). Mature human IL-10 is an 18KDa molecule (160 amino acids) with one potential N-linked glycosylation site and four cysteines which form two intrachain disulfide bridges (Maynard *et al*., 2007 and Luo *et al*., 2012).

IL-10 has been shown to inhibit cellular immune responses via a number of mechanisms. IL-10 can block the accumulation of macrophages and DC at the tumor site, down-regulate the expression of MHC class II on these cells, thus suppressing the induction of specific immune responses(Chaudhry *et al*.,2011). It has also been reported that CD4+ T cells in the presence of IL-10 during activation can differentiate T regulatory cells 1 (Tr1) that are responsible for the peripheral immune tolerance induced by IL-10 (Cai *et al*., 2007). In addition, IL-10 can also prevent the release of cytokines (e.g., IFN-γ and TNF-α) and reactive nitrogen/oxygen intermediates (e.g., NO) by macrophages and NK cells, thus inhibiting inflammatory and tumorcidal activities of these cells(Tanikawa *et al*.,2012). In addition, IL-10 can also inhibit cancer development and progression through its regulatory effects on inflammatory cytokine production as inflammation is often associated with the increased tumor angiogenesis and invasiveness. Thus, IL-10 plays a dual role in tumor-associated immune responses, either promoting antitumor immune responses or mediating tumor escape from immune surveillance (Mocellin *et al*., 2005).

Increased concentrations of IL-10 and other Th2 cytokines (e.g., IL-13) have been observed in the urine of bladder cancer patients and that urinary IL-6/IL-10 ratio might be useful as a prognostic marker of recurrence in patients

with intermediate risk superficial bladder cancer(Margel *et al*., 2011). IL-10 gene polymorphisms in bladder cancer have functions in a highly complex and coordinated manner may lead to altered production and activity of this cytokine, thus, affecting the susceptibility to bladder cancer.

1.2.7.3 Tumor Necrosis Factor

Tumor necrosis factor-α was recognized in 1986. It is encoded by gene located on chromosome 6p21.3. It is synthesized as 26 KDa (233 amino acids) membrane bound pro-peptide (pro-TNF-α) and secreted upon cleavage by TNF-α converting enzyme(Rama *et al*., 2012). The secreted form of TNF-α is a soluble 17 KDa (157 amino acids) that binds with two receptors on the cell surface: TNFRI (P55 receptor) and TNFRII (P75 receptor). It is the most important pro-inflammatory cytokine produced chiefly by activating macrophage and diverse kinds of cells such as, neutrophils, fibroblasts, mast cell, eosinophil, NK cells, T cells, neurons during acute inflammation and tumor cells that is responsible for many signaling events within the cell leading to necrosis or apoptosis (Sun and Fink,2007;Holla *et al*., 2014).

In contrast, TNF-α production levels can induce a tumor phenotype. A TNF-α tumor promotion mechanism which is based on reactive oxygen species and reactive nitrogen species which can induce DNA damage, hence facilitating tumorigenesis. TNF- α-mediated inflammation has been linked to cancer (Landskron *et al*., 2014). TNF-α is one of the major mediators of inflammation and linked to all steps involved in tumorigenesis: cellular transformation, promotion, survival, proliferation, invasion, angiogenesis, and metastasis. Tumor cells secrete their own TNF-α which further enhance the expression of other growth factors such as TGF-α and epidermal growth factor receptor (EGFR), both of which mediate proliferation (Thompson *et al*., 2015).

1.2.7.4 Macrophage Migration Inhibitory Factor

Macrophage migration inhibitory factor (MIF) is a proinflammatory cytokine shown to promote tumorigenesis discovered in 1966(Shimizu,2010). The *MIF* gene coded 12.5 kDa polypeptide and the protein consists of 115 amino acids which lie on chromosome 22q11.2 and the regulation of the gene is done by the two polymorphic sites in the promoter region .The first site consists of the CATT repeat at -794 which repeats 5-8 times and the second site is a single nucleotide polymorphism at -173(G/C)(Yuan *et al*.,2013). This site is associated with the enhanced promoter activity in some cancer cell lines and the activity proportionally increased MIF levels in serum (Xue *et al*., 2010;;Spoorthy *et al*.,2012). MIF was thought to inhibit the migration of macrophages, thus hence derived it's name and helps macrophage in its functions such as phagocytosis, adherence, spreading, and metabolism. MIF is thought to be released from monocytes/macrophage in presence of glucocorticoids which acts as the inflammatory mediator to stimulate the expression of other cytokines like TNF-α, IL-1, IL-6(Bucala and Donnelly, 2007 and Grieb *et al*., 2010). It's binding receptor was identified recently by the expression cloning as CD74 which is the cell-surface form of the MHC class II invariant chain that signal transduction requires the recruitment and activation of an additional protein CD44 (Zheng, 2012).

MIF, apart from being involved in stages of cancer, has a potential to suppress the tumor suppressor gene p53 thereby leading to an uncontrolled cell growth which over-expressed under hypoxia **(**Hypoxia is a condition where there is low availability of oxygen for the cells when the cells are under stress, they adapt themselves to that condition and continue with the proliferation**).**It plays an important role in tumor and also up-regulated. There is an increase in the

expression of matrix mettaloproteinases (MMPs) which degrade the basal membrane and the tumor cells enter into the blood circulation when they receive proper homing factors, they establish secondary tumors in different organs by interacting with the homing factors. The expression of MIF is directly proportional to the expression of MMPs (Choudhary *et al.,* 2013; Knowles and Hurst 2015).

1.2.8 Polymorphism of MIF Gene and Bladder Cancer

A DNA polymorphism is a difference in the nucleotide sequence between individuals of the same species. These differences can be single base pair changes, deletions, insertions, or even changes in the number of copies of a given DNA sequence. SNPs (single nucleotide polymorphisms) are the most common type of DNA polymorphism in humans. An example of an SNP would be if a cytosine (C) nucleotide is present at a particular locus in one person's DNA but a thymine (T) nucleotide occurs at the same locus in another person's DNA. A polymorphic variant of a gene may lead to the abnormal expression or to the production of an abnormal form of the gene; this may cause or be associated with disease (Kwok and Chen, 2003 and Cardiol, 2014).

Cancer results from a series of genetic alterations leading to a progressive disorder of the normal mechanisms controlling growth, differentiation, cell death, or genomic instability. The response of the cell to genetic injury and its ability to maintain genomic stability by means of a variety of DNA repair mechanisms are essential in preventing tumor initiation and progression (Hosgood *et al.*, 2008).

Polymorphisms of several DNA repair-related genes and some cytokine genes have been found to be associated with the risk of developing different

tumor types. Polymorphic alleles of DNA genes would predispose carriers to a higher risk of developing cancer but would not necessarily cause cancer. Therefore, possible gene–gene interaction and gene–risk factor interaction may play an important role in modifying the cancer risk associated with particular gene polymorphism in different study populations (Michiels *et al*., 2009 and Wu and Shen, 2011). The nucleotide sequences of DNAs in humans are not identical in different individuals. Nucleotide substitutions have been estimated to occur every few hundred base pairs in the human genome. Nucleotide sequence polymorphism has been detected as restriction fragment length polymorphism (RFLP)(Orita *et al*., 1989). Although RFLPs are very useful for distinguishing two alleles at chromosomal loci, they can be detected only when DNA polymorphisms are present in the recognition sequences for the corresponding restriction endonucleases or when deletion or insertion of a short sequence is present in the region detected by a particular probe. In recent years, several functional polymorphisms, particularly, SNPs have been identified in cytokines and their receptor genes that regulate levels of cytokine expression (Birdsell *et al*., 2012). These have been implicated as immune prognostic markers in diseases, including differential response to therapy and as biomarkers of graft outcome following organ and stem cell transplantation. Population distribution of cytokine gene polymorphisms (CGPs) reveals significant variations in allele frequencies in different ethnic groups and this might explain, to some extent, the observed differences in SNP associations with various diseases and immune-pathologies (Kaur and Mehra ,2012and Berno *et al*., 2014).

1.2.9 CD74

CD74 is an integral membrane protein has a molecular weight 33KD

which consists of 296 amino acids. It's gene is located on chromosome 5q32. There are four major isoforms of this protein in humans and the most common isoform of CD74 is the p33 isoform. CD74 is mainly expressed in antigen presenting cells, endothelial cells and neuroglia cells (Shachar and Haran, 2011 and Stariets *et al*., 2015). Cluster of differentiation 74 performs multiple roles in B cells, T cells, and antigen-presenting cells within the immune system. It has two main functions:

- **MHC II chaperon**: It is a restricted antigen presentation, including the prevention of MHC class Ⅱ to bind non-processed peptide and self-antigen. CD74 was originally reported to be a molecular chaperone for regulating MHC class Ⅱ folding in the rough endoplasmic reticulum (ER), where it was thought to play a major role in processing and transporting MHC class Ⅱ molecules in the immune system. Absence of CD74 results in aberrant MHC class Ⅱ-dependent antigen processing and perturbs host defenses (Stumptner *et al*., 2002).
- **CD74 as cell surface receptor**: A small proportion of CD74 is modified by the addition of chondroitin sulfate (CD74-CS), and this form of CD74 is expressed on the cell surface .The cell surface expression of CD74 is not strictly dependent on class II MHC and numerous non-class II positive cells express CD74 where it can serve as a receptor for the initiation of different signaling cascades (Binsky *et al.*, 2007) .The MIF was found to be the natural ligand of CD74. MIF binds to the extracellular domain of CD74 with high affinity and it initiates a signaling cascade

.CD74 forms a complex with CD44 which is essential for the MIF-induced signaling cascade (Richard *et al*., 2014) .

CD74 was reported as an accessory signaling molecule in cancers because of its localization on the plasma membrane in certain cell types, and its role as a surface-binding receptor for MIF, a pro-inflammatory cytokine. Indeed, it is now generally accepted that the oncogenic role of CD74 is MIF-dependent , CD74 promotes cell proliferation and motility and prevents cell death in a macrophage migration inhibitory factor dependent manner(Liu and Lin , 2014). Its roles is an accessory signal receptor on the cell surface and had the ability to interact with other signaling molecules which make CD74 an attractive therapeutic target for the treatment of cancer. In the bladder expression is increased in high-grade, invasive carcinoma of the bladder. Its expression is significantly associated with older age at diagnosis overexpression of CD74 in the intracellular space and on the cell surface could impair MHC class Ⅱ antigen presentation by tumor cells, thereby contributing to the immune escape and facilitating tumor metastasis (Choi *et al*., 2013).

1.2.10 CD 44

CD44 is a transmembrane glycoprotein that has been postulated to play important roles in a variety of biological processes in healthy and diseased tissues. CD44's encoding gene is located on chromosome 11p3and it consists of at least 21 exons. The CD44 molecule consists of three core epitopes encoded by ten exons with alternative mRNA splicing of the remaining exons generating multiple isoforms.

The standard form of CD44 (CD44s) is expressed on almost all cells and is heavily glycosylated, while variant isoforms are expressed in a cell- and tissue

specific manner(Ross *et al*., 2001; Chang *et al*., 2009r. Post-translational modifications, such as glycosylation and alternative splicing, add further to

the diversity of the function of the special CD44 isoforms (Matuschek *et al*., 2014 and Stepan *et al.,* 2015).

CD44 is a widely expressed cell surface antigen that serves as an adhesion molecule in cell-cell and cell-matrix interactions(Liao *et al*., 2014).The expression of the CD44 gene becomes disorderly in the early stages of carcinogenesis, and excessive quantities of many inappropriate alternatively spliced CD44 variants accumulate in cancer cells which promotes signaling pathways that induce tumor growth, survival as well as cancer cell invasion (Erdogan *et al*., 2008 and Wakamatsu *et al.,* 2012).

In many epithelial tumors, expression of is CD44 correlated with tumor progression, metastatic potential, and patient prognosis. In urothelial cancers, strong expressions are associated with a well-differentiated histology, and predominantly weak negative expressions are found in poorly differentiated, invasive bladder cancers(Goodison *et al.,* 1999). In addition to the expressions of CD44 and its variant forms in urothelial carcinoma tissues, detection of elevated levels of CD44 protein in exfoliated urothelial cells in the urine can be used to identify primary and recurrent urothelial tumors(Yildrim *et al.,* 2014*).*

1.2.11 p53 (Tumor Suppressor Protein)

p53 tumor suppressor gene is known as the “guardian of the genome” located on chromosome 17p13, which encodes wild-type p53 protein that consists of 393 amino acid. This protein has a role in cell cycle arrest after apoptosis, DNA damage and mitotic check point regulation(Wiman, 2007). In other words, this gene plays an important role in the control of cell proliferation.

It is tightly suppressed in normal cells by Mdm2 which inhibits the p53activity by degrading it when DNA mismatch is sensed by the cell cycle. The N terminal

end of p53 is phosphorylated there by separating the p53from Mdm2 and it carries out its function (Girardini *et al*., 2014). In the cell, p53 protein binds DNA, which in turn stimulates another gene to produce a protein called p21 that interacts with a cell division-stimulating protein (cdk2) when p21 is complexed with cdk2, the cell cannot pass through to the next stage of cell division (Grivennikov *et al*., 2010).

Gene mutations have as a result the production of an altered protein with an altered function. These altered proteins do not play their role correctly and have a prolonged half time(Siegel *et al*., 2012). Mutations of p53 are among the most frequent causal events in many cancers, and their combined inactivation has profound consequences for tumorigenesis for invasive bladder cancer (Green and Kroemer, 2009and Hai-Bin *et al*., 2013) .

There are enough studies which prove that the nuclear accumulation of p53 protein is an important prognostic indicator for bladder cancer progression. Mutant p53 can no longer bind DNA in an effective way, and as a consequence the p21 protein is not made available to act as the 'stop signal' for cell division (Takimoto *et al.*, 2002 and Madka *et al*., 2013),Thus cells divide uncontrollably, and form tumors. Therefore, p53 is one of the most important cancer-related genes and its mutation has been reported in a variety of cancers(Noroozinia *et al*., 2015). By losing the apoptotic pathway and cell cycle checkpoints p53 tumor suppressor gene has been found to be mutated in more than 50% of human cancers. The abnormality of this gene plays a fundamental role in tumor development and progression **(**Olsson *et al.*, 2012).

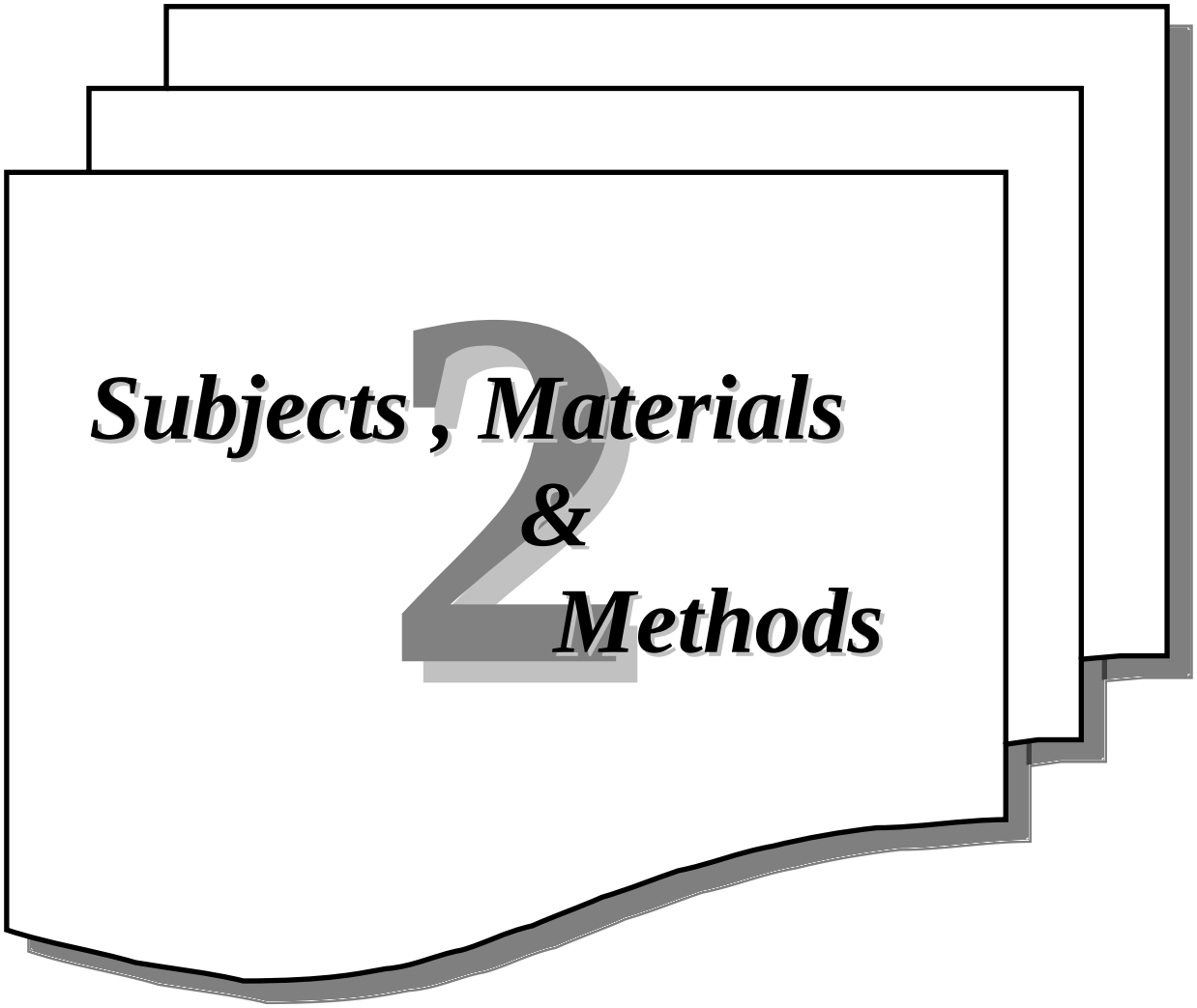
1.2.12 p21 (Cyclin- Dependent Kinase Inhibitor).

This protein is encoded by the *CDKN1A* gene located on chromosome 6p21.2 in humans. It's role as an inhibitor of cellular proliferation in response to DNA damage. It binds to and inhibits cyclin-dependent kinase activity, preventing phosphorylation of critical cyclin-dependent kinase substrates and blocking cell cycle progression. The expression of this gene is tightly controlled by the tumor suppressor protein p53, through which this protein mediates the p53-dependent cell cycle G_1 phase arrest in response to a variety of stress stimuli (Harper *et al*., 2010 and Tang *et al.*, 2015).Sometimes, p21 is expressed without being induced by p53. This kind of induction plays a big role in p53 independent differentiation which is promoted by p21. Expression of p21 is mainly dependent on stimulus provided and type of the cell. Growth arrest by p21 can promote cellular differentiation. p21,therefore, prevents cell proliferation (McKenzie *et al*., 2003)

p21 was expressed in a variety of human malignancies and is correlated with tumor progression and a poor prognosis in various carcinomas. Altered tumor suppressor gene p21 has been reported to be associated with bladder cancer progression. p21 is an independent predicator of time to recurrence and overall survival and acts synergistically with p53and associated with bladder cancer risk and death (Duggan and Williamson,2004). Alterations in p53 function can result in loss of p21 expression and may be one of the mechanisms by which altered p53 influences tumor progression. Despite regulation by tumor suppressor gene p53, loss-of-function mutations in p21 (unlike p53) do not

accumulate in cancer nor do they predispose to cancer incidence (Kausch, 2002 and Resnick *et al*., 2013).

Chapter Two



2- Material and Method

2.1 Materials

2.1.1 Apparatus &Equipments

Apparatus &Equipment	Company/Country
Autoclave	Express/Germany
Auto-vortex	Stuart/England
Compound light Microscope	Olympus /Japan
Deep Freeze	Teka/Spain
Electrophoresis System	Thermoscientific/USA
ELISA Reader	Bio Rad/ USA
Freezing Plate	Leica/Germany
Gel Documentation System	Bio Rad/ USA
Hot Plate Magnetic Stirrer	Gallenkamp/England
Incubator	Gallenkamp/England
Laminar Air Flow Capinate	ESCO/ Singapore
Micro-centrifuge	Eppendorff/Germany
Micropipettes	Eppendorf/Germany
Microwave Histo-processer	Leica/Germany
Nanodrop	Thermoscientific/USA
Oven	Gallenkamp/England
Pap Pen	Dako/Denmark
PCR Cabinet	ESCO/Singapore
Plate Shaker	Heidolph/ Germany
Portable Centrifuge	Eppendorf/Germany
Positive Charge Slide	Dako/Denmark

Apparatus &Equipments	Company/Country
Rotary Microtome	Leica /Germany
Sensitive Balance	Denver/Germany
Slide Holder	Leica /Germany
Thermal Cycler	BioRad/ USA
Water Bath	Leica/Germany

2.1.2Chemicals

Chemical	Company /Country
Catalase Reagent(H_2O_2)	Local market/Iraq
DPX	BioGenex/USA
Eosin	BioGenex/USA
Ethanol	Scharlau/Spain
Ethidium Bromide	Sigma/USA
Formalin	Biosolve/France
Glucose	BDH/England
Glycerol	BDH/England
Gram Staining Kit	Syrbio / Syria
Hematoxylin	Scharlau/Spain
Kovacs Reagent	BioMerieux /France
Methyl Red	BDH/England
NaCl and KOH	BDH/England

Chemical	Company /Country
Paraffin	Scharlau/Spain
Sodium Citrate	BDH/England
Wash Buffer	Dako/ Denmark
Xylene	Scharlau/Spain
α-Nephthol	Fluka / Switzerland
Oxidase reagent	BioMerieux /France

2.1.3 Media and Ingredients

Medium or	Company/Country
Agar agar, Blood agar	Difco/USA
Eosin Methylene Blue	Himedia / India
MacConkey agar	
Manitol salt agar	
MR-VP media	
Nutrient agar	
Peptone	
Simmons citrate agar	
Sabouraud dextrose agar	
Triple sugar Iron Agar (TSI)	
Urea Agar Base	
Yeast Extract	

2.1.4 Specific primers of *MIF* Gene (Yuan *et al* ., 2012)

Primer	Sequence	Size(bp)	Company/Country
Forward	/5- ACTAAGAAAGACCCGAGGC-/3	366	Alpha DNA / Canada
Reverse	/5-GGGGCACGTTGGTGTTTA-/3		

2.1.5Materials of DNA Purification ,PCR and RFLP

	Material	Company / Country
1-	Relia prep DNA purification Kit	Promega / USA
2-	Agarose	
3-	TBE Buffer	
4-	TE Buffer	
5-	Safe Dye	
6-	Nuclease Free Water	
7-	Master Mix	
8-	*Alu I*	
9-	DNA Marker(100 bp ladder)	

2.1.6 Kits

	Kit	Company/Country	Catalog No.
1-	Antibody Diluent	Abcam/USA	Ab64211
2-	Api 20 E kit	BioMerieux /France	20160
3-	Human IL-6 Quantikine ELISA Kit	R&D/USA	D6050
4-	Human IL-10 Quantikine ELISA Kit	R&D/USA	D1000B
5-	Human TNF-α Quantikine ELISA Kit	R&D/USA	DTA00C
6-	Human MIF Quantikine ELISA Kit	R&D/USA	DMF00B
7-	Mouse Specific HRP/DAB(ABC) Detection Kit	Abcam/USA	Ab64259
8-	Primary Antibody for CD 105	Abcam/USA	ab114052
9-	Primary Antibody for CD 44	Abcam/USA	ab6124
10-	Primary Antibody for CD 74	Abcam/USA	ab9514
11-	Primary Antibody for P^{21}	Abcam/USA	ab80633
12-	Primary Antibody for P^{53}	Abcam/USA	ab26
13-	Retrieval Solution (pH=9)	Dako/Denmark	

2.2 Methods

2.2.1 Patients and Individuals

This study included 135 Iraqi patients and subject of 32 to 85 years from both sexes (104 male and 31 female) who attended Urology Unit at Al-Yarmook Teaching Hospital and Al-Jaibachi Private Hospital in Baghdad for the period from the March to November 2014. As shown in appendix 1,was filled for each of them including: name, gender, age, smoking, alcohol intake, location and family history, taken chemotherapy or not and the healthy state of urinary tract

were carefully taken in consideration. The 135 subjects were classified into three groups after clinically diagnosed by a consultant urologist and laboratory tests.

