Baghdad University

Molecular Association Study of Epstein - Barr Virus and Human Papilloma Virus Infections in Patients with Bladder Cancer

A Thesis

Submitted to College of Medicine-Baghdad University in Partial Fulfillment of the Requirements for the Degree of Doctor of philosophy in Medical Microbiology

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March/2010 *Rabee' Awwal/1431*

بسم الله الرحمن الرحيم

نَرْفَعُ دَرَجَاتٍ مِّن نَّشَاء وَفَوْقَ كُلِّ ذِي عِلْمٍ عَلِيمٌ

صدق الله العظيم
سورة يوسف ، الآية (٧٦)

Dedication

Σ *To my Dear parents Mother and Father*

With my love

Σ *To my darling and lovely friends*

Ruqaya Mohammed and Rana M. Abdul-Karim

With my endless love and loyalty

Σ *To anyone who help me and see I deserved it*

Areej

Acknowledgments

First and foremost, I would like to thank my God, for his merciful and his beneficial for helping me to perform this work.

Then, I would like to thank my supervisor, Dr. Jasim Mouhmmed Karhoot and Dr. Alaa Ghani Hussain for guidance to accomplish this work.

I am very indebtedness to Assistant Prof Mohammad A. Al-Faham, for helping me during the certification of proposal subject for Ph. D degree.

I owe special thank and deepest gratitude to Dr. Haider Sabah Kadhim at Microbiology department /College of Medicine, Al-Nahrain University for his scientific and endless support, invaluable advice during the course of investigation and writing of this thesis. I would like to express my sincere thanks to him and I dedicated him my beast wishes.

With all my heart I thank Basim Mohammed at Microbiology department /College of Medicine and Basim Mohammed at oral pathology department /College of Dentistry and Basim Abdullah at pathology unit in Specialized Surgical hospital for their co-operation to accomplish this work.

Special thanks and full appreciation to Affaq Mohammed Ali for her great help in prepared tissue sections, I wish to her happiness in her life.

Also I would like to express my sincere thanks to Zahra'a Kassim at Department of Histology, for her help in the first step of this work. Specially teaching the techniques of ISH, IHC and providing positive control.

I am deeply thankful to staff of Computer Unit in library of the College of Medicine, Baghdad University especially Rehab Salman and Hind Mohammed Jabber for their kind help and providing useful references during my work.

My warmest thank belong to all my private friends for their support ,care and all the time spent to gather, I am grateful for them for giving me perspective to life out side the laboratory especially Suha Auda, Sana Mohammed and Fatima Talib.

Last but not least I would like to thank every one who provided any assistance even with a single word.

Areej

Summary

SUMMARY

A retrospective study included fifty formalin-fixed paraffin embedded tissue blocks from patients with transitional cell carcinoma of the bladder and 10 subjects that were age and gender matched with study groups as healthy control were included. They were collected from the archives of histopathological laboratories of Specialized Surgical Hospital as well as the archives of institute of forensic medicine in Baghdad, during the period from February 2009 till June 2009. All the samples were related to the period between March 2008 to June 2009.

A four micrometer thick tissue sections were obtained from representative area for both specimens and controls, one section was stained with Hematoxylin and Eosin then reviewed, while six sections were used to detect presence of Human papillomavirus -16, -18, Matrix metalloproteinases-2, -9, tissue inhibitor of metalloproteinase-1 and -2 by *In Situ* hybridization technique (ISH). And two sections were stained immunohistochemically for latent membrane protein-1 and tumor suppressor protein.

1- The age of patients with transitional cell carcinoma of the bladder ranged from 25-70 years with mean of 57.30 years, the highest percentage 70% was diagnosed in the age group 55-70 years, with a male to female ratio of 2.3:1.

2- The majority of transitional cell carcinoma of the bladder occur within the moderately differentiated category (62%) and the well differentiation category was the least common (8%).

3- Papillary type (56%) was significantly higher than solid type (44%). Most of transitional cell carcinoma of the bladder cases (52%) fall in muscle invasion.

4- Among transitional cell carcinoma of the bladder 22 archived tissue blocks showed positive result for latent membrane protein-1. This result constituted 44 % of total transitional cell carcinoma, whereas 18 and 7 bladder tissue blocks showed positive results for Human Papilloma Virus -16 and -18 respectively.

5- P53 over expression was documented in 46% of patients with transitional cell carcinoma of the bladder.

6- Matrix metalloproteinases-2 and -9 were localized by *In Situ* hybridization technique especially within the cytoplasm of cancer cell. It was detected in 32 out of 50 (64 %) for both of them.

7- Among transitional cell carcinoma of the bladder cases, significant correlation was reported between tissue inhibitor of metalloproteinase-1 and -2.

8- There is no latent membrane protein-1, Human papillomavirus -16,-18, tumor suppressor protein, matrix metalloproteinases-2, -9, tissue inhibitor of metalloproteinase-1 and -2 were detected in any tissues of those healthy control group.