- First group contained 73 patients of of urinary bladder carcinoma (UBC), 60 male and 13 female of 43-85 years.The tumors were graded as low or high on the basis of WHO classification criteria. The TNM stage was classified as non-invasive (Ta-T1) and invasive (T2–T4) according to the American Joint Committee on Cancer guidelines.
- Second group contained 32 patients with urinary bladder disorder (UBD), 22 male and 10 female ,of 33 to 68 years.
- Third group included 30 healthy individuals 22 male and 8 female of 32 to 63 years.

2.2.2 Sterilization methods

- **Moist heat sterilization :** Autoclave was used to sterilize media, buffers and solutions at 121 ºC (15 Ib/ in^2) for 15 minutes.
- **Dry heat sterilization:** Electric oven was used to sterilize glass wares and others by heating at 180 ºC for 2 hours.
- **Membrane Filtration:** 0.22 Millipore filter unit was used to sterilize the sugars, urea, and crystal violet after dissolving them in distilled water.

2.2.3 Samples collection

- **Urine samples**

  Midstream urine samples were collected from UBC, UBD patients and healthy. Each of the samples was collected and transported to the laboratory during 1 hour by using a cool box.

- **Blood samples**

Blood samples have been collected from all groups via venipuncture by using 5 ml plastic disposable syringes (before surgery for UBC group). Each blood sample was divided into two parts: 2 ml were put in the EDTA tube for later molecular analysis and 3 ml were allowed to clot at RT, then centrifuged for 15 minutes at approximately 5000 rpm to obtain serum which is used for measurement of some cytokines level.

- **Tissues samples**

Seventy three biopsies were collected from UBC patients after cystoscopy and radical cystectomy surgery while 10 normal bladder biopsies have been collected from forensic autopsy. Samples were fixed in 10% formalin and embedded in paraffin blocks. Slides were prepared from tissues embedded in the paraffin blocks then stained by hematoxylin and eosin before examined by the histopathologist to determine the degree of tumors differentiation. After that, 5 µm thickness sections were made from each paraffin embedded block and fixed on positively charged slides to be subjected to immunohistochemistry procedures for detection of CD markers (CD74, CD44 ,p53 and p21).

2.2.4 Media preparation

- **Blood agar (Collee *et al*.,1996).**

It was prepared according to the instructions of the company. The pH was adjusted to7 and then sterilized by autoclaving before cooled to 50 °C. After that, blood base agar supplemented with (5-10%) human blood was added and mixed well, poured in sterile petri dishes and kept at 5°C until use.

- **Peptone water (Collee *et al*., 1996).**

It was prepared by dissolving 20 g of peptone and 5 g of NaCl in 1000 ml of distilled water and divided in test tubes (5ml in each tube) before sterilized by autoclaving for 15 minutes.

- **Urea agar (Collee *et al*.,1996).**

It was prepared by adding 24 g of urea agar base to 950 ml of distilled water, pH was adjusted to (6.8-7.0) and sterilized by autoclaving then left to cool to 50 °C before adding 50 ml of 20% urea solution which was previously sterilized by filtration using 0.22 micrometer) filter units. After that, 5 ml were dispensed in sterile test tubes and left to solidify declined as slants.

- **Semi solid agar medium (Collee *et al*., 1996).**

It was prepared by dissolving 0.5% of agar in nutrients broth medium and sterilized by autoclaving. After that, 10 ml were dispensed in sterile test tubes and left to be solidify in vertical position. It was used for motility test.

2.2.5 Indicators preparation

- **Methyl red reagent (Collee *et al*., 1996).**

It was prepared by dissolving 0.1 g methyl red pigment in 300 ml of ethanol (99 %) then 200 ml of D.W was added.

- **Vogas- Proskour reagents (Collee *et al*.,1996)**

It was used with acetone formation test, and consisted of the following:-

- First solution (40 % KOH) ,it was prepared by dissolving 40 g of KOH in 100 ml of D.W.

- Second solution: It was prepared by dissolving 5 g of α-naphthol in 100 ml of absolute ethanol.

2.2.6 Isolation of bacteria

One loopfull of undiluted urine sample was spread on blood agar, eosin methylene blue and MacConkey agar plate. Plates then incubated over night at 37 ºC. This process was repeated several times for purity before use for further diagnosis steps.

2.2.7 Identification of bacteria(Atlas *et al*.,1995).

2.2.7.1 Cultural identification

Identification was made according to the shape, color, size, edges and height of the colony on the surface of agar plates.

2.2.7.2Microscopical identification

A loopfull of each of the suspected isolate was fixed on a microscopic slide then stained by Gram staining method to examine cell shape, grouping, Gram reaction and spore forming.

2.2.7.3Biochemical identification

Biochemical tests were more specific in the identification of bacteria which included many tests:

- **Indol test (Colle *et al*.,1996)**

  This test demonstrates the ability of bacteria to decompose the amino acid tryptophan to indol. Peptone water was inoculated by the isolate and incubated at 37 ºC for 24 hr. After that,0.5 ml of Kovac's reagent was added and mixed gently. A positive result was recorded by the appearance of a pink ring on surface of the broth.

- **Methyl red test (Colle *et al*.,1996).**

This test is employed to detect the production of sufficient acid during fermentation of glucose. The MR –VP medium was prepared and divided into test tubes before sterilized by autoclaving. After that, it was inoculated by the isolate ,then incubated at 37ºC for 48 hr. Five drops of methyl red reagent were added and mixed gently. Appearance of red color represents positive result, while yellow color represents a negative result .

- **Catalase test (Colle *et al*.,1996).**

This test demonstrates the presence of catalase which catalyze the release of oxygen from hydrogen peroxide This test was performed by putting a colony on a glass slide and smearing it then a drop of 3% H_2O_2 was added. The appearance of bubbles indicates a positive result.

- **Voges – Proskuar test (Colle *et al*.,1996).**

The MR-VP medium was prepared and divided into 5 ml test tubes before sterilized by autoclaving , then inoculated by isolate and incubated at 37 ºC for 48 hr. After that, few drops from VP reagent were added with mixing . Appearance of pink color during 2-5 min indicates positive test.

- **Oxidase test (Atlas *et al*.,1995)**

This test was done by adding few drops of oxidase reagent on a filter paper .Then a clump of cells was picked up from growth on slant with a sterile wooden stick and smeared on the filter paper. The development of purple color within 5 -10seconds indicates a positive result.

- **Citrate utilization (Colle *et al*.,1996)**

This test was used to detect the ability of bacteria to utilize citrate as the sole of carbon and energy source and an ammonium salts as the source of nitrogen. Simmon citrate agar was streaked by the isolate culture and incubated at 37 ºC for 24 hr. Changing the color of medium from green to blue indicates a positive result.

- **Urease test (Atlas *et al.*,1995)**

This test was used to examine the presence of urease enzyme. Urease production was detected by inoculating the surface of Christensen urea agar slants with isolate and incubated at 37 ºC for 24 hr. After incubation, appearance of pink color indicates a positive result while yellow negative result.

- **TSI test (Atlas *et al.*,1995)**

Each isolate was cultured on triple sugar iron agar slants by stapping and streaking on slant surface, then incubated for 24 hr. at 37 ºC. Changing color of medium was changed from red to yellow indicates acid production , while formation of black precipitate indicates ferric sulfate and pushing the bottom of agar to the top indicate CO_2 production .

- **Motility test (Cruckshank *et al*.,1975).**

In semi–solid agar medium, the motile bacteria (swarm) gave diffused spreading growth that was easily recognized by the naked eye. The medium was prepared by adding 0.5 % of agar to the nutrient broth and left to set in a vertical position. It is important that the final medium should be clear and transparent. The isolate was inoculated in straight line before incubated at 37°C for 24 hours. Motile bacteria would diffuse.

- **Coagulase test(Atlas *et al.*,1995).**

Human plasma in a dilution of 1:10 to normal saline (0.85% NaCl) was prepared and placed in a small tube . The isolate under test was inoculated by adding 0.1 ml of it over night culture .The tube was incubated at 37 °C and examined for coagulation after 1-3 hours. Conversion of plasma into soft gel was observed by tilting tube to horizontal position.

2.2.8 Identification of bacteria by Api 20E & Api Staph Kit

Identification of the isolates was also carried out by subculturing representative colonies from MacConkey agar plates and Manitol salt agar plate on api 20 E and api 20 Staph microtubes systems respectively. This system is designed for the performance of 20 standard biochemical tests. Each test in this system is performed within a sterile plastic microtube which contains the appropriate substrate and affixed to an impermeable plastic strip (Gallery). Each gallery contains 20 micro tubes (each of them consists of a tube and a cupules section).

2.2.9Preparation of buffers and solutions

- **Formalin solution** (10%):

It was prepared by mixing 100 ml of (37-40%) with 900 ml of D.W.

- **Sodium citrate buffer (10mM Sodium Citrate, 0.05% Tween 20, pH 6**

A quantity of 2.94 g Tri-sodium citrate (dihydrate) was dissolved with 950 ml D.W. pH was adjusted to 6.0 then 0.5 ml of Tween 20 was added. The volume was completed to 1000 ml with D.W, mixed well and stored at room temperature for 3 months or at 4°C.

- **DAB solution**

Thirty microliters of DAB Chromogen were added to 1.5 ml (50 drops) of DAB substrate, mixed by swirling and kept in dark until use.

2.2.10 Maintenance of bacterial strains

Maintenance of bacterial isolates was performed according to (Maniatis *et al*.,1982).

- **Short term storage**

Isolates of bacteria were maintained for period of few weeks on agar plates. The plates were tightly wrapped with parafilm and stored at 4 ºC until use.

- **Medium term storage**

Isolates of bacteria were maintained for few months. Such cultures were prepared in small screw capped bottles containing 5-7 ml of nutrient agar as slants and stored at 4 ºC until use.

2.2.11 DNA extraction from blood

The ReliaPrep™ Blood gDNA Miniprep System has been used for the purification of the genomic DNA from blood samples as follow:

- The blood sample was mixed at least 10 min. at room temperature. Frozen blood samples were thawed completely before mixing for 10 minutes.
- Twenty µl of Proteinase K (PK) Solution was dispensed into a 1.5 ml micro-centrifuge tube and 200µl of blood was added before briefly mixed.
- An aliquot of 200µl of Cell Lysis Buffer (CLD) was added to the tube, capped and mixed by vortexing for at least 10 seconds (this vortexing step was essential for obtaining good yields), then incubated at 56°C for 10 min

and a ReliaPrep™ Binding Column was placed into an empty Collection Tube.

- The tube was removed from the heating block. Then 250µl of Binding Buffer (BBA) was added and mixed by vortexing for 10 seconds (the lysate should be dark green at this point). This vortexing step was essential for obtaining good yields.
- The content of the tube was added to the ReliaPrep™ Binding Column, caped and placed in a micro-centrifuge for 1 min at 12,000 rpm.
- The binding column was checked to make sure the lysate has completely passed through the membrane. If lysate was still visible on top of the membrane, the column was centrifuged for another minute (The sample can be centrifuged at alower speed, if desired the centrifugation time was increased accordingly to ensure that the lysate completely passed through the membrane).
- The collection tube containing the flow was removed, and the liquid was discarded as hazardous waste.
- The binding column was placed into a fresh collection tube. Then 500µl of Column Wash Solution (CWD) was added to the column, and centrifuged for 3 min at maximum speed. The flow was discarded (If any of the wash solution remains on the membrane, the column was centrifuged for another minute).
- Step 8 was repeated twice for a total of three washes.
- The column was placed into a clean 1.5ml micro-centrifuge tube.
- Twe hundreds µl of Nuclease-Free Water was added to the column and centrifuged for 1 min at maximum speed.
- The ReliaPrep™ Binding Column was discarded, and elute was saved.

2.2.12 Estimation of DNA concentration and purity (Green and Sambrook, 2012)

The concentration of DNA was measured by Nanodrop system. According to the Nanodrop manual, 1 µl of each DNA sample was used, and DNA purity was measured depending on the ratio of sample absorbance at wave lengths 260 and 280 nm.

2.2.13 Agarose gel electrophoresis(Green and Sambrook, 2012)

Agarose gel was used for the separation of DNA fragments. 1% was used for checking genomic DNA bands and 1.2% concentration was utilized to confirm the size of the PCR products. Gel was run horizontally in 1 X TBE buffer. Samples of DNA mixed with loading buffer (2:5v/v), and in this steps for checking PCR product, loading buffer was not used because of the green master mix reaction buffer which contains a compound which leading to increase the density of the sample with blue and yellow dyes that works as a loading dye when reaction products were analyzed by gel electrophoresis. Electrophoresis buffer was added to cover the gel and run for 1-2 hours at 5 Volt /cm. Agarose gel was stained with Ethidium bromide 0.5 µg/ml for 20 – 30 minutes. The Results were visualized by gel documentation system.

2.2.14 Preparation of primers solution

The lyophilized primer was dissolved using deionizer distilled water(DDH_2O) to obtain 100 pmol/µl in the master tube, then 10 pmol/µl was prepared as a working solution by transferring 10 µl from the master to another tube and the volume was completed to 100 µl by adding DDH_2O.

2.2.15 Amplification of DNA (*MIF* $_{-173}$gene)

In order to amplify the target gene, PCR was used with specific primer. PCR reactions were performed in 25 µl volumes in PCR tubes under sterile conditions, all the volume of the reaction mixture was completed to 25 µl with using DDH_2O and the master mix which contained optimum concentrations of reaction requirements($MgCl_2$ 1.5 mM, Taq polymerase 1 U, each dNTPs 200 µM) has been used. All amplification experiments included a negative control blank which contained all PCR material with the exception of template DNA. Mixture of reaction was listed in table (2-1).

Table(2-1): Components of master mix for detection of *MIF* $_{-173}$gene

Material	Volume(µl)
Master Mix	12.5
Forward primer	1
Reverse primer	1
D.W	8.5
Template DNA	2
Total	25

The mixture was centrifuged for 3 seconds to collect the drops from wall to ensure the final volume of the reaction of certain material which is 25 µl , then the extracted DNA was subjected to the amplification as illustrated in table (2-2). The product (366 bp) of this amplification reaction was detected by electrophoresis of the samples with 1.2 % agarose for 2 hour (70 volt), and using the 100bp DNA ladder as a size marker to determine the size of the amplified fragment.

Table (2-2) PCR program for detection of *MIF*$_{-173}$ gene

Step	Temperature(°C)	Time(min.)	No. of Cycle
Initial Denaturation	95	5	1
Denaturation	95	1	35
Annealing	60	1	
Extension	72	1	
Final Extension.	72	5	1
Final Hold	4	10	

2.2.16 Polymorphism of *MIF* gene -173G>C by RFLP

The PCR product was a 366 bp fragment that contained both the polymorphic and a non polymorphic *AluI* site. Amplified PCR product (20 µl) was digested in a 25 µl final reaction volume using 5 µl of reaction buffer and 10 units of *AluI* restriction enzyme at 37°C for 3 hours. The digested fragments were separated by 3% agarose gel then stained with Ethidium bromide. Since G/G genotype cannot be cut off by *AluI* restriction enzyme at -173 and two fragments were obtained (268 and 98bp), while genotype C/C was digested by *AluI* at -173, and three fragments were obtained (62, 206 and 98 bp) on the other hand genotype G/C was digested by *AluI* at -173, and four fragments were obtained 62, 98, 206 and 268) bp (Yuan *et al.*,2012).

2.2.17 Estimation of serum cytokines

Sandwich ELISA was used to estimate IL-6, IL-10, TNF-α and MIF in sera of UBC, UBD and healthy controls.

A-Principle of assay : This assay employs the sandwich enzyme immunoassay technique which is designed for quantitative measurement

of human cytokine (IL-6, IL-10, TNF-α and MIF). A monoclonal antibody specific for each cytokine has been pre-coated onto a microplate. Standards and samples pipetted into the wells, when mixing or reconstituting solutions, always avoid foaming. Any cytokine present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human cytokine is added to the well. Following a wash to remove any unbound antibody enzyme reagent, a substrate solution added to the wells should remain colorless until being added to the plate and color develops in proportion to the amount of cytokine bound in the initial step. The color developed in the wells will turn from blue to yellow upon addition of the stop solution. Wells that are green in color indicate that the stop solution has not mixed thoroughly with substrate solution. The color development is stopped and the intensity of color is measured.

B-Kits contents

- Four polystyrene96 well microplates (12 strips of 8 wells)have been used. Each plate was coated with a monoclonal antibody against human particular cytokine.
- Cytokine conjugate: polyclonal antibody against human cytokine conjugated to horseradish peroxidase .
- Standard: recombinant human IL-6, IL-10, TNF-α and MIF in a buffered protein base with preservatives (lyophilized).
- Assay Diluent: buffered protein base with preservatives.
- Calibrator Diluent: concentrated buffered protein base with preservatives

- Wash Buffer Concentrate: 25-fold concentrated solution of buffered surfactant with preservative.
- Color Reagent A: stabilized hydrogen peroxide.
- Color Reagent B: stabilized chromogen (tetramethylbenzidine).
- Stop Solution: 2 N sulfuric acid.
- Plate Sealers: Adhesive strips.

C-Assay procedure

Before carrying out the assay procedure of (IL-6, IL-10, TNF-α and MIF), all reagents and samples were left at room temperature before use for 30 minutes to equilibrate(the high concentrations of MIF are found in saliva.

- All reagents, working standards, and samples were prepared.
- One hundred µl of assay Diluent was added each well.
- Fifty µl of standard and sample were added to appropriate well. Plate was covered with the adhesive strip and incubated for 2 hours at room temperature.
- The plate was washed four times with wash buffer (400 µl) using a squirt bottle, manifold dispenser, or auto-washer. Any remaining wash buffer was removed by inverting the plate and blotting it against clean paper towels.
- Two hundreds µl of cytokine conjugate was added to each well. The plate was covered with a new adhesive strip and incubated for 2 hours at room temperature on the shaker.
- The plate was re-washed four times again.
- Two hundreds µl of substrate solution was added to each well and incubated for 30 min at room temperature in dark.

- Fifty µl of Stop Solution was added to each well. The color in the wells should change from blue to yellow.
- The optical density of each well was measured within 30 minutes, using a microplate reader set to 450 nm.

- **D-Calculation of results**

The samples results were calculated by interpolation from standard curve that was performed in the same assay as that for the samples by using standard curve fitting equations for IL-6, IL-10, TNF-α and MIF. The standard curve was made by averaging the readings for each standard concentration, and subtracting the average zero standard optical density (OD). The standard curve was drawn by plotting the mean absorbance for each standard on the y-axis against the concentration curve on the x-axis as follow.

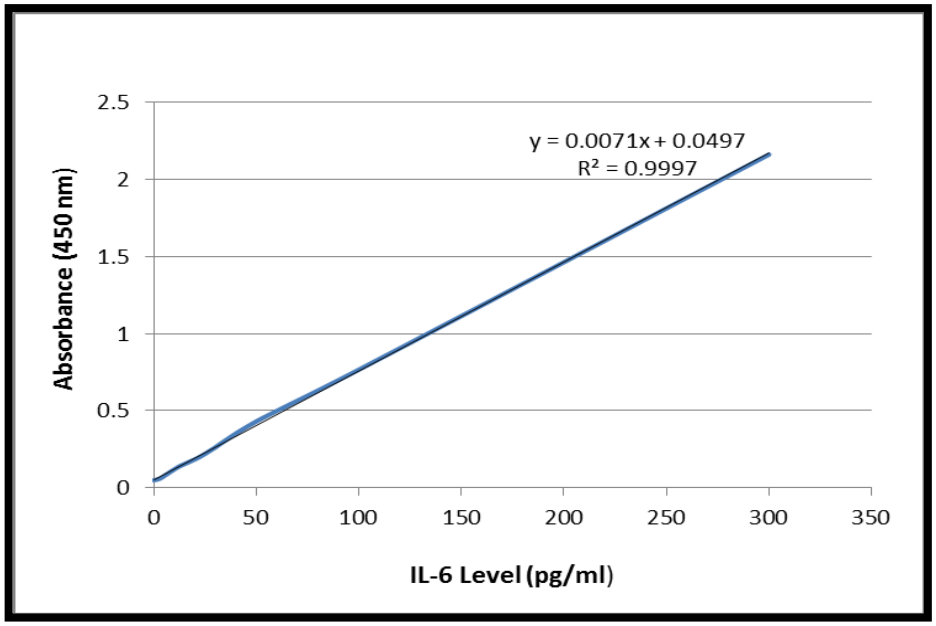


Figure (2-1):Standard curve of IL-6

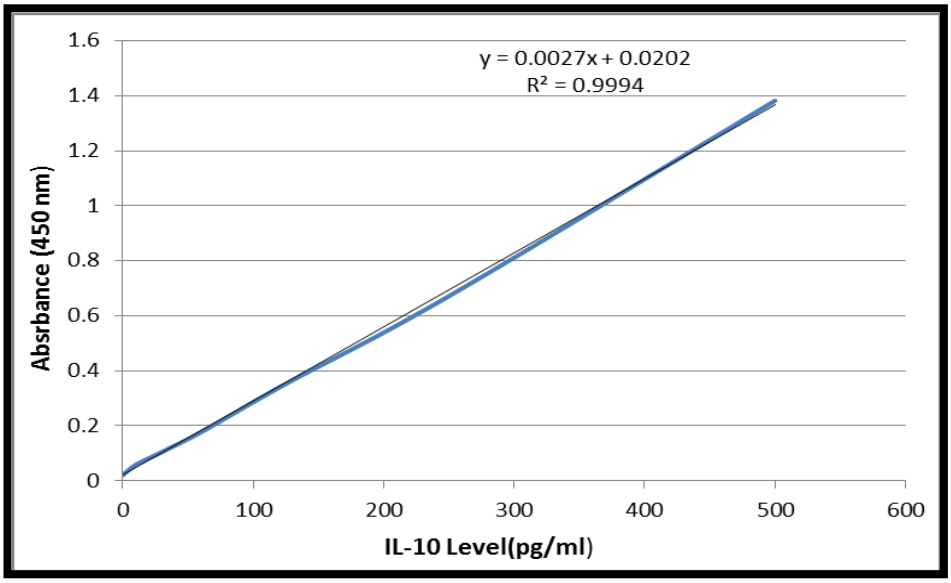


Figure (2-2):Standard curve of IL-10

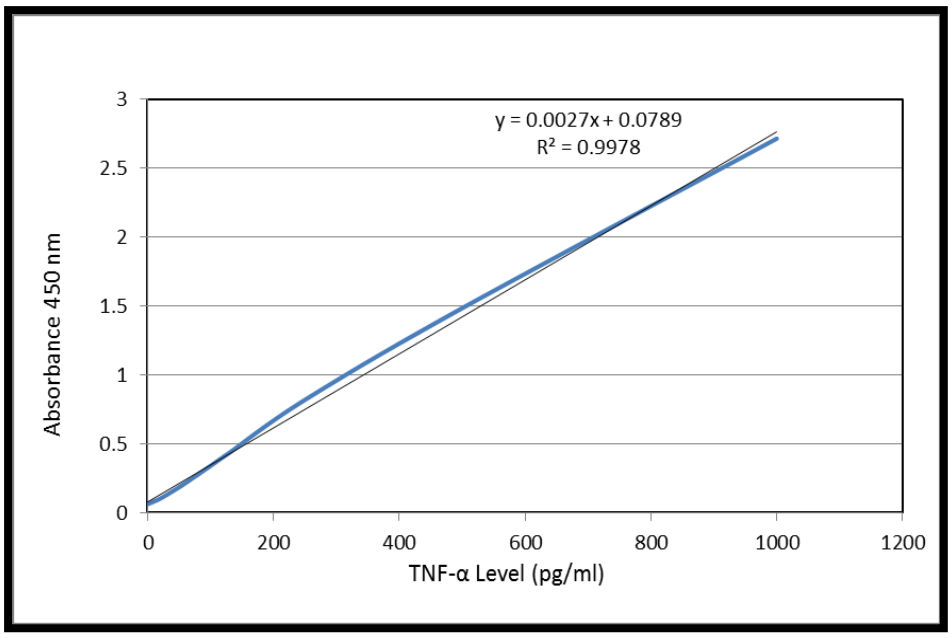


Figure (2-3):Standard curve of TNF-α

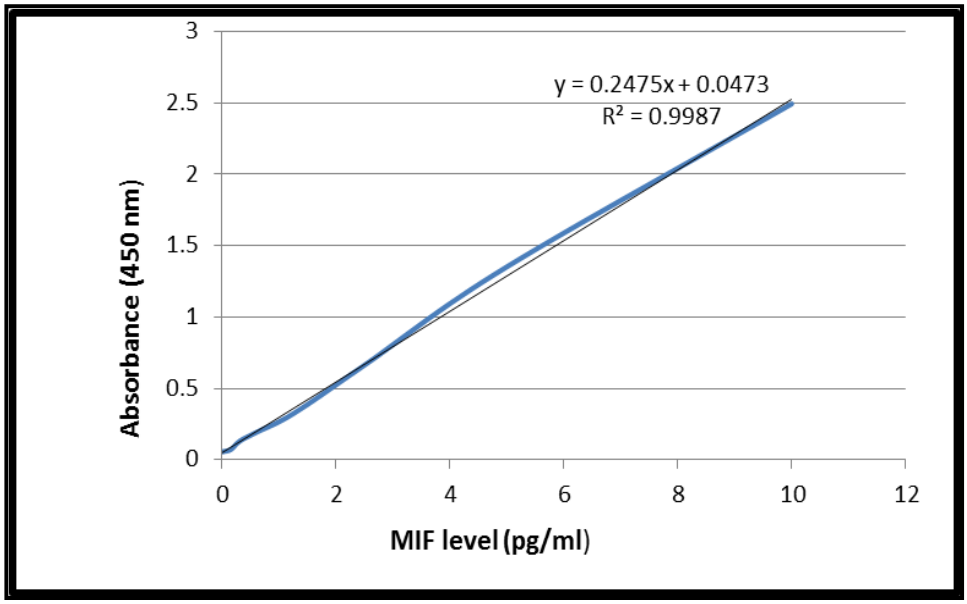


Figure (3-4):Standard Curve of MIF

2.2.18 Detection of CD74, CD44, p53 and p21 paraffin embedded tissue by Immunohistochemistry technique

- **Preparation of tissue sections**

Paraffin embedded sections of bladder tumor were cut into 5µm thickness by using an electronic microtome then were applied to positively charged slide and left over night at room temperature to dry.

- **Principles**

Immunohistochemistry is a method for demonstrating the presence and location of antigen in tissue sections by using antibodies that are highly specific to recognize only the target antigen. This is especially useful for assessing the progression and treatment of diseases such as cancer. The immunohistochemical staining is used for the visualization of tissues antigens by sequential reaction of a specific primary antibody to its corresponding antigen (CD74, CD44, p53and p21) in tissue samples. The specific antibody is located by a biotin-conjugated

secondary antibody. This step is followed by the addition of a streptavidin-enzyme conjugate that binds to the biotin present on the secondary antibody. The specific antibody, secondary antibody, and streptavidin-enzyme complex are then visualized with an appropriate substrate/chromogen

A. Deparaffinzation

Before proceeding with the staining protocol, the slides must be deparaffinized. Incomplete removal of paraffin can cause poor staining of the section. Sections were deparaffinized in three changes of xylene for 4 min of each.

B. Rehydration

Sections were rehydrated by immersing the slide sequentially for 3 min. in each of xylene , absolute ethanol,90%EOH,70%EOH,50%EOH**,** tap water and PBS buffer. The slides were kept in the buffer until being ready to perform antigen retrieval.

C. Antigen Retrieval

Most formalin-fixed tissues requires an antigen retrieval step before immunohistochemical staining can proceed. This is due to the formation of methylene bridges during fixation, which cross-links proteins and therefore masks antigenic sites. Buffer heat- -induced epitope retrieval was performed by placing a steel holder that carries the slides in Tris-EDTA Buffer (pH=9) for p53 and CD74 and in Sodium Citrate buffer (pH=6) for CD44 and p21 then placing the container in a microwave histoprocesser at (850 w) for 20 min. After cooling for 10-15 minutes at (4°C), the slides were removed and washed by wash buffer for 10 minutes.

D. Protocol

- The tissue sections were determined by drawing a circle around them by pap pen.
- Enough drops of Hydrogen Peroxide Block were added to cover the sections, incubated for 10 min and washed 4 times in PBS buffer.
- Protein Block was applied and incubated for 10 min at room temperature to block nonspecific background staining, then washed 3 times in PBS buffer.
- Diluted mouse monoclonal primary antibody at ratios (1/200 for CD44 and p21, 1/100 and 1/50 for p53 and CD74) were added respectively was added to tissue sections and incubated for 20 min in humidity chamber. Incubation period was 30 min for CD74 primary antibody.
- Sections were washed 4 times in PBS buffer; Biotinylated Goat Anti-Mouse was applied and incubated for 15 min in humidity chamber at room temperature.
- Sections were washed 4 times in PBS buffer; Streptavidin Peroxidase was applied and incubated for 10 min humidity chamber at room temperature.
- Sections were rinsed 4 times in PBS buffer. Two hundreds µl of DAB solution was added and incubated 10 minutes at room temperature
- Sections were rinsed 4 times in PBS buffer, and counterstain was added for 2 min at room temperature and washed in tap water.
- The slides were dehydrated and mounted with DPX and coverslip. Then, slides were examined by compound light microscope 10X, 20X and 40X. Results were compared with positive control which were determined according to the leaflet. Positivity was assessed semi- quantitatively by the

intensity and percentage of staining. Score was determined for CD74 and CD44 according to following scale when membrane of the cell has been stained with a brown color (Choi *et al*., 2013 ;Keymoosi *et al*., 2014)

i-Score 0 (negative): (none of the cells revealed positively for the marker)

ii-Score 1 (weak positive (+1): number of positive cell represents 10% or less of total (few scatter ≤10%)

iii-Score 2 (moderate positive (+2): the positive cells 11≤30%.

iv-Score 3(strong positive (+3): the positive cells31 ≤50 %.

v-Score 4 (very strong (+4): the positive cells more than 50%.

While score of p53 and p21 was determined according to following scale when nuclei was stained by brown color (Wang *et al*., 2014 and Stein *et al*., 2015).

1-Score 0 (negative): positive cell less than 10%

2-Score 1 (weak positive (+1): positive cell 10 <25%.

3- Score 2 (moderate positive (+2): positive cell 25-50%.

4-Score 3 (strong positive(+3): positive cells 50-75% .

5-Score 4 (very strong (+4): more than 75%.

2.2.19 Statistical Analysis

The Statistical Analysis System- SAS (2012) program was used to affect the different factors of the study parameters. Serum level of cytokines was statically analyzed , their data were given as mean ± Standard error(S.E.) and differences between means were assessed by ANOVA(Analysis of Variance)followed by LSD (Least Significant Difference) or Duncan test.

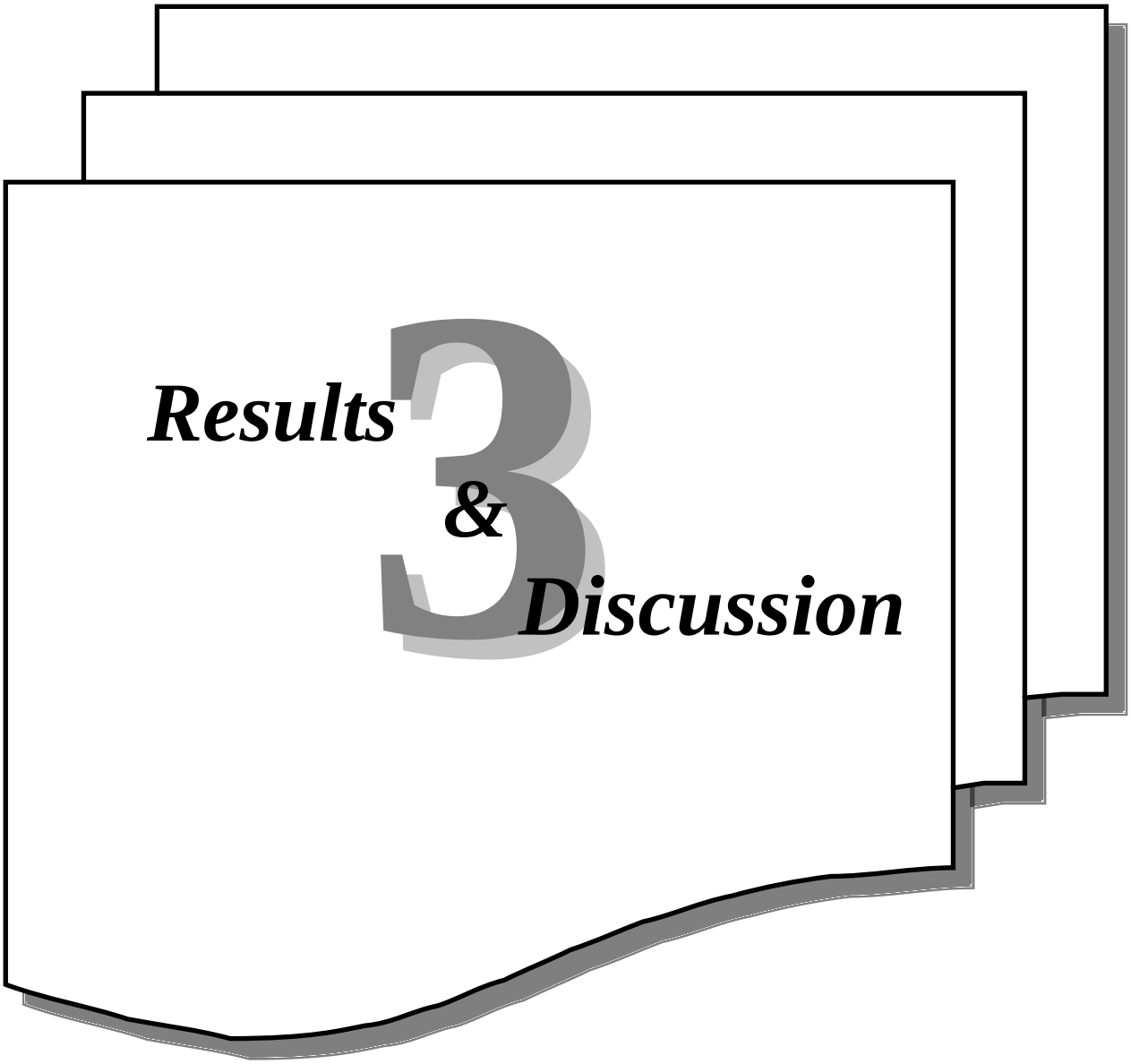
Genotypes of MIF_{-173} were presented as percentage frequencies and significant differences between their distribution in patients and control were

assessed by two-tailed Fisher's exact probability .In addition relative risk(RR) ,etiological fraction (EF) and preventive fraction (PF) were also estimated to define the association between genotypes with the disease. The RR value can range from less than one (negative association)to more than one (positive association). If the association was positive , the EF was calculated while if it was negative, the PF was given . These estimation was calculated by using the WINPEPI computer programs.

Allele frequencies were calculated by direct gene counting method ,while significant departure from Hardy-Weinberg(H-W) equilibrium was estimated using H-W calculator for two alleles.

Chi-square test was used to significantly compare between the percentage of observed and expected frequencies .

Chapter Three



3.Results and Discussion

3.1 Gender Distribution

One handed and thirty five Iraqi subjects have been included in this study; 104 (77%) males and 31(23%) females. Gender distribution of the investigated groups was shown in Figure (3-1). Seventy three subjects were UBC patients 60(82%) male and 13(18%) female and male: female ratio was 4.61:1 .Thirty two subjects with different UBD 22 male (68.75%) and 10 female (31.25) % and male: female ratio was 2.2:1 Thirty healthy subjects 22 male (73.3%) and 8 female (26.7%) and male: female ratio was 2.75:1 Sixty seven (91.8%) out of the 73 UBC patients were presented with macroscopic hematuria and 6 patients (8.2%) presented with microscopic hematuria.

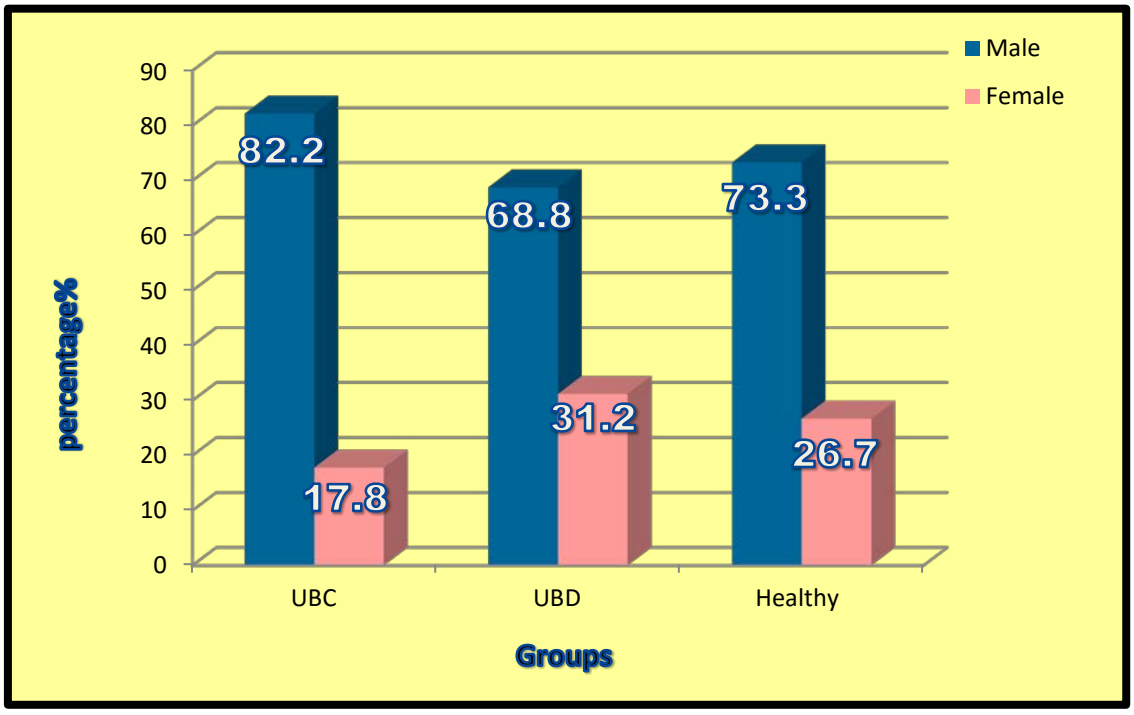


Figure (3-1): Distribution of UBC, UBD and healthy control according to Gender .

Results showed that prevalence of BC was generally higher in males than in females.This results agreed with the result recorded by Mousa,(2013)

who reported that the incidence of bladder cancer was higher in male than female with rates of 74.1% and 25.9 % in males and female, respectively. Yeh *et al*.,(2015) found that bladder cancer incidence in males was around three fold higher than in females, but the 5 year survival rate was lower in female bladder cancer patients which indicates that gender differences may be a factor affecting bladder cancer incidence and invasion. The majority of bladder cancer occurs in males and there was a 14-fold variation in incidence internationally. The highest incidence rates are found in the countries of Europe, North America, and Northern Africa. Egyptian males have the highest mortality rates which is twice as high as the highest rates in Europe and over 4 times higher than that in the United States(Walsh *et al*., 2012). Smoking and occupational exposures are the major risk factors in western countries, whereas chronic infection with *Schistosoma hematobium* in the developing countries, particularly in Africa and the Middle East. A majority of bladder cancer associated with schistosomiasis are squamous cell carcinoma .Bladder cancer is caused by genetic abnormalities and external risk factors, including carcinogen exposure, age, alcohol, inflammation, infection and radiation. Environmental carcinogens can enter the system and cause bladder cancer from inhalation or through skin absorption (Ahmedin *et al*., 2011).

3.2 Age distribution

The risk of bladder cancer increases with age and the mean age at diagnosis was 65.2 year rang from (43-85) for UBC patients. The results of in table (3-1). The results show that the age group (61-70) year had the maximum number of UBC patients while no patients was recorded in the age group less than 40 years. Such findings declared that the older individual was more susceptible and had a great chance of bladder carcinoma. This result

agreed with result reported by Alwadi (2011) who recorded > 60 years was the highest risk group for BC.

BC incidence was strongly related to age ,about 9 out of 10 people with BC were older than 55 years , with the highest incidence rates being in older men and women. Age-specific incidence rates rise gradually in ages 50-54 of both males and females, with a sharper rising in males from age 60-64, to peak in both sexes in the 85+ age group (Grossman *et al*.,2005; Cheung *et al*.,2013).

Table(3-1):Distribution of UBC,UBD and healthy control according to age.

Group	UBC		UBD		Healthy		Total	
	No.	%	No.	%	No.	%	No.	%
20-30	0	0.00	1	3.1	0	0.00	1	0.7
31-40	0	0.00	6	18.8	2	6.7	8	6
41-50	10	13.7	12	37.5	11	36.7	33	24.4
51-60	14	19.2	7	21.8	13	43.3	34	25.2
61-70	30	41.1	6	18.8	4	13.3	40	29.7
71-80	13	17.8	0	0.00	0	0.00	13	9.6
81-90	6	8.2	0	0.00	0	0.00	6	4.4

BC is primarily found in older people, with approximately 80 % of new cases occurring in individuals of 60 years or older (Jemal *et al*.,2005).

Siegel *et al* ., (2012) reported that the medium age of bladder cancer patients at diagnosis was 73 years for male and 74 years for female, and the treatment of bladder cancer varied by stage and patient age, while 56.8 year was the mean age reported by Abdul Muhsin (2008) and 55.3 year was the mean age in Africa reported by Taha and Zahrani(2012).

3.3 Smoking

The results revealed that 78 (57.8%) subjects in this study were smokers and 57 (42.2. %) were non smokers. Fifty five (75.3%) out of the 73 UBC patients were smokers. This result reflect the big risk of smoking that increases the incidence of urothelial carcinoma of the bladder as shown in table (3-2). This result came in accordance with the result of Maximilian *et al*.,(2013)who recorded that smoking was the most common risk factor with the incidence rate(75%) which accounted for approximately half of all UBCs. Occupational exposure to aromatic amines and polycyclic aromatic hydrocarbons are other important risk factors while the impact of diet and environmental pollution is less evident.

Table(3-2):Incidence rate of Smoking in UBC, UBD patients and healthy individuals .

Group	Smoker		Non Smoker		Total	
	No.	%	No.	%	No.	%
UBC	55	75.3	18	24.7	73	54.1
UBD	21	65.6	11	34.4	32	23.7
Health	2	6.7	28	93.3	30	22.2
Total	78	57.8	57	42.2	135	100

*($P<0.01$).

Smoking is recognized as the most important risk factor for UBC and is

estimated to account for 50% of the tumors. There was a direct pathophysiologic link between tobacco and UBC. Tobacco contains aromatic amines, such as β-naphthylamine, and polycyclic aromatic hydrocarbons known to cause UBC. These are renally excreted and exert a carcinogenic effect on the entire urinary system (Neal *et al*., 2011and Rianne *et al*.,2011).Cigarette smoking is common and the leading causative factor associated with UBC which depends on duration and intensity of current smoking.The differences in incidence rates between genders are frequently attributed to different historical smoking patterns (James *et al*.,2010)

3.4 Results of Urine Culture

One hundred and thirty five midstream urine samples have been obtained from patients and healthy individuals then diagnosed by cultural, morphological and biochemical tests for checking the presence or absence of urinary tracts infections. The results of urine culture showed that 96 (71.1%) of them gave positive urine cultures and 39 (28.9%) were negative. Positive urine cultures were detected in 64 (87.7%) of UBC patients, 9(12.3%) urine samples were negative culture and all the urine samples obtained from UBD subjects 32 (100%) gave positive cultures, while all the 30urine samples (100%)of healthy individuals gave a negative culture as shown in Figure(3-2).

The results revealed that UBC patients suffering from immunosuppression state caused increasing incidence of UTIs. AL-Shukr (2005) and AlChalabi (2007) found that the percentages of positive urine cultures in UTIs were (93.75%) and (94.1%) ,respectively. While AL-Jabouri (2005) found that the percentage of positive culture from urine samples as (77.3 %). The variation in the percentage may be due to differences in size of

samples, the seasons and medications before sampling and geographical location of the study .

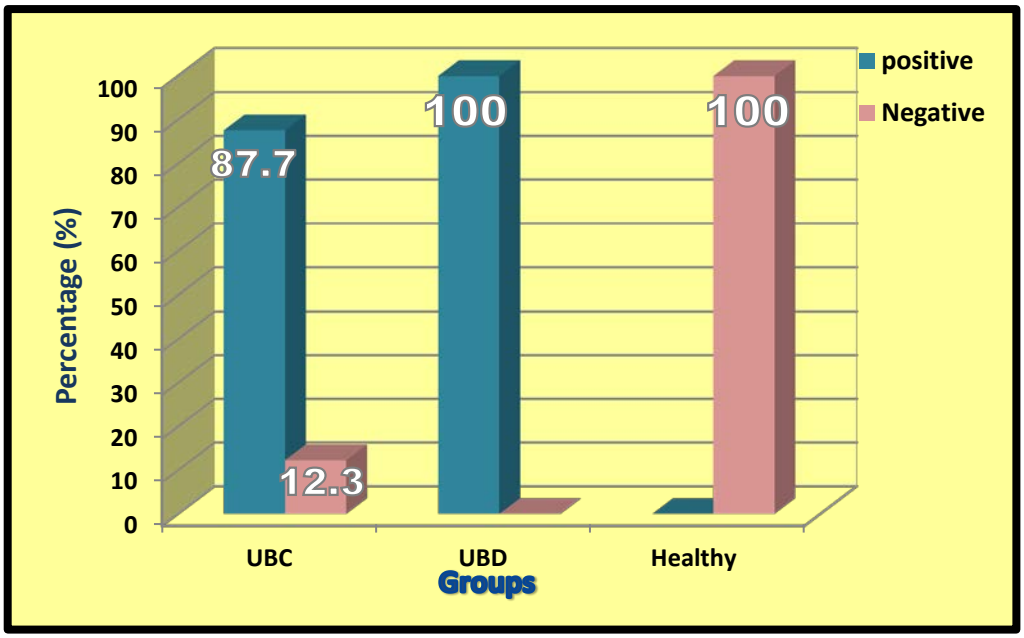


Figure (3-2): Prevalence of UTIs in UBC,UBD and healthy individuals.

The results of Gram staining and microscopicy examination of UBC and UBD urine samples showed that 9 (9.4 %) isolates were identified as Gram positive bacteria ,85 (88.5%) Gram negative bacteria and only 2 (2.1%) isolates were yeasts as shown in Fig (3-3).

Al- Hemdouy(2005) found that G-ve bacteria were the dominant and G+ve represents the second agent that causes UTIs. The results are compatible with another Iraqi study by Al-Wadi (2011) who found that G-ve bacteria were responsible for (86 %) of UTIs cases, while G+ve were about (14 %). This results were closed to Fadhil *et al*.,(2013) who found that (8.9 %) of the isolates from midstream urine samples were classified as Gram positive ,bacteria and (91.1 %) isolates as Gram negative bacteria .

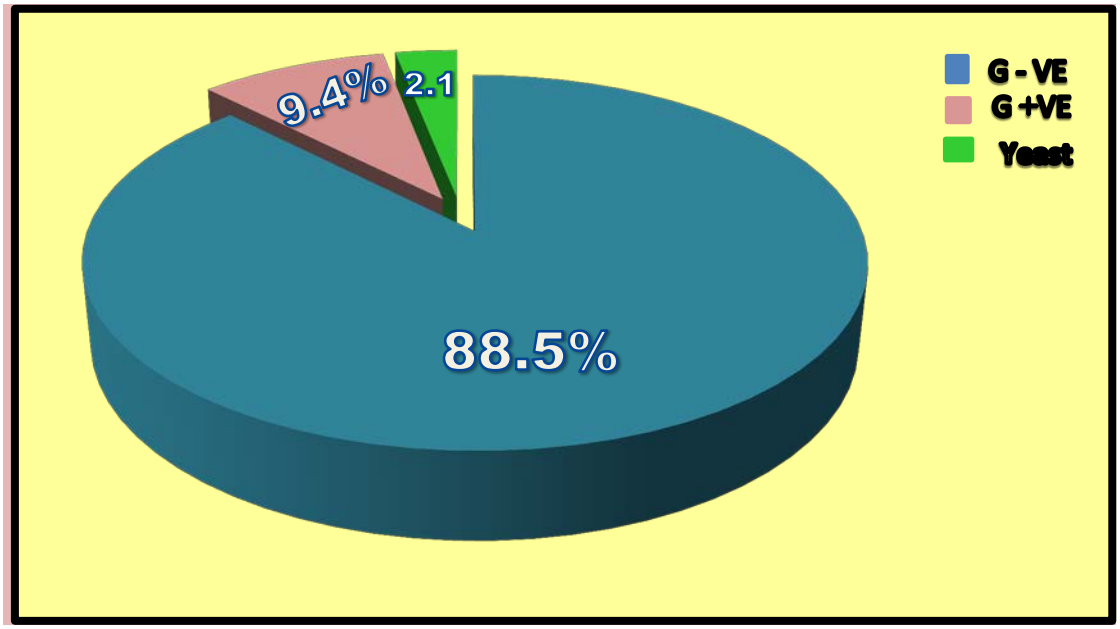


Figure (3-3): Percentage of bacteria and yeast isolated from urine of UBC and UBD

The results showed that all UTIs cases were caused by a single infectious agent as follows: 2 (2.1%) isolates were identified as *Candida albicans*,1(1%) *Citrobacter sp.*, 1(1%) *Streptococcus fecalis*, 7 (7.3 %) *Pseudomonas sp.*, 9(9.4%) *Staphylococcus aureus*, 14(14.6%) *Klebsiella sp.*, 19(19.8%) *Proteus sp.* and 43(44.8%) isolates identified as *E.coli* as shown in table(3-3). According to such findings , members of *Enterobacteriacea* family are considered to be main causative agent of UTIs, specially *E.coli* which is able to cause UTI because of it's expression of several virulence factors that are responsible for it's pathogensity.

The present results were closely to those reported by Al-Sukar (2005) who found that *Klebsiella sp.*(11%) and *Proteus sp.*(8%) representing the second and third causative agents of uncomplicated UTIs after *E.coli* (22%) .

Table(3-3):Percentages of microbial species isolated from urine samples of UBC,UBD patients.

Isolate	patients				Total	
	UBC		UBD		No.	%
	No.	%	No.	%		
Candida albicans	2	2.7	0	0.0	2	2.1
Citrobacter sp.	0	0.0	1	3.1	1	1
Escherichia coli	33	45.2	10	31.3	43	44.8
Klebsiella sp.	9	12.3	5	15.6	14	14.6
Proteus sp.	12	16.4	7	21.9	19	19.8
Pseudomonas sp.	3	4.1	4	12.5	7	7.3
Staphylococcus aureus	5	6.8	4	12.5	9	9.4
Streptococcus fecalis	0	0.0	1	3.1	1	1
Total	64	66.7	32	33.3	96	100
Chi-square(χ^2)	10.094 *				--	---

*($p \leq 0.01$)

Qiao *et al* .,(2015) found that the most frequently identified pathogens causing UTIs in the Chinese population was *E.coli* which is characterized by high resistance and strong pathogenicity, while Fadhil (2012) reported that 65% of patients with bladder cancer had UTIs, and *Staphylococcus aureus* was the most common microorganisms isolated from the urine of bladder cancer patients which represented (34 %)while *E.coli* was found to be the second common microorganism (30.7%) then *Proteus sp*. (15.3%), *Klebsiella sp*. (11.5%) and (7.6%) of microorganism was *Pseudomonas sp*.

Urinary tract infections are one of the most commonly diagnosed infections in hospitals and the microorganism causing UTI vary in their susceptibility to the antimicrobials from place to place and time to time. Complicated UTIs such as abnormalities or malformation in urinary tract are the most common infection diagnosed all

over the world, and the use of catheter is commonly associated with this type of UTIs because of using for different periods made it susceptible to contamination by microorganisms such as bacteria and fungi (Prakash and Saxena,2013)

Patients with complicating factors such as urinary catheter can suffer from UTIs caused by *Klebsiella* and *Proteus* as the main causative agents because *Proteus* is swarming and *Klebsiella* forms a capsule(Lo *et al*.,2008) While *Pseudomonas sp.* (as third agents) causes complicate UTIs because of their resistance to antibiotics obtained by hospitalized patients. other species of bacteria may contribute to the contamination of catheter and led to infection but with a low frequency(Amine *et al*.,2009).

Milojevic *et al*.,(2015) suggested that the stress of the bacteria is insufficient to induce tumors but may be sufficient to augment neoplastic changes induced by bacterial infection. The cytokine network induced by bacterial infections may play a significant role in cell proliferation, inflammation and induced urothelial hyperplasia (Zachary *et al*.,2013). Furthermore ,there is a possibility that cytokines such as TNF-α, MIF and IL-8 which have chemotactic and angiogenic activity may be involved in the development of the marked vascular proliferation in the stroma and aggregation within the epithelium which are characteristic of the tumors observed in the present and previous studies (Dase *et al*.,2014).

3.5Stage and grade of UBC

Seventy three bladder tumors samples were collected from Iraqi patients after bladder cystoscopy and cystectomy then diagnosed by a consultant histopathologist according to WHO. Cystoscopy of bladder has done when urologist confirmed the presence of bladder mass after performing

the required laboratory tests, ultrasonic examination or computerized tomography scan. The most important tests were general urine examination, urine culture and in some cases urine cytology for checking the presence of blood in urine(hematuria), microbial infection and abnormal cell. Referred to the histopathologist diagnosis, all tumor samples were classified as UCC. Results showed that grade of tumors was characterized as 40(54.8%)of high grade and 33(45.2%)of low grade, while according to stages of tumors 12(16.4%) Ta, 28(38.4%)T1 invaded subepithelial connective tissue,22(30.1%) T2 invaded muscle,7(9.6)T3 invaded perivesical tissue and 4(5.5%) T4 invade other organ as shown in table (3-4).

Table (3-4):Classification of UBC according to stage and grade of tumors

Grade	Stage										Total	
	Ta		T1		T2		T3		T4			
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Low	12	36.4	21	63.6	0	0.0	0	0.0	0	0.0	33	45.2
High	0	0.0	7	17.5	22	55	7	17.5	4	10	40	54.8
Total	12	16.4	28	384	22	30.1	7	9.6	4	5.5	73	100

This result agreed with Chen *et al*.,(2013) who recorded that UCC of the bladder was the most common form of bladder cancer which including non-muscle invasive, muscle invasive, and metastatic lesions.

Sanjeev *et al*.,(2009) reported that approximately 70% of the patients had NMIBC and the remaining 30% had muscle-invasive tumors. Approximately 90 % of BC were urothelial carcinomas, the remaining 10% non urothelial (SCC or AC) and the cystoscopy remains the mainstay of

diagnosis and surveillance. Up to 85% of the patients with bladder cancer present with disease confined to stage Ta:non invasive papillary carcinoma or stage T1:invade subepithelial connective tissue.

Stage and grade are very important to determine treatment because the non –muscle-invasive tumors should be treated totally differently from muscle-invasive tumors. In the non–muscle-invasive disease, TURBT paired with adjuvant intravesical chemotherapy or immunotherapy is the choice treatment while in the muscle invasive disease, cystectomy is the most appropriate curative option (Antoine *et al*., 2009).

On the other hand, Aldousari and Kassouf (2010) reported that the overall rate of recurrence for non muscle invasive bladder tumor was ranged from 60% to 70%, and the overall rate of progression is 20% to 30%. Ta tumors (which are mostly low grade) were rarely progress to a higher stage but they tend to recur and the risk of progression to muscle invasion was strongly associated with tumor grade. Thus, the suggested grade is a better prognostic indicator of progression and mortality than recurrence. The remaining 30% of bladder cancers were muscle invasive and generally require surgery to remove the bladder (cystectomy) and the surrounding organs. Around 1 per 10 bladder cancers (10%) have grown into the muscle layer of bladder. There is a higher risk that the cancer could spread to other areas of the body than with early bladder cancer. In T2 stage, the cancer has spread into (or invaded) the muscle layer of the bladder about (50%) diagnosed while T3 stage cancer has grown through the muscle layer and intothefatbeneth.

Forty five (61.6%)out of the 73 patients had the bladder tumor for the first time (newly diagnosed) while 28(38.6%) patients suffered from recurrent bladder tumor after chemotherapy treatment as shown in Figure (3-4).

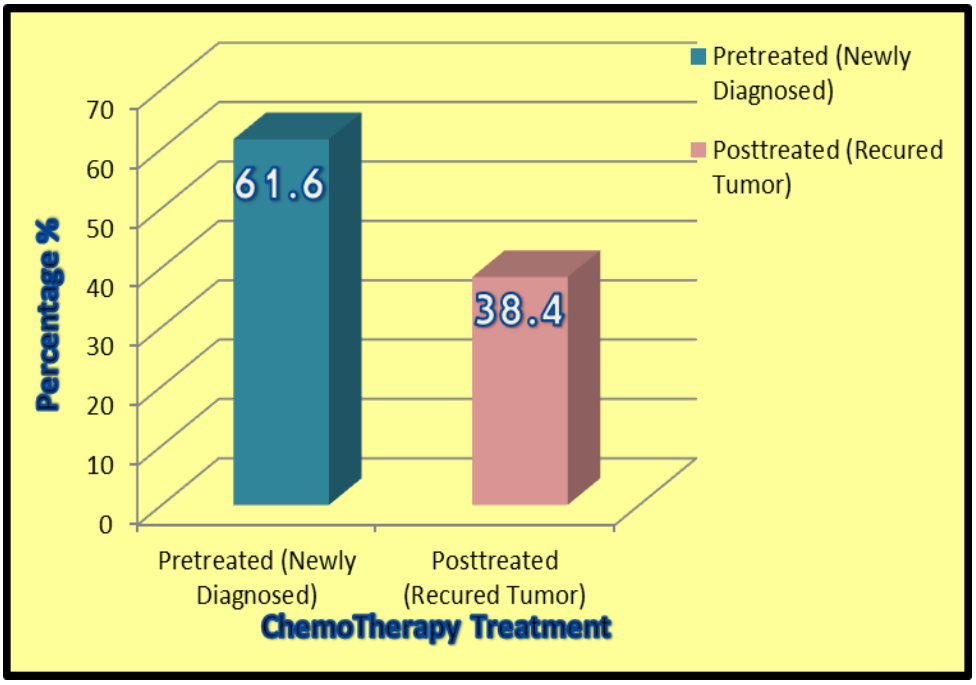


Figure (3-4): Classification of UBC patients according to chemotherapy.

Unfortunately, treatment was less successful for tumors that have grown deeper into the bladder wall when about 25% of T4 metastatic stage of BC has spread to another part of the body. About (10%) have already spread by the time they are diagnosed (Apolo *et al.*, 2014).Bladder cancer is the most expensive solid tumor to treat mainly due to the high recurrence rate of its non– muscle-invasive form (confined to the urothelium(Ta) or lamina propria (T1)). Many non–muscle-invasive bladder cancer are amenable to treatment with transurethral resection of bladder tumor (TURBT) alone.

Nowadays, histologic grade and stage are considered to be basic parameters that determine the prognosis of UBC. However, histological grades have a significant role in the biological behavior and tendency for recurrence (Beltran *et al.*, 2004; Perlis *et al.*, 2013 and Yildırım *et al.*, 2014). Incomplete transurethral resection of bladder cancer is common, as demonstrated in 26% patients and may be a risk factor for recurrence and particularly local recurrence (Jancke *et al.*, 2012).

3-6 Serum level of cytokines

The importance of secreted cytokines and growth factors in the development and promotion of malignancies is large. Many different soluble, extracellular gene products participate in processes that collectively contribute to the growth and survival of a developing neoplasm. These secreted molecules can, directly or indirectly, play a central role in the uncontrolled tumor cell division, angiogenic stimulation or suppression of tumor cell immune surveillance (Walsh *et al.,* 2012).

3-6-1 Serum Level of IL-6

The IL-6 level in sera has been obtained from the blood samples of the investigated groups measured by Sandwich ELISA . The results revealed that IL-6 levels were higher in patients with UBC and UBD than in the healthy controls with mean levels of (90.14 , 61.5 and 8.4) pg/ml, respectively with significant differences (P≤ 0 .05) as shown in table (3-5) .

Table(3-5): Serum levels of IL-6 in UBC,UBD and Healthy control .

Group	Mean ± S.E of IL-6 pg/ml	Sig. between Groups	p -value
UBC	90.14±7.29 a	(LSD =16.063)	0.0001
UBD	61.5±5.09 b		
Healthy	8.4±0. 45 c		

This result was close to Abdulmohymen and Ashoor (2010) who reported that IL-6 is a pleiotropic cytokine with varied systemic functions . It is secreted by a number of different cell types and has been implicated in various disease processes including bladder cancer which implicated in proliferation pathways, because it acts with other factors.

In this study ,the mean of serum levels in sera of the bladder cancer patients was elevated to reach (389.13pg/ml) when compared to serum IL-6 levels of non-cancerous patients (260.37 pg/ml) thus, suggested that it plays a significant role in bladder carcinoma.

According to the results shown in table(3-6), of this study, it is shown that the highest mean levels of IL-6 was observed in the sera of UBC patients had T4(237.47pg/ml)when compared to patients had T3 stage with mean level(155.70pg/ml) thenT2(91.99pg/ml),T1 (70.48 pg/ml) and Ta(45.24) with a significant difference.

Table(3-6):Serum levels of IL-6 in UBC patients at different stages.

Stage	No.	Mean ±S.E of IL-6pg/ml	Sig. between Groups	p value
Ta	**12**	45.24±5.06 e	(LSD = 14.533)	0.0001
T1	**28**	70.48±7.04 d		
T2	**22**	91.99±9.59 c		
T3	**7**	155.70±29.29 b		
T4	**4**	237.47±26.31 a		

Tsui *et al*.,(2013) reported that IL-6 is a multifunctional cytokine found to be high level in serum of the patients with bladder cancer than in the healthy control which may contribute to tumor cell proliferation, differentiation and associated with a number of biological functions in bladder cancer. The IL-6 levels were positively correlated with the size of tumors and depth of tumor invasion, but no significant correlation was found between serum IL-6 level and lymph node metastasis. The mean level of IL-6 was increased proportionally with the late stage of bladder tumor which means an overexpression of IL-6 in the invasive tumor stage.

Kirti *et al.*,(2015) documented that the function of IL-6 in the pathogenesis and development of cancer showed positive association between serum IL-6 concentrations and the bladder cancer stage. IL-6 level in UBC patients and controls estimated which by ELISA, showed higher levels in UBC subjects than those of no bladder tumors specially in the sera of patients with more advanced invasive stage (T2-T4) which expressed a high level than sera of patients had early stage tumors (Ta-T1).Thus significantly correlation with the clinical stage was reflected (Chen *et al.*,2013).

The relationship between mean levels of IL-6 in the sera of patients and grade of urinary bladder tumors reflected a positive correlation due to the highest level of IL-6 observed in the sera of bladder cancer patients with a high grade (118.16 pg/ml) and in the sera of patients had low grade (59.98pg/ml) with a significant difference as shown in table (3-7).

Table (3-7): Serum level of IL-6 of tumor at different grade

Grade	No.	Mean ± IL-6 pg/ml	Sig .between Groups	P value
Low	33	60.28± 5.89	(LSD = 17.94)	0.00163
High	44	114.77 ±11.01		

The results in the above table agreed with those of Gaballah *et al.*,(2015) who reported that the high grade urothelial carcinoma had mean serum levels of IL-6 (106.7pg/ml) which was significantly higher than that of in low grade carcinoma(58.6 pg/ml).

Yeh *et al.*, (2015) compared IL-6 expression in muscle-invasive and non-muscle invasive bladder cancer samples and their data revealed that the expression level of IL-6 was significantly correlated with higher clinical grade, higher recurrence rate after treatment and reduced survival rate.

Baharlou *et al*.,(2015) concluded that the mean level of IL-6 in sera of UBC patients increased with highly grade invasive stage than in low grade non invasive tumors.

3-6-2 Serum Level of IL-10

Interleukin-10 is an immunosuppressive cytokine produced by various leukocytes. Tissue epithelial cells can also secrete some of these cytokines and expressed by cells of the innate and the adaptive immune system with their different functional roles. IL-10 has been shown to inhibit various immune functions, such as antigen presentation, cytokine production, macrophage activation and antigen-specific T-cell. It has been proposed that IL-10 plays a key role in the oncogenetic and metastatic ability of neoplasms (Chau *et al*., 2000 ; Ouyang *et al*., 2011and Chan *et al*.,2013)**.**

The results illustrated in table (3-8) demonstrated that the serum level of IL-10 was significantly higher in patients with UBC (35.84pg/ml) and with UBD (27.95pg/ml) when compared to the healthy individuals (19.26pg/ml),thus which play an important role in the progression of tumor .

Table(3-8): Serum Level of IL-10 of UBC,UBD and healthy individuals

Group	Mean ± S.E of IL-10 pg/ml	Sig. between Groups	p value
UBC	35.84± 1.83 a	(LSD = 5.026)	0.00293
UBD	27.95± 1.17 b		
Healthy	19.26±0.87 c		

This result agreed was closed to that reported by Ikeguchi *et al*., (2009) who found that IL-10 may be produced as an anti-inflammatory cytokine downregulate the host immune response with a higher mean level (58.8pg/ml) in UBC than in healthy controls (21.7pg/ml).

Results in tables (3-9)and (3-10) showed that significant higher levels were recorded in stage T4 (68.6)pg/ml followed by (40.57 , 37.19, 31.43 and 29.97)pg/ml in stage of T3, T2, T1 and Ta, respectively. Also, high grade showed significantly higher level (40.98)pg/ml than low grade (29.61) pg/ml as shown in table (3-9) and (3-10)

Table(3-9):Serum levels of IL-10 in patients with UBC at different stages.

Stage	No.	Mean ±S.E of IL-10 pg/ml	Sig. between Groups	P value
Ta	12	29.97±1.81d	(LSD = 5.318)	0.0017
T1	28	31.43±1.35 d		
T2	22	37.19±3.29 c		
T3	7	40.57±7.82 b		
T4	4	68.60±15.26 a		

Table (3-10)Serum level of IL-10 in patients with UBC at different grade.

Grade	No.	Mean ±SE of IL-10pg/ml	Sig .between Groups	P value
Low	33	29.61±1.01	(LSD = 6.819)	0.0116
High	40	40.98±3.02		

BC is a common urologic malignancy dominated by a Th2 polarized immunopathologic response. Bacillus Calmette–Guérin (BCG) has been used to treat non-muscle-invasive bladder cancer for more than 30 years. However, the current BCG therapy is associated with a high disease recurrence and progression as well as a lack of therapeutic response in some patients(Luo *et al.,* 2012). IL-10 plays an important regulatory role in bladder cancer immunosurveillance and BCG immunotherapy, blocking IL-10 activity could enhance BCG induction of Th1 immunity and therapeutic control of bladder cancer (Redelman *et al*., 2014).

3-6-3 Serum Level of TNF-α

Tumor necrosis factor alpha is the most important proinflammatory cytokine involved in the different functions and survival of many cells. It is produced by diverse kinds of cells, such as neutrophils, NK cells, T cells , B cells and tumor cells but mainly macrophage. It has been reported to play an important role in the pathogenesis of cancer(Leibovici *et al*.,2015).TNF-α plays a vital role in inflammation and many studies have show that it is a tumor promoter and an active contributor to carcinogenesis. Expression studies have consistently shown aberrant high concentrations of TNF-α in advanced tumors. The TNF-α 308G/A SNP is associated with an increased expression of TNF-α which may account for the over-representation of the variant allele in invasive UBC patients (Zhou *et al*., 2011).

The results demonstrated in table (3-11) showed that serum level of TNF-α was significantly higher for UBC and UBD patients when compared to the controls with a mean levels (36.74, 15.31 and 10.15 pg/ml) respectively.

Table (3-11): Serum level of TNF-α of in UBC, UBD and healthy individuals.

Group	Mean ± S.E of TNF-α pg/ml	Sig. between Groups	p value
UBC	36.74±2.25 a	(LSD = 3.977)	0.00041
UBD	15.31±0.81 b		
Healthy	10.15±0.55 c		

These results are in harmony with Metwally *et al*.,(2011) who found a significant increase in TNF-a level (37.7 pg/ml) in sera of bladder cancer patients versus normal controls (8.7 pg/ml).

Fan *et al*.,(2012) recorded that serum IL-6 and TNF-α concentrations in UBC patients were significantly higher than those in the control group which increased according to the severity of the disease, and differed greatly among different types of the disease.

Results in table (3-12) showed that TNF-α levels were correlated with the clinical staging of urinary bladder carcinoma with higher levels in T3(54.65 pg/ml) ,T4 advanced-stage patients (51.92 pg/ml) while low levels (17.71, 30.43 and 46.68 pg/ml) in Ta, T1 and T2, respectively with significant differences .

Table (3-12):Serum level of TNF-α in UBC patients at different stages

Stage	No.	Mean ± TNF-α pg/ml	Sig .between Groups	P value
Ta	12	17.71±1.08 d	(LSD = 5.932)	0.0029
T1	28	30.43±3.18 c		
T2	22	46.68±3.78 b		
T3	7	54.65± 3.79 a		
T4	4	51.92±2.73 ab		

TNF-α is a very well-known cytokine frequently seen in several types of cancer, and many studies reported that the cancer stage and grade were significantly associated with the GA genotype in the TNF-α promoter region (Kakehi *et al*.,2010). Moreover, the serum concentration of TNF-α was significantly higher in bladder cancer patients who had an advanced invasive stage and has been implicated in tumor invasion and metastasis (Chen*etal*.,2013).

The relationship between sera mean level of TNF-α and tumor grade of UBC patients showed a highly significant increase in the mean level in UBC

(47.35pg/ml) with high grade as compared to a low grade(23.87)pg/ml as shown in table (3-13).

Table (3-13): Serum levels of TNF-α in UBC patients at different grade

Grade	No.	Mean ± TNF-α pg/ml	Sig .between Groups	P value
Low	33	23.87±2.66	(LSD =7.732)	0.0013
High	40	47.35±2.54		

These results are compatible with Zhu *et al*., (2012) who found TNF-α as a critical mediator of inflammation, TNF-α represents one of the most important potential links between chronic inflammation and cancer. It expressed an increased serum level for high grade tumors when compared to low grade tumor s and normal urothelium. Besides, they found that the expressional change of TNF-α was associated with angiogenesis of bladder tumor.

Jasim and khalil (2014) reported that the high grade UBC patients show significant increase in TNF-α level as compared with low grade patients characterized by significant differences among different stages such results came in accordance with a previous study by Maria Sofra *et al*.,(2013)who observed an increase serum level of TNF-α proportionally with advanced invasive stage. It was important to note that increased TNF-α expression has been reported in recurrent, larger bladder tumors as well as in tumors that show progression in grade and stage .

3-6-4 Serum Level of MIF

Macrophage Migration Inhibitory Factor (MIF) is an unique mediator that participates in all of the pro-tumorigenic processes and is overexpressed in most tumor types beyond inflammatory and immune responses.

MIF is reported to be overexpressed in a large variety of human neoplasia which have been shown significantly higher levels of MIF protein than their non-cancerous cell counterparts (Takahashi *et al*., 2007 and Choudhary *et al*., 2013) .

According to the results illustrated in table (3-14) after determination of MIF serum level by sandwich ELISA for subjects of the investigated groups. The mean level of serum MIF in UBC patients was significantly higher than that observed in UBD patients and healthy control (55.57 and 39.08 *vs*. 18.53) pg/ml ,respectively.

Table (3-14): Serum level of MIF in UBC, UBD and healthy individuals.

Group	Mean ± S.E of MIF pg/ml	Sig. between Groups	p value
UBC	55.57±2.97 a	(LSD = 6.721)	0.001
UBD	39.08±2.37 b		
Healthy	18.53±2.26 c		

Yuan *et al*., (2012) who found that the concentration of serum MIF was higher in patients had bladder cancer than in normal subjects especially in muscle invasive bladder cancer. It also agreed with Grieb *et al*., (2012) reported that several actions of MIF may promote oncogenesis or tumor progression in different cancers and described as a potentially predictive biomarker in bladder cancer which expresses high serum levels of MIF when compared to the serum level of healthy.

MIF is the initial inflammatory mediator stimulates expression of other cytokines such as TNF-α and IL-1 via suppression of the anti-inflammatory actions of glucocorticoids (Siegler *et al*.,2007). MIF has the potential role which inhibits the action of the tumor suppressor gene p53. Macrophages lacking MIF are sensitized to p53-dependent activation-induced apoptosis

while cells containing MIF are significantly more resistant.In the tumor microenvironment, bypass of p53 by high concentrations of MIF expressed intrinsically by transformed cells or provided by the surrounding inflammatory cells which enhance cell proliferation, extend lifespan, create a deficient response to genotoxic damage and allow for the accumulation of oncogenic mutations(Nishihira *et al*., 2003 and Bach *et al*., 2009).

Regarding the relationship between the serum mean levels of MIF and tumor stages of UBC patients, results in table (3-15) show that the highest level was observed in the sera of UBC patients who had T3(91.48 pg/ml),then T4(86.62 pg/ml), T2(64.21 pg/ml) and T1(43.06 pg/ml) while Ta (37.63 pg/ml).

Table (3-15): Serum level of MIF in UBC patient at different stages.

Stage	No.	Mean ±S.E of MIF pg/ml	Significant between Groups	P Value
Ta	12	37.63 ± 5.06 c	(LSD = 8.963)	0.0012
T1	28	43.06 ± 4.04 c		
T2	22	64.21±3.66 b		
T3	7	91.48 ±5.19 a		
T4	4	86.62±6.84 a		

MIF plays a central role in the uncontrolled tumor cells division, angiogenic stimulation or suppression of tumor cell immune surveillance. The MIF and the MIF receptor (CD74), when they bound, they initiate survival pathways and cell proliferation thus, were highly expressed in invasive stages than the non invasive tumors (Bai *et al.*,2012). Overexpressed in most tumor types has been shown to promote malignant cell transformation , inhibit tumor cell-specific immune cytolytic responses and strongly enhance neovascularization (Morris *et al.*,2014).

Result illustrated in table (3-16) showed that the mean serum level of MIF is significantly elevated with the higher grade of advanced stage (69.26 pg/ml) than the low grade of the primary stage (38.98 pg/ml).

Table (3-16): Serum level of MIF in UBC patient at different grades.

Grade	No.	Mean ± MIF pg/ml	Sig .between Groups	P value
Low	**33**	38.98±3.66 b	(LSD = 9.205)	0.0001
High	**40**	69.26 ±3.17 a		

The above result disagreed with Ys *et al*., (2011) who reported that the expression of MIF protein was found predominantly in tumor cell and inversely correlated with tumor stage and grade. The expression of MIF in non muscle invasive bladder cancer was more frequent than in the muscle invasive disease**.**

3-7 Correlation between cytokines level in UBC patients.

According to the correlation coefficient between some parameters, there were several correlations between those biomarkers involved in bladder cancer as shown in table (3-17).Expression of MIF had a significant strong positive correlation with expression of other investigated cytokines IL-6, IL-10 and TNF-α (r=0.52, 0.69 and 0.67), respectively . Among other cytokines , IL-10 expression was also found to be positively correlated with IL-6 and TNF-α (r= 0.68 and 0.48), respectively .Also a positive association was observed between IL-6 and TNF-α expression(r=0.59)($p \leq 0.001$).

This result agrees with that reported by Conroy *et al.,(* 2010) who found that MIF was the original cytokine, described almost 50 years ago and

has been revealed to be an important player in pro-inflammatory diseases and has specific biological activities related directly to cancer growth or contributing towards a microenvironment favoring cancer progression such as induce production of other inflammatory cytokine like IL-6,IL-8andTNF-α. Cytokines modulate the functional activities of individual cells and tissues both under normal and pathogenic condition.

Table(3-17):Correlation between (MIF,IL-10,IL-6&TNF-α) level in UBC.

Cytokine parameters	Correlation coefficient (r)	P-value
MIF & IL-10	0.52 *	0.0001
MIF & IL-6	0.69 *	0.0001
MIF & TNF-alpha	0.67 *	0.0001
IL-10 & IL-6	0.68 *	0.0001
IL-10 & TNF-alpha	0.48 *	0.0001
IL-6 & TNF-alpha	0.59 *	0.0001

* ($P<0.01$).

Cytokines such IL-6 and IL-8 are now being regarded as main molecules in the progression pathway of chronic inflammatory process to carcinogenesis. Therefore, it evaluated the expression of different cytokines (TNF-α, IL -6, IL-8 and VEGF) in bladder cancer. TNF-α is released in response to infection and inflammation produced by activated macrophages and lymphocytes which found overexpression of TNF-α with the advance stage and lymph nodal metastasis of the cancer(Chaturmohta *et al.,* 2015) .

TNF-α showed a positive linear correlation with IL-6 with the invasion of the cancer and it expressed a higher serum level compared to the healthy control(Kerschbaumer *et al.,* 2012). Besides, serum levels of IL-6 were found to be highly elevated and positively correlated to tumor load which indicates that it has significant role in carcinogenesis of UBC and altered gene expression of IL-6 enhances tumor growth (kang *et al*., 2013)

IL-10 was elevated mostly in advanced disease. The increased levels of IL-10 were associated with significantly poor survival of patient(Landskron *et al.*, 2014). Cytokine production capacity varies among individuals and depends on cytokine gene polymorphisms which is associated with altered protein levels and/or transcription rates might influence cancer susceptibility by altered inflammatory responses(Seifarta *et al.*,2005).

3-8 *MIF* Gene Polymorphism at -173

Macrophage migration inhibitory factor is an inflammatory factors which may promote carcinogenesis. It is derived from T-cell, known as a member of the transforming growth factor-β (TGF-β) superfamily and it plays an important role in the etiology of bladder cancer. MIF_{-173} locus polymorphism might contribute to the genetic susceptibility to bladder cancer(Yuan *et al*., 2012). The -173G/C polymorphisms in the *MIF* promoter region are associated with altered levels of *MIF* gene transcription and may be involved in the predisposition to develop risk of bladder cancer (Hizawa *et al*., 2004 and Yang *et al*.,2013).

In a hospital-based case–control study of 73 patients with bladder cancer and 62 cancer-free controls , the *MIF* polymorphism was genotyped . Genomic DNA was extracted from peripheral blood of all 135 subjects then amplified by PCR with using specific primer for -173 locus that gave the PCR product (366) bp as shown as in Figure (3-5). The PCR product was a 366 bp fragment that contained both the polymorphic and a non polymorphic *AluI* site. The results showed that G/G genotype gave two fragments (268- and 98-bp),C/C genotype gave (62-,206-and 98-bp) and four fragments as (62-, 98-, 206- and 268-bp) after digestion of G/C genotype by *AluI*. as shown in figure (3-6).

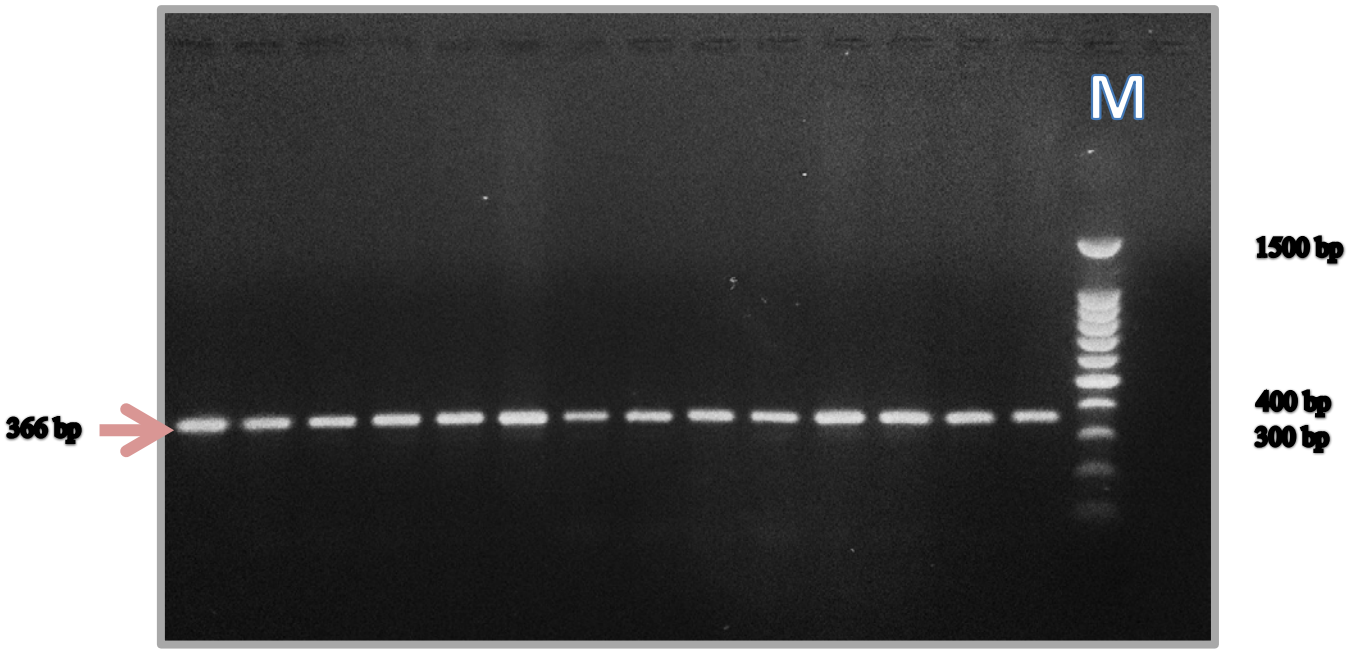


Figure (3-5) Gel electrophoresis for amplification of human *MIF* gene. Electrophoresis was performed on 1.2 % agarose gel and run with a 70 volt current for 2 hrs. Lane M is a (100 bp) ladder.

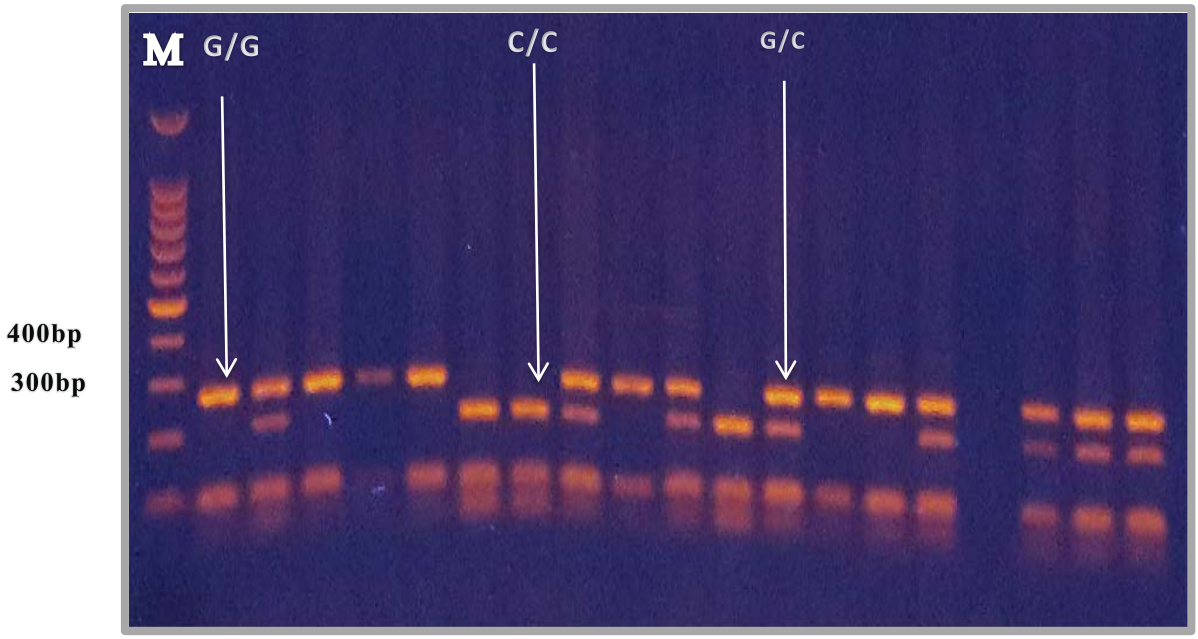


Figure (3-6):Gel electrophoresis of *MIF*$_{-173}$polymorphism(RFLP by *AluI*);M: DNA Marker;G/G:Homozygous for absence of *AluI* restriction site (268bp 98bp); C/C: Homozygous for *AluI* restriction site (206bp, 98bp,62Heterozygous for *AluI* restriction site (268bp,206bp,98bp,62bp).

The frequency of genotypes distribution and alleles of the groups are presented in table (3-18). The GG, GC, and CC genotype frequencies were 61.9%, 31.6% and 5.5%, respectively among the UBC cases and 56.3, 37.5, and 6.2%, respectively, among the UBD cases while represent 53.3 %, 33.3% and 13.4% respectively among healthy cases.

Table(3-18):Genotypes distribution of *MIF*$_{-173}$ among UBC,UBD and healthy control.

Genotype	Groups					
	UBC		UBD		Healthy	
	No.	%	No.	%	No.	%
GG	45	61.6	18	56.3	16	53.3
GC	24	32.9	12	37.5	10	33.3
CC	4	5.5	2	6.2	4	13.4
Total	73	100	32	100	30	100

The results nearly agreed with the results reported by Yuan *et al.* (2012) who found that individuals with GC/CC genotype had a significantly decreased risk of bladder cancer than those with GG genotype and the CC genotype frequency of *MIF* was lower among the cases. The GG, GC, and CC genotype frequencies were 63.4, 30.5, and 6.1%, respectively, among the cases and 50.7, 43.2, and 6.1%, respectively, among the controls.

The human *MIF* gene is located on chromosome 22q11.2 which contains 3 exons and 2 introns. Gene mapping has shown that the *MIF* gene displays polymorphism, including microsatellite polymorphism and single nucleotide polymorphism, at 4 sites (Das *et al*., 2014). G/C polymorphism is located at -173, T/G polymorphism is located at +254,C/G polymorphism

locates at +656, and a CATT repetitive sequence is located at -794,−173G/C polymorphism was be associated with cancer risk.(Yuan *et al.*, 2013 and Zhang *et al*., 2015).

The genotypes and allele frequencies of *MIF* polymorphism and its association with risk of bladder cancer are summarized in table (3-19).

Table (3-19):Observed numbers alleles frequencies(Hardy-Weinberg equilibrium) of the *MIF* at -173 SNP in UBC,UBD and Control .

Group		MIF-173 Genotype or Allele						H-W (P≤)
		GG		GC	CC	*G*	*C*	
UBC	Observed	No.	46	23	4	115	31	N.S
		%	61.9	31.6	5.5	78.77	21.23	
	Expected	No.	45.29	24.42	3.29	Not Estimated		
		%	62	33.45	4.5			
UBD	Observed	No.	18	12	2	48	16	N.S
		%	56.25	37.50	6.25	75	25	
	Expected	No.	18	12	2	Not Estimated		
		%	56.25	37.50	37.50			
Control	Observed	No.	16	10	4	42	18	N.S
		%	53.33	33.33	13.34	70	30	
	Expected	No.	14.7	12.6	2.7	Not Estimated		
		%	49	42	9			

Makhija *et al*. (2007) reported that the $MIF_{-173}C$ alleles was over expressed in acute pancreatitis patients. Their data all suggested that the

polymorphisms might affect on MIF protein expression and activity thus the variation in MIF may play a role in etiology of bladder cancer. Although, how the *MIF*$_{-173}$ G/C polymorphisms affect the inflammatory factor activity remains to be investigated, some studies suggested that the polymorphisms may affect the protein functions.

The frequency of GG genotype was increased as well as G allele which had positive association with RR value1.41 and EF value 0.18 with no significant differences while GC and CC genotypes were decreased as well as C allele, both of them had negative association with RR value 0.98 and 0.38respectively.Two negative association PF value 0.07 for G/C and 0.08 for C/C as shown in table (3-20).

Table (3-20):Statistical evolution of association between *MIF* $_{-173}$ genotypes or alleles and urinary bladder carcinoma.

MIF$_{-173}$ Genotype or Allele	Statistical Evolution			
	Relative Risk	Etiological Or Preventive Fraction	Fisher's Exact Probability	95% Confidence Intervals
G/G	1.41	0.18	0.510	0.60-3.28
G/C	0.98	0.07	0.569	0.40-2.42
C/C	0.38	0.08	0.170	0.09-1.59
G	1.59	0.29	0.123	0.81-3.12
C	0.63	0.11	0.123	0.32-1.24

*There was No Significant differences between groups of patients and control in the distribution of *MIF* genotypes -173 frequencies.

Single nucleotide polymorphisms in inflammation genes have been shown to alter their expression and functions. A G/C SNP in the promoter

region -174 of IL-6 was shown to affect transcription and alter plasma IL-6 levels. The A-allele of an IL-8 SNP in the promoter region (T-251A) has been associated with the increased IL-8 production by lipopolysaccharide-stimulated whole blood. A G-to-A transition in the promoter region (308) of the TNF-α gene results in a higher expression of TNF.

The results in table (3-21)showed that MIF level in GG was (62.69) pg/ml significantly higher in UBC. A significant decrease was recorded in UBD and healthy (37.98 and 16. 61 pg/ml) respectively. In GC genotype, MIF level was (52.51) pg/ml followed by (38.16) pg/ml in UBD and (22.38) pg/ml in healthy with significant differences. A significant increase was recorded in CC genotype in UBC and UBD (53.54 and 54.60) pg/ml respectively in comparison to healthy (16. 57) pg/ml with significant differences. Meyer-Siegler *et al* .,(2007) reported that the concentration of MIF is higher in bladder cancer tissue than in normal bladder tissue especially in muscle invasive bladder cancer tissue and in functional promoter.

Table (3-21): Association between different genotypes and MIF level in UBC , UBD and healthy.

Genotype	Group (Mean level of MIF Mean± SE) pg/ml			Significant between group	P-value
	UBC	UBD	Healthy		
GG	62.69 ± 5.52	37.98 ± 2.46	16.61 ± 0.79	13.685 *	0.0027
GC	52.51 ± 3.57	38.16 ± 4.51	22.38 ± 3.21	18.702 *	0.0001
CC	53.54 ± 6.54	54.60 ± 16.50	16.57 ± 1.78	18.963 *	0.0001
LSD value	15.355 NS**	19.050 NS**	11.163 NS**		

***(Significant)** Not Significant**

The *MIF*-173 is situated in the 50 flanking region of *MIF* gene, which is strongly associated with protein production. Also, *MIF* allele defined by -173C SNP was associated independently with prostate cancer and independently with elevate levels of circulating MIF. The human *MIF* gene had a single nucleotide polymorphism (SNP; G to C transition) in the 50-flankingregion at position -173,which was associated with susceptibility to adult inflammation.

3- 9 Immunohitochemical staining of Bladder Tumor Tissues.

Ten normal urothelium tissues have been taken from forensic autopsy and tumors tissues of 73 patients had bladder cancer which were collected for studying the expression of some tumors markers (CD74, CD44, p53 and p21) by using immunostaining standard protocols.

3.9.1 CD74 IHC Score

CD74 is an integral membrane protein which consists of 296 amino acids and has a molecular weight of 33 kDa. It is mainly expressed in antigen presenting cells, endothelial cells and neuroglia cells and had two main functions as MHCII chaperon or as cell surface receptor for MIF (Gil-Yarom *et al* ., 2014).

In order to study the expression of CD74 molecules, staining was done by using anti-CD74 clone then comparing the CD74 expressions. The results in table (3-22) showed that 50(68.49) % exhibited positive staining with different scores and 23(31.51%) gave negative staining with highly significant differences, while all the bladder tumors free tissue expressed a negative staining.

These results agree with the results recorded by choi *et al.,* (2013) who found the immunohistochemical staining of 192 (56.1%) cases from 342 urothelial carcinoma showed CD74 positivity while 150 (43.9%) showed a negative result and CD74 was negatively or faintly stained in the cytoplasm of

non-neoplastic normal urothelial cells.

Table (3-22): CD74 expression in UBC and control.

Group	CD 74 Expression				Total		P-Value
	Positive		Negative				
	No.	%	No.	%	No.	%	
UBC	50	68.49	23	31.51	73	88	0.0027 *
Control	0	0.00	10	100	10	12	
Total	50	60.2	33	39.8	83	100	

*** (Significant).**

The results revealed that the positive score +3 gave the highest frequency (26.1 %) then score +2 represented(17.8%) followed by score +1 (16.4%) while score +4 represented the lowest frequency (8.2%) and negative score represented (31.5%) with significant differences as shown in table (3-23). It was also noticed that some tumors had necrosis, thus membrane of the cancerous cells did not express the CD74 clearly.

Table (3-23): Frequency of CD74 Scores in UBC and control.

CD 74 Score	UBC		Control		Total	
	No.	%	No.	%	No.	%
Scorer 0	23	31.5	10	100	33	39.8
Score +1	12	16.4	0	0.0	12	14.5
Score +2	13	17.8	0	0.0	13	15.6
Score +3	19	26.1	0	0.0	19	22.9
Score +4	6	8.2	0	0.0	6	7.2
Total	73	88	10	12	83	100
p-value	0.0001 *					

*** (Significant).**

In addition, the result in table (3-24) showed that a higher positive expression of CD74 was 20 (40%) out of 50 observed in stage T2 and 16(32 %) in stage T1. A lower positive expression was recorded in stage T3, Ta and T4 7(14.8 %), 4(8 %) and 3(6%) respectively. The highest negative expression was 12 (52.2 %) out of 23 in stage T1 and 8(34.8%) in stage Ta followed by 2(8.7%), 1(4.3%) in stage T1 and Ta followed by (8.7, 4.3 and 0) % in T2, T4 and T3 respectively with significant difference**.** The results revealed that early malignancy showed a highly expression of CD74 and a reduced progress as noticed in some tumors which had necrosis, thus membrane of cancerous cells not expressed the CD74 clearly.

Table (3-24): Association between CD74 Expression and UBC Stages.

Stage	CD74 expression Score										Total positive	
	Negative		+1		+2		+3		+4		No	%
	No.	%	No.	%	No.	%	No	%	No.	%		
Ta	8	34.8	1	8.3	3	23.1	0.0	0.0	0.0	0.0	4	8
T1	12	52.2	8	66.7	4	30.8	4	21.1	0.0	0.0	16	32
T2	2	8.7	1	8.3	5	38.5	8	42.1	6	100	20	40
T3	0	0.0	2	16.7	0.0	0.0	5	26.3	0.0	0.0	7	14
T4	1	4.3	0.0	0.0	1	7.6	2	10.5	0.0	0.0	3	6
Total	23	31.5	12	16.4	13	17.8	19	26.1	6	8.2	50	100
P value	0.001*											

Chi square=16.529 ;* (Significant)

CD74 expression is increased in the invasive carcinoma of the bladder. Its expression was significantly associated with older age at diagnosis. The result also agree with the results reported by McClelland *et al*., (2009) who found that CD74 expression primarily located on the malignant cells in the tumor would suggest that MIF might be working through antagonism of

apoptotic pathways, or by autocrine regulation of angiogenic factor expression. In many tumors, the malignant cells themselves formed and advanced invasive tumor strongly expressed CD74 and larger proportions of CD74-negative tumors were stage I-II.

Table (3-25) revealed that a higher positive expression was recorded in high grade 36 out of 50 (72) % and14 (28) % in low grade. The highest negative expression in low grade was 19 out of 23 (82.6) % and 4 (17.4) % in high grade. as shown in Figure (3-7), (3-8) (3-9)and (3-10) .

Table (3-25): Association between CD74 Expression and UBC Grade.

Grade	CD74 expression Score										Total positive	
	Negative		+1		+2		+3		+4			
	No	%	No.	%	No	%	No	%	No.	%	No.	%
Low	19	82.6	10	83.3	4	30.8	0	0.0	0	0.0	14	28
High	4	17.4	2	16.7	9	69.2	19	100	6	100	36	72
Total	23	31.5	12	16.4	13	17.8	19	26.1	6	8.2	50	100
P value	0.001*											

Chi square=18.092; *(Significant)

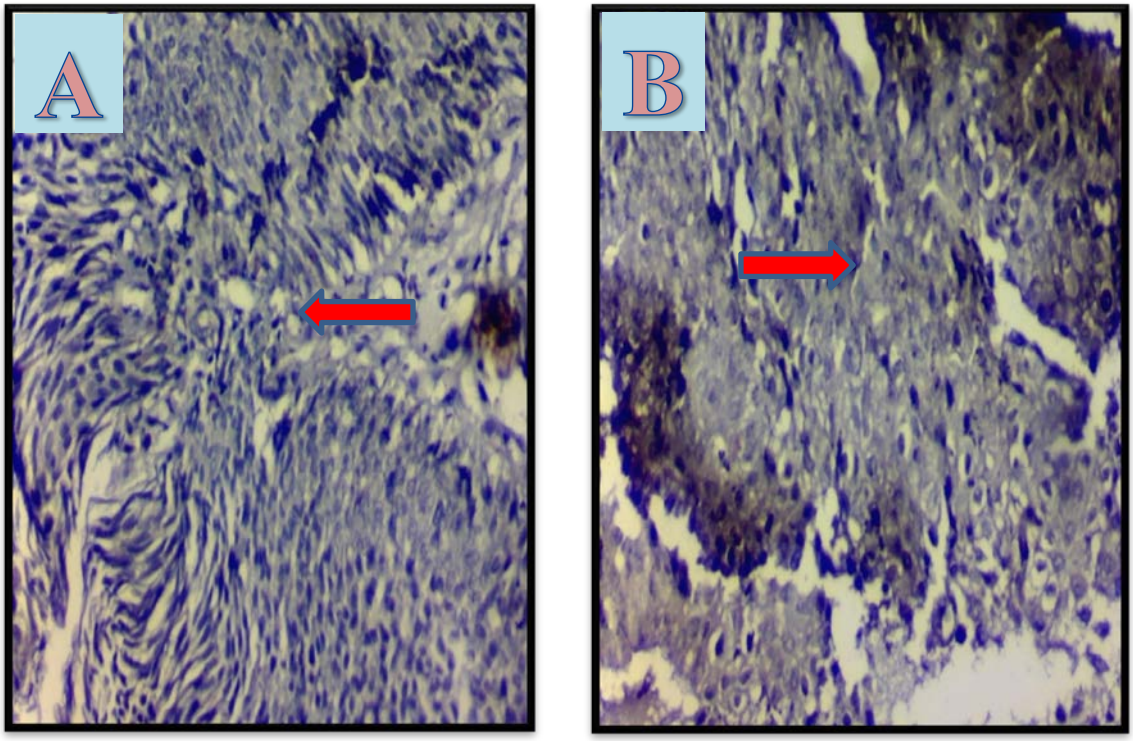


Figure (3-7): Negative Immunohistochemistry staining of CD7. A: Non Invasive UCC (Stage T0, Low Grade) (10X) B: Invasive UCC (Stage T2, High Grade) (10 X).

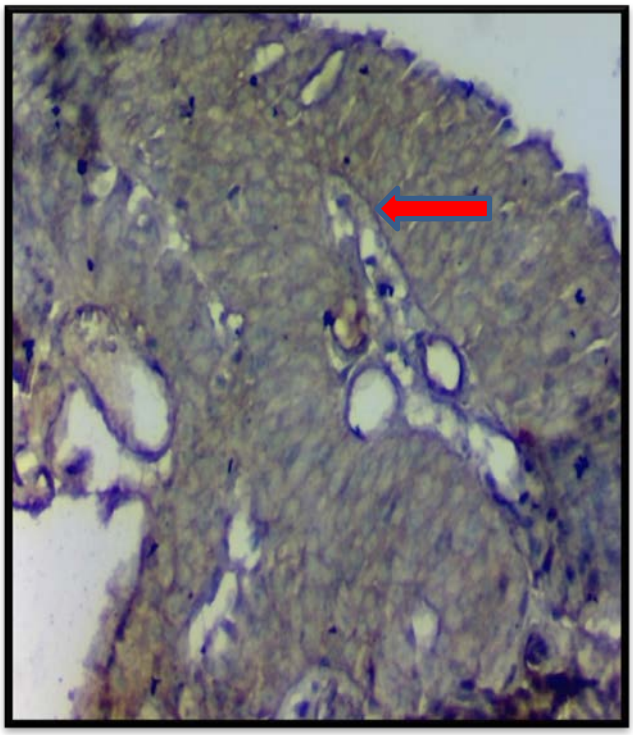
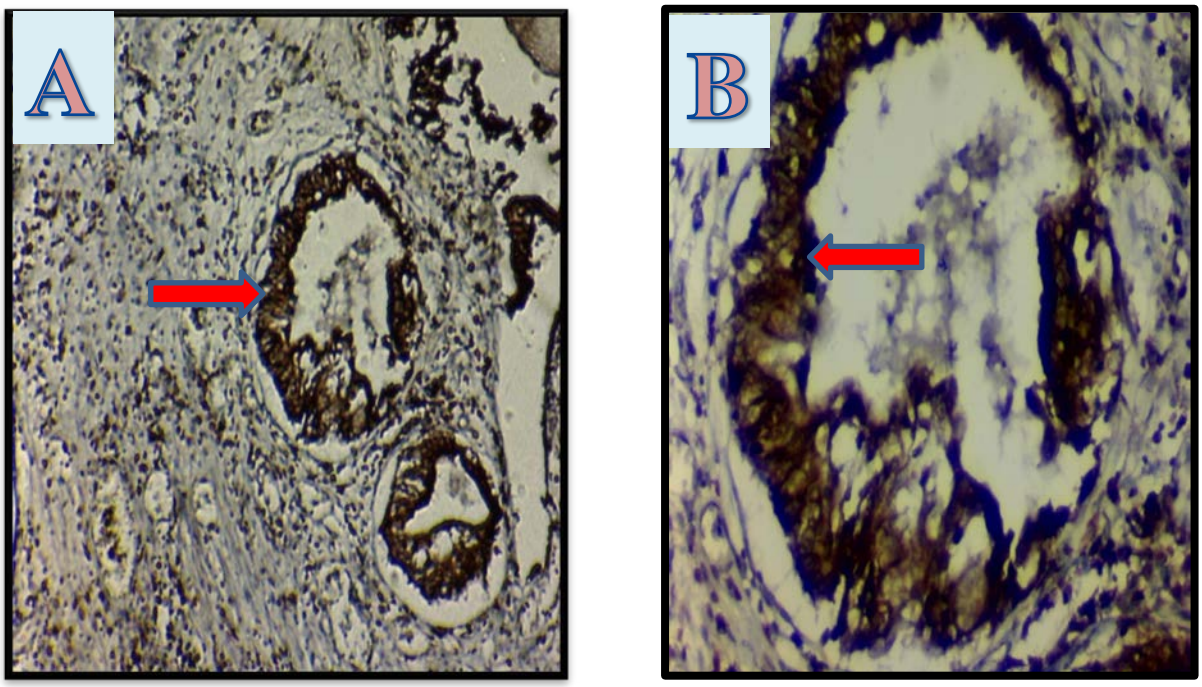


Figure (3-8):Positive immunohistochemistry staining of CD74 in non Invasive UCC (Stage T1, Low Grade) (10X).



Figure(3-9):Immunohistochemcal staining of CD74 (Invasive UCC stage: T2, High Grade)expressed positive staining (Score +3). A: Section under 10 X B: Section under 40 X.

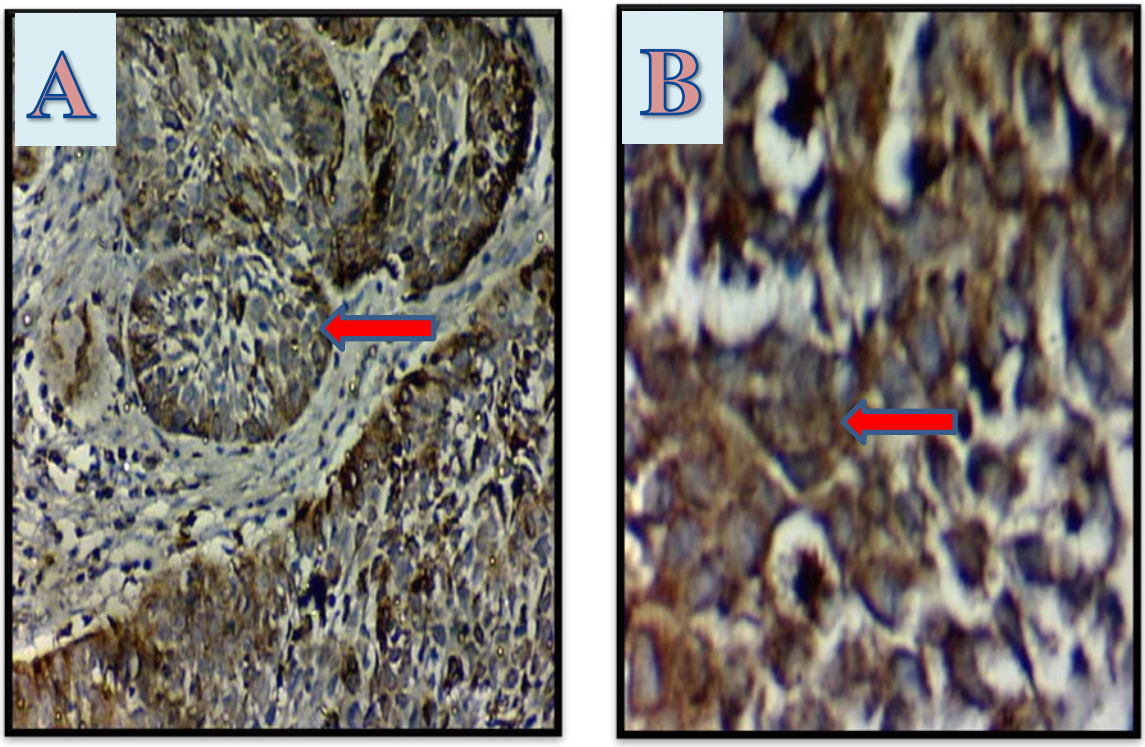


Figure (3-10): Immunohistochemistry Staining of CD74 (Invasive UCC stage: T2, High Grade) expressed positive staining (Score +3). A: Section under 10 X B: Section under 40 X.

Mahdi and Mohaymen (2009) studied the Immunohistochemical staining for CD74 on gastric biopsies from patients infected with H. *pylori* and found that the expression of CD74 was evident in uninfected tissue, but the infected tissue showed a marked increase in CD74 expression in 47 (73.4%) out of the 64 patients were positive for *H. pylori* infection patient biopsies were examined for CD74 expression. The results showed that 44 out of 47 (93.6%) were found to have a high CD74 expression .

In cancer cells, immunoreactivity for CD74 mostly located on the membrane and in the cytoplasm. There is growing evidence that MIF and it's receptor CD74 is involved in cancer genesis and progression. Currently, there is a general consensus that MIF promotes tumor growth by several mechanisms; it stimulates cancer cell proliferation, inhibits induction of p53-dependent apoptosis and increases production of vascular endothelial growth factor (Richard *et al*., 2014).

CD74 was reported as an accessory signaling molecule in cancers because of its localization on the plasma membrane in certain cell types, and its role as a surface-binding receptor for MIF a pro-inflammatory cytokine. Indeed, it is now generally accepted that the oncogenic role of CD74 is MIF-dependent (Liu and Lin, 2014).

3.9.2CD44 IHC Score

CD44 is a transmembrane glycoprotein that has been postulated to play important roles in a variety of biological processes in healthy and disease. Tissues showed highly positive immunohistochemical expression in bladder tumor tissues, 64 (87.7%) out of 73 gave positive results of staining with different scores as shown as in table (3-26) and 9(12.3%) (one with a low grade tumor and eight with a high grade tumor) gave a negative result while 9(90%) out of ten normal urothelium gave a positive expression with highly significant differences.

The results agreed with results reported by Sugino *et al*., (1996) who found that in normal bladder tissue, CD44 protein was strongly expressed on the plasma membranes of the basal cells of the urothelium and demonstrated that the overexpression of the CD4 locus previously recorded in early malignancy of the bladder and other organs progressively diminishes as the tumor invades deeper into the bladder wall. Also compatible with results reported by Stephan *et al*., (2015) who found that in bladder carcinomas, the strongest reaction to CD44 was seen in the basal epithelial region next to the basement membrane, suggesting an important role in the attachment between basal cell and basal lamina.

Table (3-26): CD44 expression in UBC patients and control.

Group	CD 44 Expression				Total		P-Value
	Positive		Negative				
	No.	%	No.	%	No.	%	
UBC	64	87.7	9	12.3	73	88	0.0001*
Control	9	90	1	10	10	12	
Total	73	88	10	12	83	100	

Chi square =15.063 ;* (Significant).

The score+2 represented the highest frequency (32.9%) then score +3 represented (31.5%) followed by score +1(13.7) and score +4 represented the lowest frequency(9.6%) while the negative score represented (12.3%) as shown in table (3-27) .Some tumors had necrosis thus membrane of the cancerous cells not expressed the CD44 clearly. Score +2 was the highest percentage 50% for the control followed by score +3and score +1represented (30 and 10%) respectively.

Yildırım *et al*., (2014) explained that CD44 expression in TCC showed an inverse correlation between tumor stage and CD44,+1(11%),+2 (56%) and

+3(33%) with highly significant differences. In addition, CD44 expression was reduced in poorly differentiated and invasive TCC as compared to noninvasive tumors.

Table (3-27): Frequency of CD44 Score in UBC and control.

CD 44 Score	UBC		Control		Total	
	No.	%	No.	%	No.	%
Score 0	9	12.3	1	10	10	12
Score +1	10	13.7	1	10	11	13.3
Score +2	24	32.9	5	50	29	35
Score +3	23	31.5	3	30	26	31.3
Score +4	7	9.6	0	0.0	7	8.4
Total	73	100	10	100	83	100
P value	0.00361*					

Chi square = 15.217; * (Significant).

The results in table (3-28) demonstrated that the highest positive expression of CD44 was 28(43.8%) out of 64 in T1 and 19(28.1%) of stage T2 followed by 10 (15.6%), 6(9.4%) and 2 (3.1%) of Ta, T3 and T4 respectively with highly significant differences. Moreover, the highest negative CD44 expression was4 (44.5) % out of 9 recorded in T2 and 2(22.2) % in Ta and T4 followed by (11.1 and 0)% in T3and T1, respectively.

The results have been explained in table (3-29) showed that the positive expression of CD44 was 31 (48.4) % out of 64 tumors as a low grade and high grade33 (51.6) % with no significant difference while the negative expression was 2(22.2%) in a low grade and 7 (77.8%) high grade as shown in figure (3-11),(3-12), (3-13) and (3-14).

Table (3-28): Association between CD44 Expression and UBC Stages.

Stage	CD44 expression Score										Total of positive	
	Negative		+1		+2		+3		+4		No.	%
	No.	%	No.	%	No.	%	No.	%	No.	%		
Ta	2	22.2	0	0.0	3	12.5	6	26.1	1	14.3	10	15.6
T1	0	0.0	2	20	9	37.5	13	56.6	4	57.1	28	43.8
T2	4	44.5	6	60	9	37.5	1	4.3	2	28.6	18	28.1
T3	1	11.1	2	20	2	8.3	2	8.7	0	0.0	6	9.4
T4	2	22.2	0	0.0	1	4.2	1	4.3	0	0.0	2	3.1
Total	9	12.3	10	13.7	24	32.9	23	31.5	7	9.6	64	100
P value	0.001*											

Chi Square=16.394; * (Significant).

Table (3-29): Association between CD44 Expression and UBC Grade

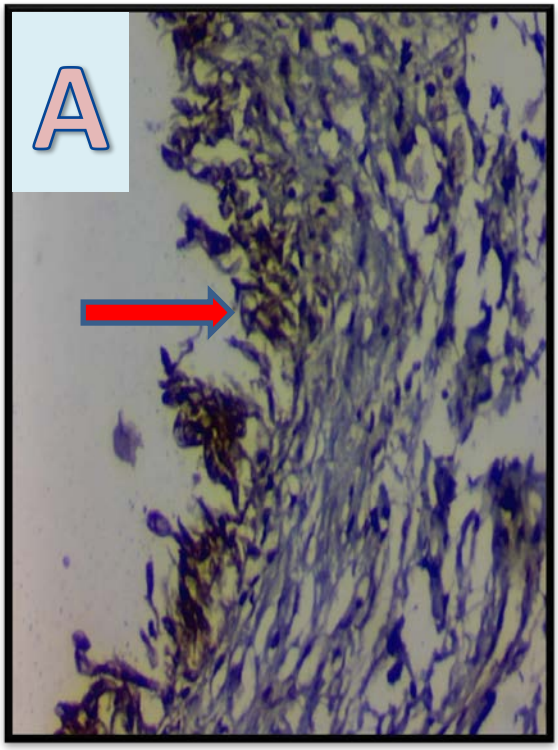
	CD44 expression Score										Total of positive	
	Negative		+1		+2		+3		+4		No.	%
	No.	%	No.	%	No	%	No.	%	No.	%		
Low	2	14.3	2	14.3	10	37.5	16	85.7	3	42.9	31	48.4
High	7	85.7	8	85.6	14	62.5	7	14.3	4	57.1	33	66
Total	9	12.3	10	13.7	24	32.9	23	31.5	7	9.6	50	100
P value	0.461*(NS)											

Chi square=15.784; * Not Significant

Although higher expression was not detected in Ta tumors compared to T1, there were significantly more extensive positive areas in noninvasive tumors than the deeply invasive tumors (Stavropoulos *et al.*, 2001).

Results of CD44 expression were agreed with previously study by

Mahmood *et al.*, (2015)who reported that there was a high association between the expression level of CD44 and tumor type, grade and lymph node metastasis and may play important roles in cancer progression and metastasis. Also, agreed with Erdogan *et al.*, (2008) in that the higher CD44 expression was revealed in a low grade and noninvasive tumors and all tumors diagnosed as T1 stage showed 100% positive expression for CD 44.



Figure(3-11):Positive Immunohistochemical staining of CD44 (Normal Urothelium):A: Section under 10X B: Section under 40X.

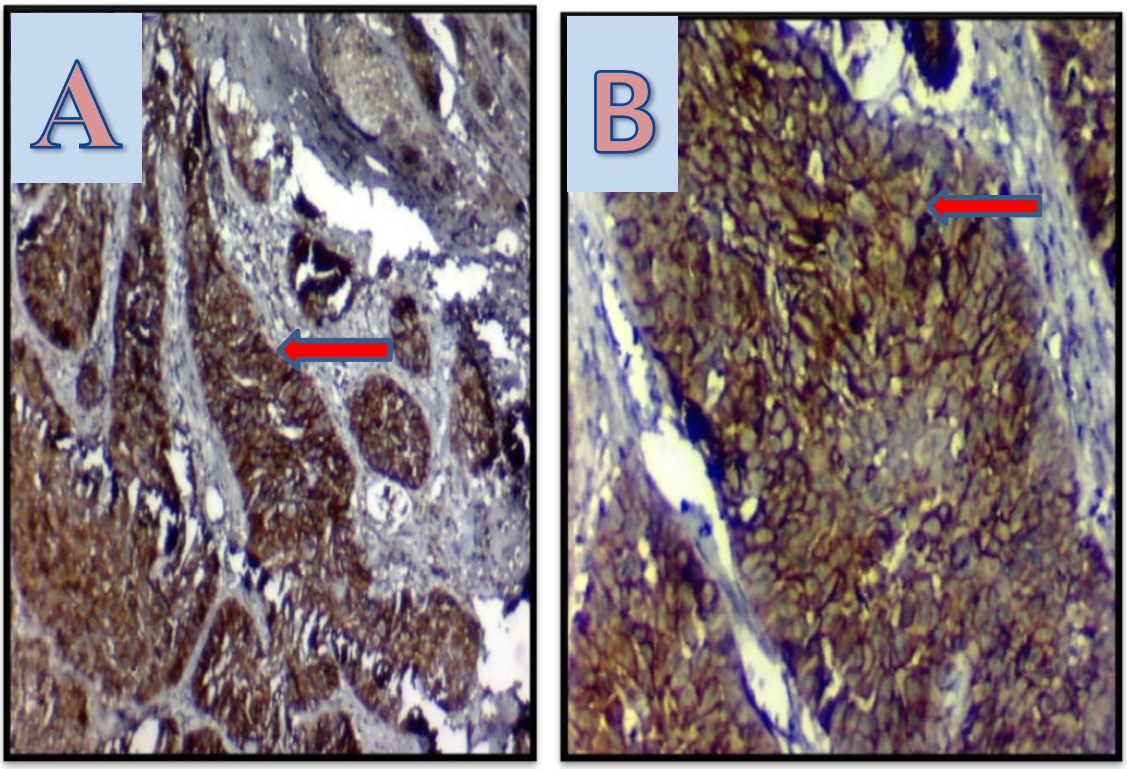


Figure (3-12): Positive Immunohistochemical staining of CD44 (Invasive UCC, StageT2, High Grade, Score 4+).A: Section under 10X B:Section under 40X).

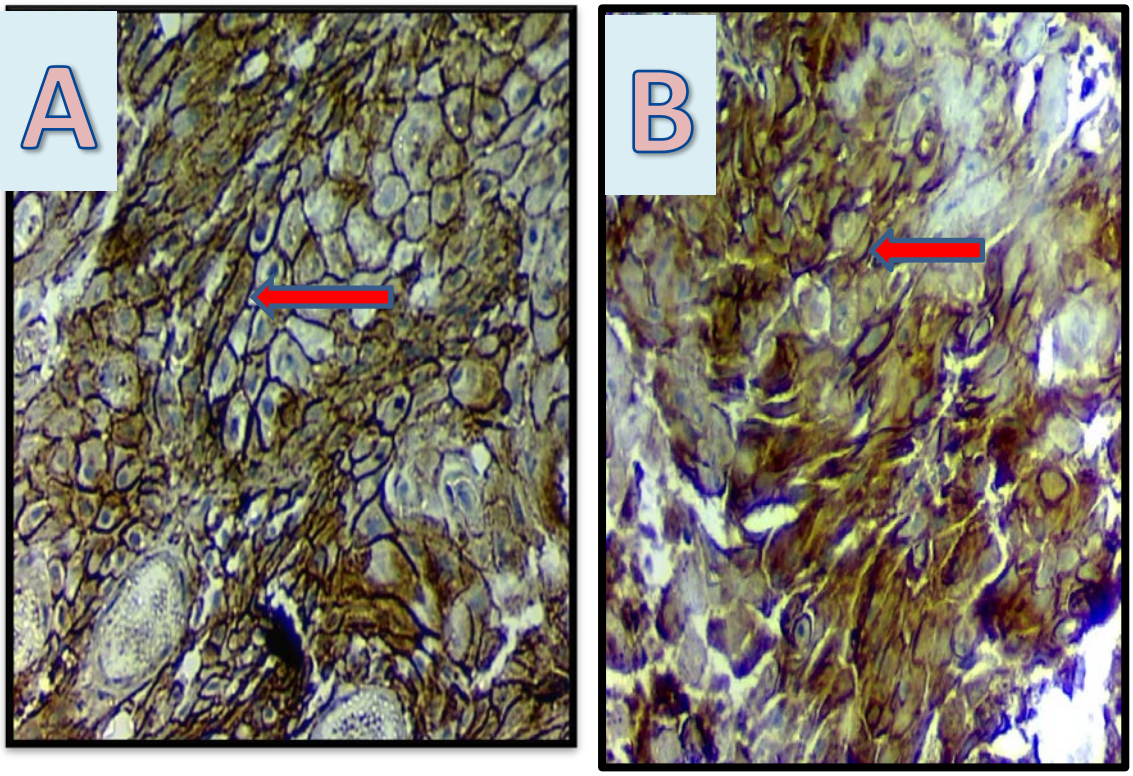
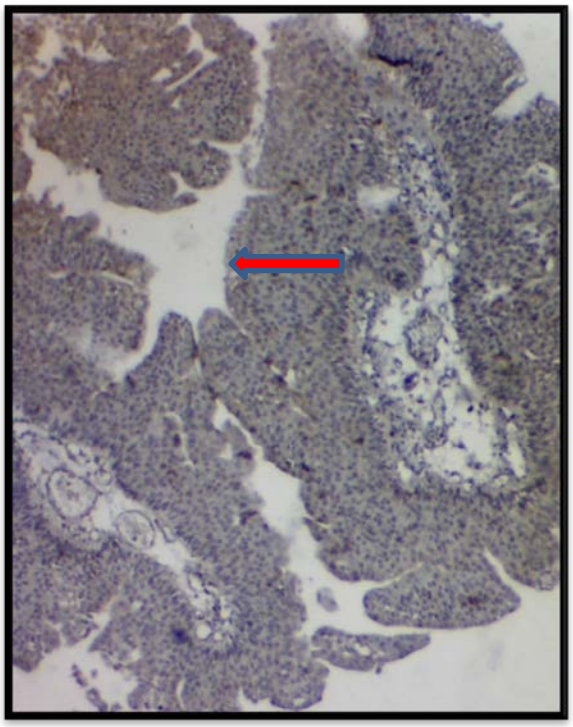


Figure (3-13): Positive Immunohistochemical staining of CD44 (Invasive UCC, StageT2, High Grade, Score 4+). A: Section under 10X B:Section under 40X.



Figure(3-14):Negative Immunohistochemical staining of CD44 (Stage T1,Low Grade).

3.9.3 p53 Immunohistochemistry Score

The tumor suppressor protein p53 plays a pivotal role in preventing damaged or abnormal cells from becoming malignant and loss of its function associated with a majority of human cancers. p53 activity is not required during normal cell growth and the protein must be kept at low levels and inactive. Analysis of 73 bladder tumors and 10 specimens of normal bladder tissues revealed that 42(57.5%) gave positive staining which were exclusively nuclear and 31(42.5%) gave a negative staining result while expression of p53 was nil in all normal transitional epithelium specimens as shown in table (3-30).

The result agree with the results reported by Shahrokh *et al*., (2004) who found that 56% of bladder tumors had a positive nuclear expression of p53 seemed to be the strongest predictor of bladder cancer associated with the later stages of bladder cancer clinical progression . p53 protein plays key roles in controlling tumor progression and loss of normal p53 function that can be

sufficient to predispose tumor cells to gain metastatic properties. In contrast, dominant p53 mutants that have gained oncogenic functions can actively drive metastasis through a variety of mechanisms.

Table (3-30): p53 **expression in UBC and Control.**

Group	p53 Expression				Total		P-Value
	Positive		Negative				
	No.	%	No.	%	No.	%	
UBC	42	57.5	31	42.5	73	88	0.894NS*
Control	0	0.0	10	100	10	12	
Total	42	50.6	41	49.4	83	100	

Chi square =16.577; *NS: Non-significant

Many muscle-invasive tumors arise from preexisting urothelial carcinoma in situ, a high-grade intraepithelial neoplasm and often exhibit alterations in the p53 tumor suppressor genes (Deletion, mutation, altered protein localization, protein accumulation). However, mutations of tumor suppressor gene are involved in the transition from in situ tumors to invasive tumors because mutation in p53 not only disrupts its function but also prevents normal ubiquitination leading to nuclear accumulation of p53 protein that can be detected immunhistochemically(Yeudall,2014; Girardini *et al.*, 2014 and Rosenberg and Hahn,2015).

The expression profile of p53 protein in tissues of UBC patients which had different stages of tumors showed differences scores among these various tissue summarized in table(3-31).Score +3 reflected a high frequency 17(23.3%) and15(20.5) for score +4 then 9(12.3%) score +2 and 1(1.4%) for score +1.

Table (3-31): Frequency of p53 **Scores in UBC and control.**

p53 Score	UBC		Control		Total	
	No.	**%**	**No.**	**%**	**No.**	**%**
Scorer 0	31	42.5	10	100	41	49.4
Score +1	1	1.4	0	0.0	1	1.2
Score +2	9	12.3	0	0.0	9	10.8
Score +3	17	23.3	0	0.0	17	20.5
Score +4	15	20.5	0	0.0	15	18.1
Total	73	100	10	100	83	100
P value	**0.001***					

Chi squar =15.062; **(Significant)

In addition, the results in table (3-32) showed a higher significant positive expression of p53 which observed in tumors had stage T2 (22(52.4%) out of 42) which had no negative expression , 5 out of 7 in stage T3(5 (11.9%)out of 42) and all T4 tumors gave strong positive expression (4(9.5%)out of 42), 10 (23.8%) in stage T1 and the lower positive expression was recorded 1(2.4%)in Ta while the highest negative expression was 18(58.1%) out of 31 in stage T1 and 11(35.5%)in stage Ta followed by 2(6.4%) in stage T3 respectively with significant difference .

In normal cells, the p53 tumor suppressor gene plays a critical role in induction of programmed cell death during cellular stress and DNA damage. In many cancers, p53 is mutated, leading to under expression or loss of function; hence, p53 has emerged as an important target for chemoprevention and therapy thus suggests that p53-modulating agents can serve as potential chemo-preventive agents for invasive urothelial cancers. The reported p53 mutation rate in bladder cancer was in the range of 30%-58% and p53 immunoreactivity was observed in 34% of the total cases and expression was

not significantly related to stage because tumors P53 expression was in a descending order. (Madka *et al.,* 2013).

Table (3-32): Association between p53 Expression and UBC stages.

Stages	p53 expression Score										Total of positive	
	Negative		+1		+2		+3		+4		No.	%
	No	%	No	%	No.	%	No.	%	No.	%		
Ta	11	35.5	0	0.0	0	0.0	1	5.9	0	0.0	1	2.4
T1	18	58.1	2	100	6	66.7	1	5.9	1	7.1	10	23.8
T2	0	0.0	0	0.0	3	33.3	10	58.8	9	64.3	22	52.4
T3	2	6.4	0	0.0	0	0.0	4	23.5	1	7.1	5	11.9
T4	0	0.0	0	0.0	0	0.0	1	5.9	3	21.5	4	9.5
Total	31	42.5	2	2.7	9	12.3	17	23.3	14	19.2	42	100
P value	0.00537*											

Chi square =16.713;*(Significant)

Tawfeeq and Al-Talib,(2012) who found that p53 tumor suppressor gene has been found to be mutated in more than 50% of human cancers. It has attracted the interest of numerous researchers and gene encoding p53 mediates a major tumor suppression pathway that is frequently altered in human cancers. Puzio-Kuter *et al*.,(2015) reported that bladder cancer represents a serious health problem worldwide. Inactivation of p53 promotes tumorigenesis in human bladder and p53 wild-type (undetectable) in noninvasive papillary tumors while p53 alteration ,p53 nuclear over overexpression and/or gene mutations were frequently observed in invasive bladder tumors .

Table (3-33) revealed that the highest significant positive expression was recorded in high grade 36(85.7%) out of 42 and 6(14.3%) low grade. The

highest negative expression in the low grade was 27out of 31(87.1%) and 4 (12.9%) in high grade as shown as in figure (3-18),(3-19), and(3-20) .

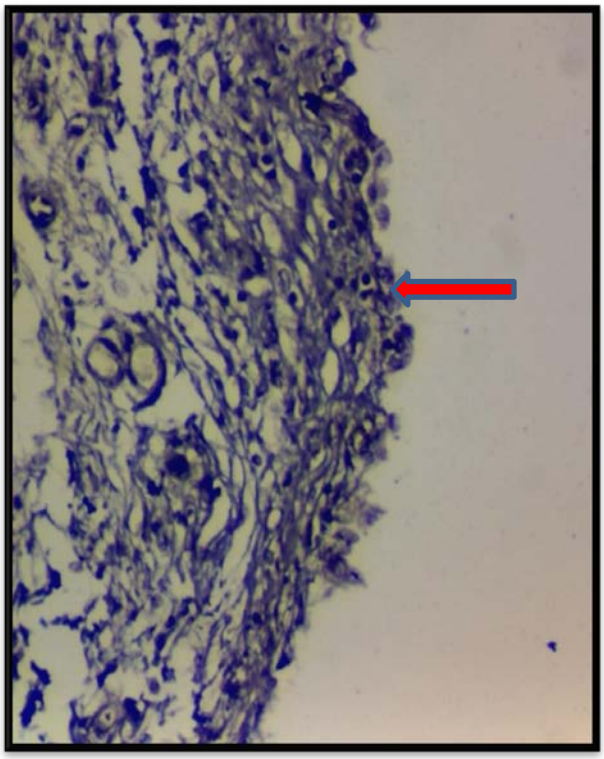
Table (3-33): Association between p53 Expression and UBC grade.

Grade	p53 expression Score										Total of positive	
	Negative		+1		+2		+3		+4		No.	%
	No.	%	No.	%	No.	%	No	%	No.	%		
Low	27	87.1	2	100	3	33.3	1	5.9	0	0.0	6	14.3
High	4	12.9	0	0.0	6	66.7	16	94.1	14	100	36	85.7
Total	31	42.5	2	2.7	9	12.3	17	23.3	14	19.2	42	100
P value	0.00627*											

Chi square =14.694; *(Significant).

The results expressed a significant relation between p53 and grade accepted with other study by Nima and Aziz (2011) who found that the positive expression of p53 was more common among patients with a high grade (76%) than low grade(34%) of histological tumor and Charf *et al*.,(2013) who found that strong expression p53 score +3 and score +4 significantly correlated with the high tumor grade.

However, these results were in disagreement with Toyoaki *et al* (2010) who showed that the overall expression of p53 protein had no statistically significant relation with different clincopathological variables like :age, gender, histological type, tumor grade and stage. This difference may be due variation in technique for enhancing epitope expression and non uniform methodology for p53staining.



Figure(3-15):Negative Immunohistochemical staining of p53 **(Normal Urothelium) (10X).**

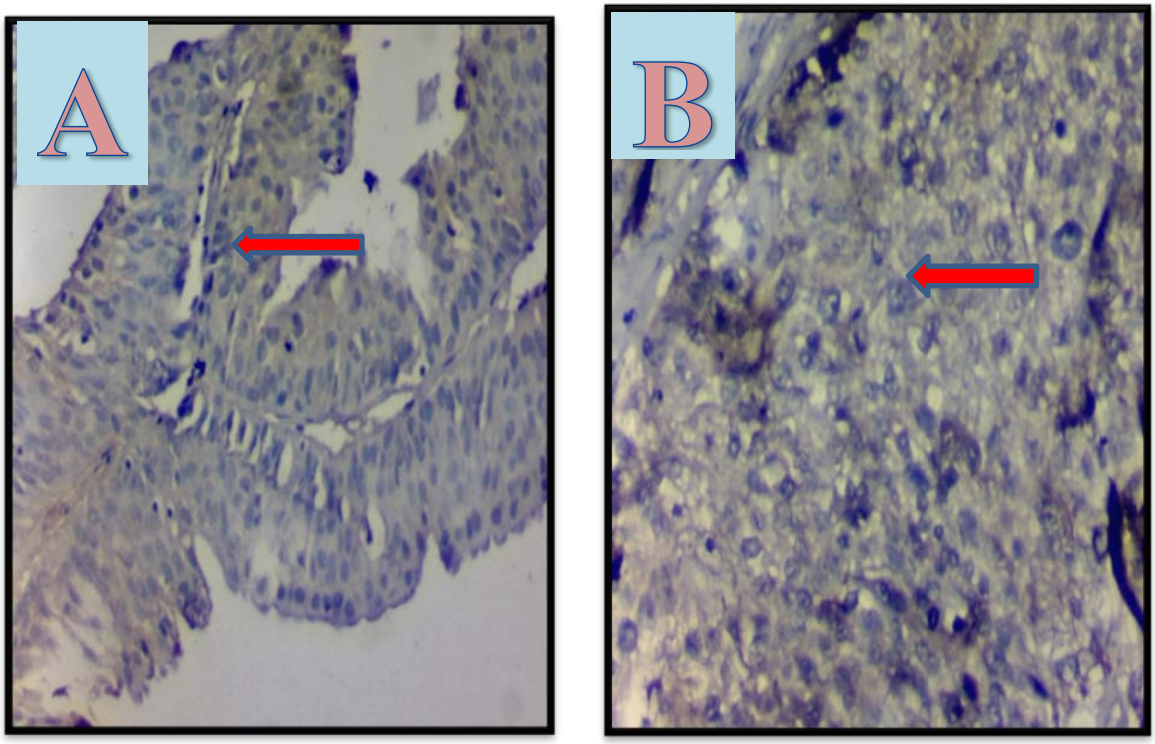
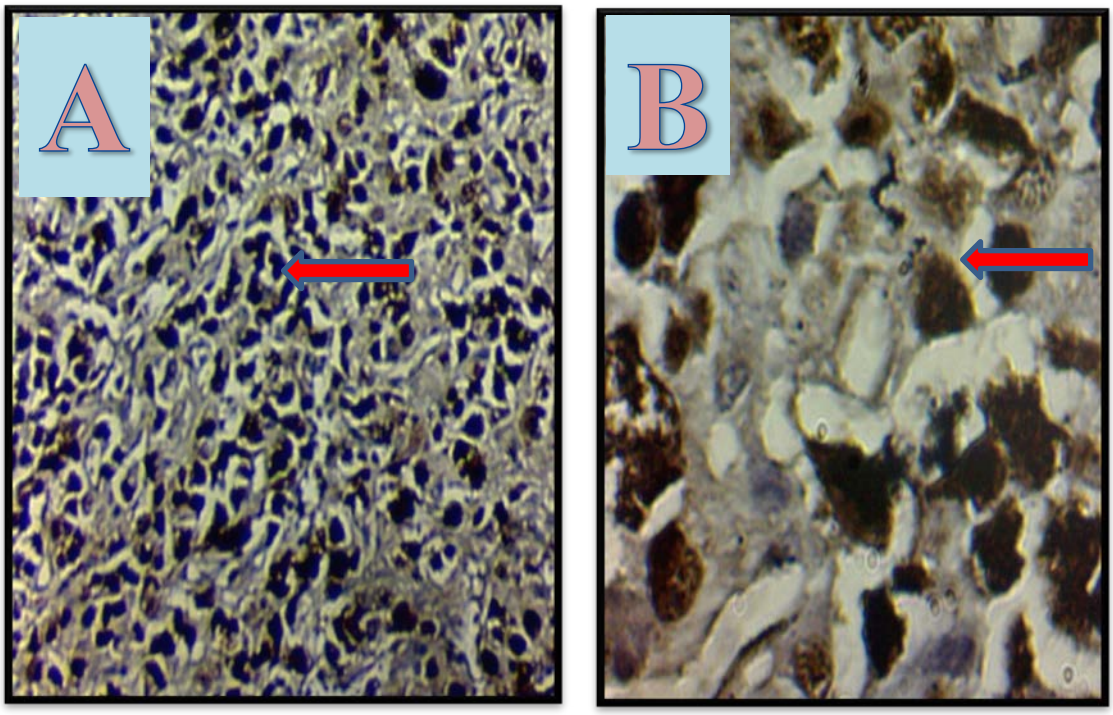


Figure (3-16): Negative Immunohistochemical staining of p53 **A: Non Invasive UCC (Stage T1,Low Grade) B: Invasive UCC (Stage T3, High Grade) (10 X).**



Figure(3-17):Positive Immunohistochemical staining of p53 **(Invasive UCC, Stage 4, high Grade)A: Section under10X B: Section under 40X**

3.9.4 p21 Immunohistochemistry Scores

p21(cyclin-dependent kinases Inhibitor) suppresses tumors by promoting the cell cycle arrest in response to a variety of cellular and environmental signals to promote tumor suppressor activities to various stimuli which inhibits the CDK activity required for the firing of replication of origins and for the activity of proteins directly involved in DNA synthesis by an unidentified mechanism. p21 also protects cells from apoptosis and allows DNA repair because an active cell cycle is required to sense these agents and trigger apoptosis.

The results in table (3-34) showed that 51(69.9%) out of 73 urinary bladder tumors gave a positive nuclear staining for p21 with different scores as shown in figure (3-23)and (3-24) while 22 (30.1%) gave negative staining result and all ten normal urothlium consistently demonstrated no p21 immunoreactivity, providing a negative staining pattern as shown in Figure(3-21) and Figure (3- 22).

Table(3-34): p21 expression in UBC and Healthy Control Groups

Group	p21 Expression				Total		P-Value
	Positive		Negative				
	No.	%	No.	%	No.	%	
UBC	51	69.9	22	30.1	73	88	0.0026*
Control	0	0.0	10	100	10	12	
Total	51	61.4	32	38.6	83	100	

Chi square =17.262 ; *(Significant)

In term, of p21 score frequency of distribution illustrated in table (3-35),UBC patients with score +2 represented the highest frequency 17 (23.3%) ,score +3 represented 16(22 %) and score +4 represented 12(16.4 %) while score +1 gave the lowest percentage 6 (8.2%) with significant differences.

Table(3-35):Frequency of p21 **Immunohistochemistry expression Scores**

P^{21} Score	UBC		Control		Total	
	No.	%	NO.	%	NO.	%
Score 0	22	30.1	10	100	32	38.6
Score +1	6	8.2	0	0.00	6	7.2
Score +2	17	23.3	0	0.00	17	20.5
Score +3	16	22	0	0.00	16	19.3
Score +4	12	16.4	0	0.00	12	14.4
Total	73	100	10	100	60	100
p-value	0.00271*					

Chisquare=15.335 * (Significant).

The result agree of the present study with the results reported by Stein *et al*., (2015) who found that (36%) of bladder tumors were p21 negative and (64%) were p21-positive while normal urothelium had no p21 immunoreactivity, providing a negative nuclear staining.

Expression of the cyclin dependent kinase inhibitor p21 provides important prognostic information in patients with UCC of the bladder. Patients with tumors that maintained p21 expression demonstrated a statistically significant decreased rate of tumor recurrence and a statistically significant increased overall survival in comparison with those whose tumors had lost expression of p21 and statistically significant association between p21 expression and tumor progression was observed at all pathologic stages.

The results demonstrated in table (3-36) showed that the highest positive expression of p21 was19 (37.3) % out of 51 in T1 and 16 (31.4) % in T2 respectively followed by 8(15.7) % in Ta, 4(7.8) % in T3 and T4 respectively with highly significant differences. Moreover, the highest negative p21 expression was 9 (41) % out of 22 recorded in T1 and 6(27.3) % in T2 followed by4 (18.1)% in Ta and 3 (13.6)% inT3.

p21 is a positive marker for invasive cancers, but is a negative prognostic marker in superficial cancers p21 is an independent predictor of time to recurrence and overall survival and acts synergistically with p53 and associated with bladder cancer progression and risk of death. p21 combined with p53 is a strong predictor of progression. Advanced stage tumors were present in patients of older age and poorly differentiated tumors were presented in the advanced stage (Duggan and Williamdson, 2004 and Simsek *et al*., 2013).The results in table (3-37) revealed that a higher positive expression was recorded in high grade 29(56.9%) out of 51 and 22(43.1%) out of 51 in low grade with significant differences while the negative expression in low grade was 11(50%)out of 22 and 11(50%) in high grade.

Table(3-36):Association between p21 expression and UBC stages.

Stage	p21 expression Score										Total of positive	
	Negative		+1		+2		+3		+4		No.	%
	No	%	No.	%	No	%	No.	%	No.	%		
Ta	4	18.1	3	50	1	6.2	1	5.6	3	27.3	8	15.7
T1	9	41	3	50	6	37.5	8	44.4	2	18.2	19	37.3
T2	6	27.3	0	0.0	3	18.8	7	38.9	6	54.5	16	31.4
T3	3	13.6	0	0.0	2	12.5	2	11.1	0	0.0	4	7.8
T4	0	0	0	0.0	4	25	0.0	0	0	0.0	4	7.8
Total	22	30.1	6	8.2	16	21.9	18	24.7	11	15.1	51	100
P-value	0.0013*											

Chi square =16.536; *(Significant)

Table (3-37): Association between p21 Expression and UBC grade.

Grade	p21 expression Score										Total of positive	
	Negative		+1		+2		+3		+4		No.	%
	No.	%	No.	%	No.	%	No.	%	No.	%		
Low	11	50	6	100	5	31.3	7	38.9	4	36.4	22	45.2
High	11	50	0	0.0	11	68.7	11	61.1	7	63.6	29	54.8
Total	22	30.1	6	8.2	16	21.9	18	24.7	11	15.1	51	100
P-value	**0.043***											

Chi square=16.519

Migaldi *et al*., (2015) found that Positive p21 staining (>5% positive nuclei) was observed in 68 of the 96 (71%) tumors. p21 expression was neither associated with tumor stage nor with tumor grade. Expression of p21

did not correlate with tumor grade nor with tumor stage in Primary superficial bladder cancers. Only cells with a clear nuclear staining were considered positive. Patients with tumors that maintained p21 expression demonstrated a statistically significant decreased rate of tumor recurrence and a statistically significant increased overall survival in comparison to those whose tumors had lost expression of p21 while Olsson *et al.* ,(2012) on the other hand , noted by comparison that 76% of the tumors exhibited abnormal p21 expression, some of which were stages Ta and T1,but the majority were stage T2 or higher.

p21 can protect against apoptosis in response to other stimuli such as those induced by growth factor deprivation, p53 overexpression or during the differentiation of monocytes. p21 binds to and inhibits the activity of proteins directly involved in the induction of apoptosis Furthermore, p21 can mediate the upregulation of genes encoding secreted factors with anti-apoptotic activities and suppresses the induction of pro-apoptotic genes. Additionally, p21 is misregulated in human cancers, but its expression, depending on the cellular context and circumstances, suggests that it can act as a tumor suppressor or as an oncogene. Loss of p21 expression is statistically significant and independent predictor of bladder cancer progression. Maintenance of p21 expression appears to abrogate the deleterious effects of p53 alterations on bladder cancer progression (Abbas and Dutta ,2010)**.**

Shahrokh *et al.*,(2004) reported that altered expression of p21 is associated with an increased risk of bladder cancer progression and death after adjusting for the effects of pathologic stage, grade, invasion, and lymph node metastases. Alteration of p21 occurred in more than half of pT1 bladder tumors and remained equally frequent throughout all stages.

The role of cell cycle regulators in bladder cancer progression seems to be a complex accumulation of genetic alterations, from which p21 seems to

be associated with the early stages of bladder cancer clinical progression and p53 and pRB/p16 seem to be associated with the later stages of bladder cancer clinical progression while Tang *et al*.,(2015) who found that high p21 expression in 78.3% was associated with tumor grade and stage but not associated with other data, such as age. It has been proposed that progressive cancers accumulate p21 due to impairment of the p21-induced inhibitory pathway or mutations in the gene, resulting in the overproduction of abnormal genes .

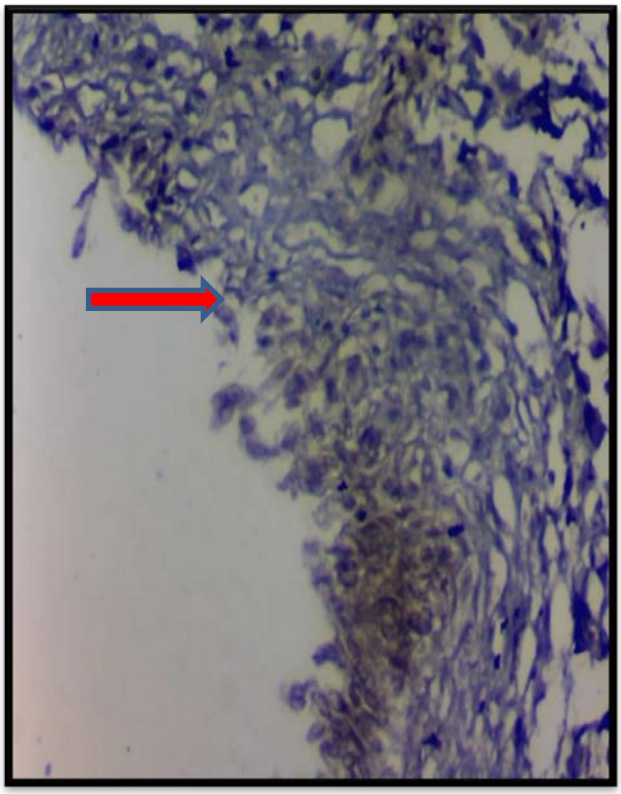


Figure (3-18): Negative Immunohistochemistry Staining of p21 **(Normal Urothelium).**

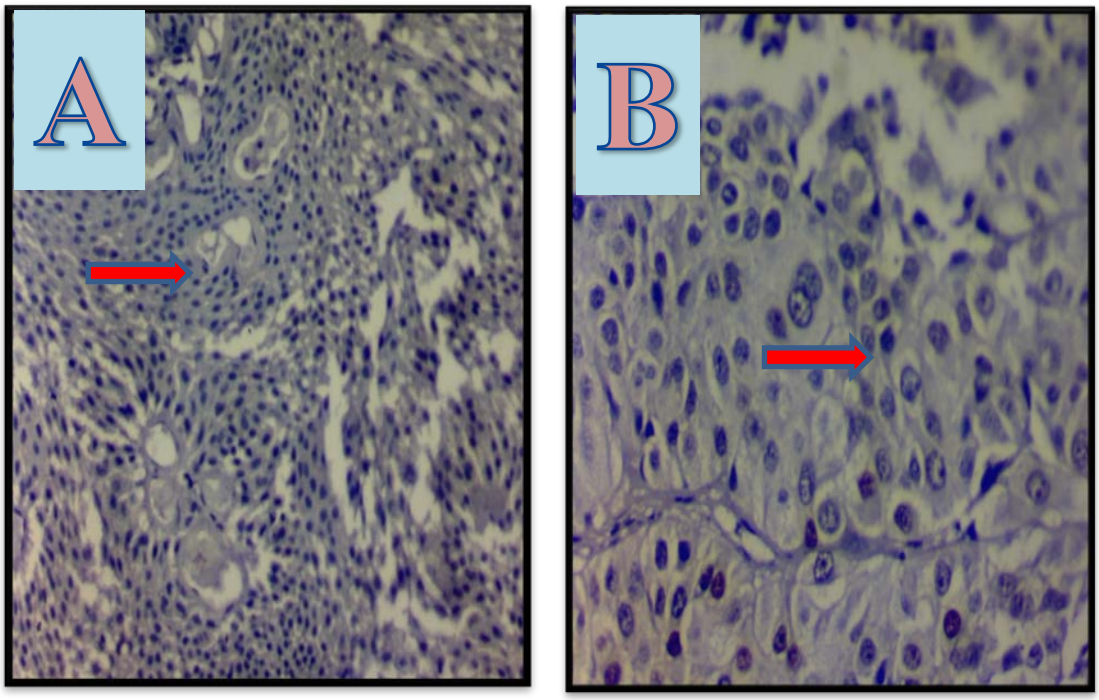


Figure (3-19): Negative Immunohistochemistry Staining of p21**A: Non Invasive UCC (Stage T1, Low Grade)B: Invasive UCC (Stage T2, High Grade) (10X).**

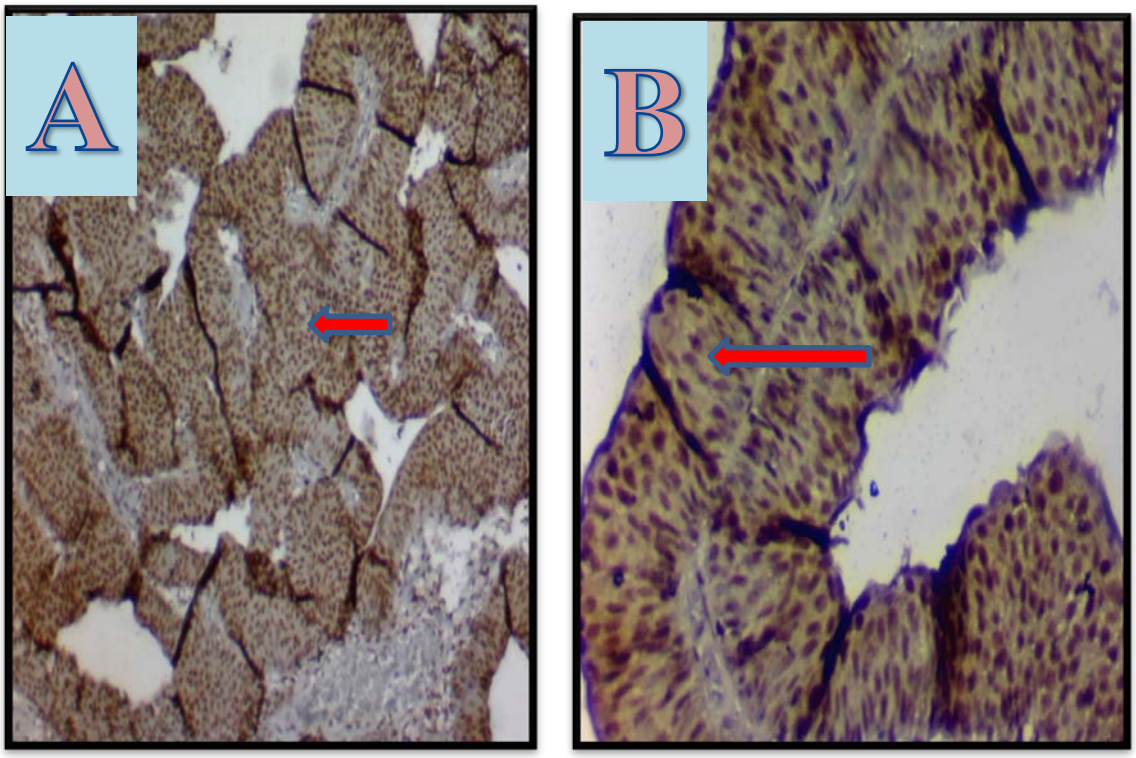


Figure (3-20): Positive Immunohistochemical Staining of p21 **(Non Invasive TCC, Stage T1, Low Grade, Score 2+). A. Section under 10X, B. Section under 40X.**

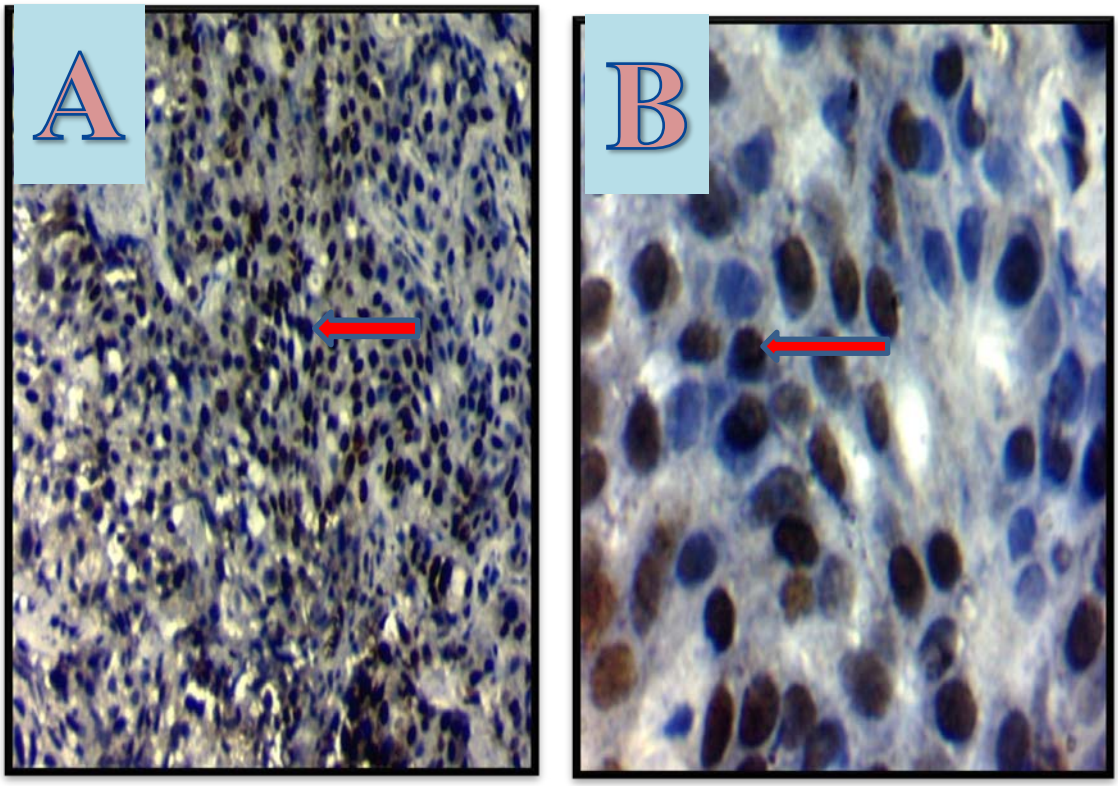
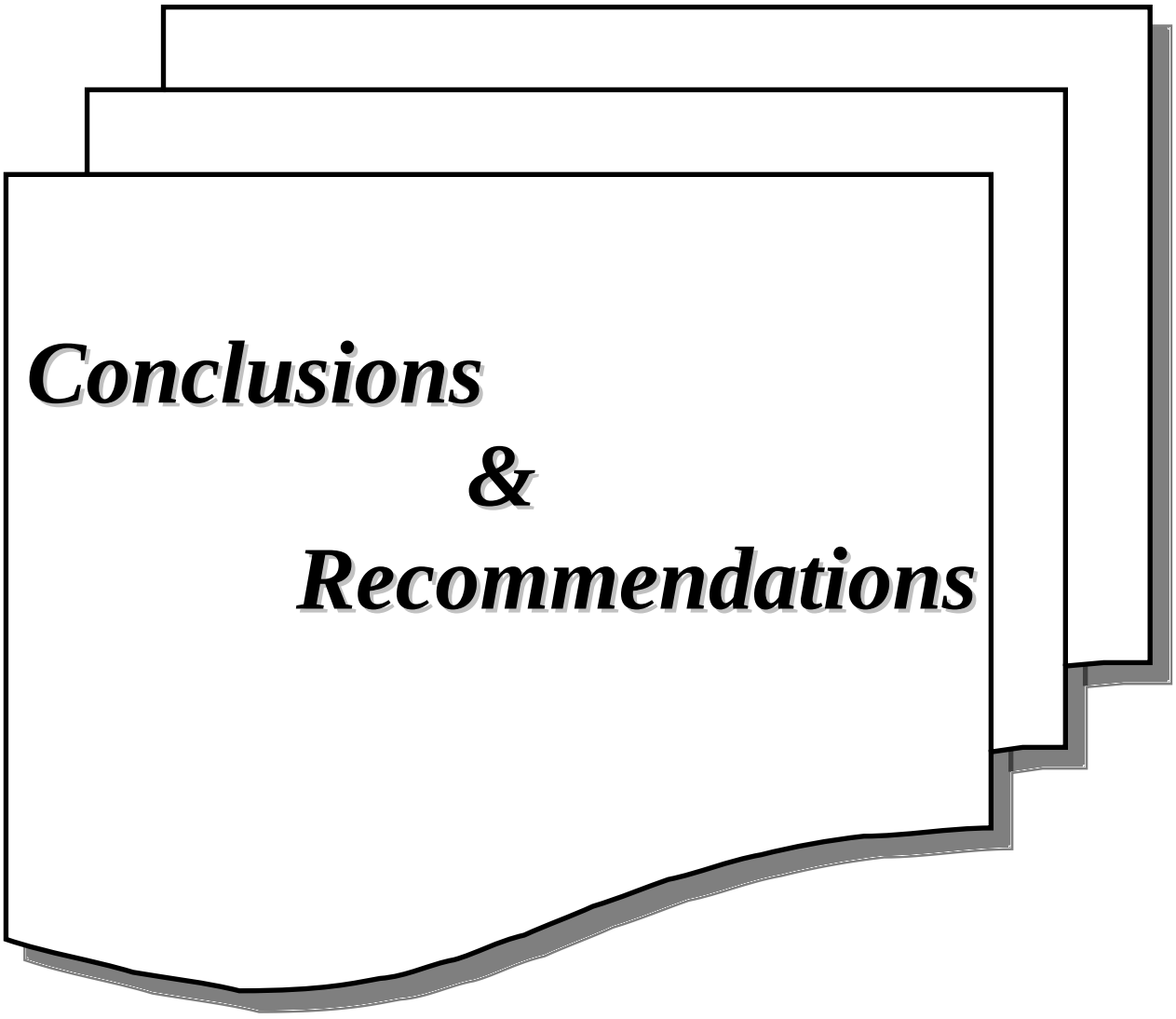


Figure (3-21): Positive Immunohistochemical staining of p21 **(Invasive UCC, Stage T2, High Grade, Score 4+).A: Section under10X. B: Section under 40X**

Chapter Four



Conclusions & Recommendations

Conclusions and Recommendations

Conclusions:

1- More than 90% of the bladder cancers were urothelial cell carcinomas (UCC) and approximately 70% of bladder tumor present as non-muscle-invasive bladder cancer (NMIBC). Nearly 60% - 70% of these tumors recurred, and 25% progressed into a higher stage or grade.

2- Males to female ratio was 4.61:1 and the bladder cancer risk increased with age. The age of diagnosis ranged was (43-85).

3- Cigarette smoking was the most well-established risk factor for bladder cancer and bacterial infection may play a significant role in secreted cytokines and growth factors participate in the development and promotion of malignancies.

3- *MIF* $-_{173}$G>C polymorphism may played a role in the etiology of bladder cancer and that individuals with GC/CC genotype were with lower bladder cancer risk than G/G genotype.

4- Cytokine level varied among individuals in a positive correlation with tumor stage and grade might influence cancer susceptibility by altered inflammatory responses.

5- Overexpression of MIF with higher expression of its receptor CD74 and CD44 promote malignant cell transformation in urinary bladder carcinoma.

6- p53 and p21 nuclear overexpression were positively correlated with the advanced high grade and increased risk of bladder cancer progression.

Recommendations:

1. Studies with high number of samples are necessary to provide new insight on bladder cancer related to *MIF* polymorphism.

2. The pro-inflammatory cytokine migration inhibitory factor and its receptor CD74 have been proposed as possible therapeutic targets in several cancers.

3. Studying the polymorphism effect of other cytokines and other SNPs of *MIF* gene by Sequencing Technique.

4. Investigation the role of bacterial infection in cancer induction and progression.

5. Additional need of more selective cancer biomarkers for the clinicopathological evaluation of tissue or biopsy material of cancer.

6. Investigation the role of immunotherapy as potential agent for immunity against bladder cancer.

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الخلاصة

هدفت هذه الدراسة الى تركيز الضوء على العلاقة بين تعدد النمط الوراثي للجين المسؤول عن تثبيط هجرة خلية البلعم في الموقع -173 من منطقة الحفاز وخطورة الاصابة بسرطان المثانة في المجتمع العراقي .فقد تم استخدام تقنية PCR-RFLP لدراسة الانماط الوراثية ،مع قياس مستوى بعض الحركيات الخلوية (IL-6, IL-10,TNF,MIF) في مصل الدم باستعمال تقنية الاليزا بالاضافة الى تحديد التعبير النسيجي لبعض معلمات الاورام النسيجية (CD74، CD44، p53 ، p21)وقد جمعت عينات الدم والادرار والنسيج من المرضى الراقدين في مستشفى اليرموك التعليمي ومستشفى الجبية جي الخاص والمتطوعين الاصحاء للفترة من اذار ولغاية تشرين الثاني لعام 2014.

شملت الدراسة مائة وخمسة وثلاثون شخصا عراقيا من بغداد (104 من الذكور و31 من الاناث) قسموا الى ثلاثة مجاميع : المجموعة الاولى تضمنت 73 مريض مصاب بسرطان المثانة (60 ذكر و 13 انثى) والمجموعة الثانية تضمنت المصابين بامراض مختلفة في المثانة (22 ذكر و10 اناث) في حين تضمنت المجموعة الثالثة الاصحاء البالغ عددهم 30 شخص (22ذكر 8 اناث) وبعد ان اجريت الفحوصات المختبرية على عينات الادرار وجد انه 64(87.7%) من مرضى السرطان اعطت عيناتهم نتيجة زرع موجبة للاصابة بالتهابات المجاري البولية و 9(12.3%)اعطت عيناتهم نتيجة زرع سالبة اما بالنسبة لافراد المجموعة الثانية فقد اعطت جميع عيناتهم32(100%) اعطت نتيجة زرع موجبة في حين عينات الاصحاء اعطت نتيجة سالبة بنسبة 100% وعند اجراء الفحص المجهري بعد تصبيغ كرام وجد انه 9(9.4%)هي بكتريا موجبة و 85(88.5) هي بكتريا سالبة و 2(2.1%) هي خمائر.فقد اوضحت النتائج انه 2(2.1%) *Sterptococcus fecalis*(%1) ;1*Citrobacter sp*. (%1)1;*Candida albicans* 19 (%14.6)14;*Staphylococcus aureus*(%9.4)9; *Pseudomonas sp* (%7.3)7 *Proteus sp*(%19.8);*Klebsiella* و 43(44.8%) كانت *E.coli*.

كانت جميع الاورام التي اخذت من المرضى بعملية ناظور المثانة ورفع المثانة شخصت من قبل استشاري انسجة مرضية وصنفت وفق اساسيات منظمة الصحة العالمية للعام (2004) من نوع UCC 40(54.8%)ورم من مجموع 73 تتصف بكونها High grade و33(45.2%) هي Low

grade .بينما وفق مرحلة الورم12(16.4%)كانتTaو 28(28.4%) من نوع T1الذي يتميز موقع الورم بكونه مخترق لنسيج المثانة الرابط و 23(30.1%)من نوع T2الذي يصل الى عضلة المثانة و 7(9.6%)من نوع T3و 4(5.5%)من نوع T4 بالاضافة الى ذلك فقد كان 45(61.6%) مريض من المجموع الكلي لمرضى سرطان المثانة هم مصابين للمرة الاولى وغير معالجين في حين البقية 28(38.4%)هم من اصحاب الورم المتكرر في المثانة ومتعاطين للعلاج الكيماوي.

اما بالنسبة الحركيات الخلوية فقط اظهرت النتائج اختلافا واضحا في تركيزها في مصل الدم بين المجاميع مع وجود فروقات معنوية واظهرت التراكيزها تزايدا طرديا مع تقدم مرحلة الورم ونوع الخلية السرطانية تحت المجهر وكان الارتباط موجب بين الحركيات الخلوية الاربعة المدروسة.

اظهرت النتائج ان النمط الوراثي G/Gيعطي قطعتين (268,98) والنمط الوراثي G/Cيعطي اربع قطع (268,206,98,62)في حين انه النمط الوراثي C/Cيعطي ثلاث قطع (206,98,62) بعد عزل الدنا من عينات الدم وتضخيمه باستعمال بادئ متخصص بتقنية تفاعيل انزيم البلمرة المتسلسل ومن ثم تقطيعه باستعمال الانزيم القاطع(AluI) واجراء الترحيل الهلامي.اجري التحليل الجيني للحركيات الخلوية ووجد ان تردد النمط الجيني GG ونسبة الاليلG % (61.9 and 78.77) على التوالي بالنسبة لمرضى سرطان المثانة ,وبتردد %(56.3 and 75) للمرضى الذين يعانون من مشاكل في الكلية في حين كانت التردد %(53.3and 70)على التوالي لمجموعة السيطرة. ولم تظهر مجموعة مرضى سرطان المثانة انحرافا معنويا عن توازن Hardy-Weinberg. واظهرت النتائج وجود اختلاف معنوي لمستوى عامل تثبيط هجرة خلية البلعم في الانماط الجينية الثلاثة واظهر النمط الجيني GG اعلى مستوى(62.69 pg/ml).

اظهرت النتائج (68.49%)50 من الانسجة المرضية اعطت نتيجة تعبير موجبة ل CD74و 64(87.7%)اعطت نتيجة موجبة ل CD44 في حين 42(57.5%) اعطت نتيجة موجبة ل P53و 51(69.9%) اعطت نتيجة موجبة بالنسبة ل P21اما بالنسبة للانسجة الطلائية الطبيعية فانها اعطت نتيجة سالبة التعبير لثلاث معلمات وهي CD74,P53 , P21بنسبة 100%في حين اعطت تعبير موجب بنسبة (90%) CD44. فضلا عن مشاهدة تعبيرمعنويا موجبا للمعلمات الاربعة في مرحلة الورم T1, T2و high grade.

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كلية العلوم/ قسم التقانة الأحيائية

التعدد الاليلي لجين العامل المثبط لهجرة خلية البلعم وبعض السمات المناعية في مرضى سرطان المثانة

الاطروحة

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