List of Contents

List of Tables

List of Figures

List of Abbreviations

- APC = Antigen presenting cells
- AIDS = Acquired immunodeficiency syndrom
- AJCC = American joint committee on cancer
- APK = Activated protein kinase
- BCR = B cell receptor
- BM = Bone marrow
- cDNA = complementary DNA
- CD 21,40 = Cluster of differentiation 21,40
- c-erb-B-1,-2 =Neu oncogen encoded protein closely homologous to EGFR
- c-myc = It is oncogen encodes the transcription factor
- CMV = Cytomegalovirus
- CR2 = Complement receptor 2
- DAB =Diaminobenzide
- DPX =Distyrene, plasticizer and xylene
- DW = Distilled water
- DDW = Deionized distilled water
- DNA = deoxyribonucleic acid
- E = Early
- EBNA = EBV nuclear antigen
- EBERs = EBV-encoded small nonpolyadenylated RNAs
- EBV = Epstein-Barr virus
- ECM = Extracellular matrix
- E2F3 = Early 2 factor 3(transcriptional factor)
- EGFR = Epidermal growth factor receptor
- FGFR3 = Fibroblast growth factor receptor-3
- G1 = The phase in which the cell gears up for division
- G2 = The space before mitosis
- HLA = Human leukocyte antigen
- H&E = Hematoxylin and eosin
- HPV = Human papillomavirus
- HSV-1,-2 = Herpes simplex virus-1,-2
- IARC = International agency for research on cancer
- ICR = Iraqi cancer registry
- IFN-γ = Interferon gamma
- IHC = immunohistochemistry
- IL = Interleukin
- IR 1 = Internal repeat 1
- ISH = *In situ* hybridization
- .KDa = Kilo dalton
- Ki-67 = Proliferation index
- L = Late
- .LMP-1,2 = Latent membrane protein,1,2

• LCR = Long control region
• LOH = Loss of heterozygosity
• Mdm-2 = Murine double minute 2 gene
• MHC = Major histocompatibility complex
• MMP = Matrixmetallo proteinase
• mRNA = messenger RNA
• MT-MMP = Membrane type-matrixmetallo proteinase
• N =Number
• NF-kB = Nuclear factor kappa B
• Nm = Nino meter
• p = Short arm of the chromosome
• P53,P21,P16, = Tumor suppressor gene
• PBS = Phosphate buffer saline
• PCNA = Proliferating cell nuclear antigen
• PCR = Polymerase chain reaction
• PKR = Protein kinase R
• PTLD = Post-transplant lymphoproliferative disease
• q = Long arm of the chromosome
• Rb = Retinoblastoma
• RNA = Ribonucleic acid
• SCC = Squamous cell carcinoma.
• TCC = Transitional cell carcinoma.
• TCR = T cell receptor
• TGF-β1= Tumor growth factor beta 1
• Th1,2 = T helper cell 1,2
• TIMP-1,2 = Tissue inhibitor metalloproteinase-1,-2
• TNF-α = Tumor necrosis factor-alpha
• TNM = staging system (T:tumor, N:lymph node, M:metastases)
• TR = Terminal repeat
• UICC = Union Internationale control cancer
• UL = Unique sequences long
• uPA = Urokinase –type plasminogen
• URR = Upper regulatory region
• US = Unique sequences short
•VAF 1 = Wild type p53 associated fragment 1
•VEGF = Vascular endothelial growth factor
• WHO =World health organization

Chapter One

Introduction

Introduction

Urinary bladder cancer is one of the most common cancers worldwide, with the highest incidence in industrialized countries (Tracey *et al.*, 2007). More than 90% of bladder cancers are transitional cell carcinomas (TCC). About 5% of bladder cancers are squamous cell carcinomas (SCC). There are also uncommon bladder cancers, such as adenocarcinoma and small cell carcinoma, which are responsible for less than 2% of all bladder cancers (De Vita *et al*., 2005).

Transitional cell carcinoma of the urinary bladder is the second most common tumor of the genitourinary tract. It is also the second most common cause of death from these cancers (Williams *et al*., 2001).

Many agents including radiation, chemicals and viruses, have been found to induce human cancer (De Villiers, 2003). Viral factors are the most important class of the infectious agents associated with human cancers (Mao *et al*., 2003). It was estimated that 17-20 % of worldwide incidence of cancers attributable to a viral etiology (Cliffard *et al*., 2003).

Epstein Barr virus is one of the viruses that have some unclear and controversial points in its ability to trigger the development of certain tumors (Vokes and Liebowitz, 1997). Like Burkitt's lymphoma, nasopharyngeal carcinoma, hodgkin's disease, gastric carcinoma and post-transplant lymphoprolifereative disease (Thornhill, 2008).

Human Papilloma Viruses (HPVs) are DNA viruses that have specific tropism for saqumous epithelial (Munoz *et al*., 2003). More than 100 types of HPVs have been reported, which are classified as low-risk and high-risk types according to their associations with malignant tumors. High-risk HPVs encode two oncogenes, E6 and E7, which an play important role in carcinogenesis. Early 6 has two zinc finger domains and interacts with tumor

suppressor p53 and degrades it to escape from apoptosis and to disrupt cell cycle checkpoint machinery (Yoshida *et al*., 2008).

The p53 protein is a major factor involved in cell-cycle regulation, the loss of p53 ability to regulate cell proliferation may lead to neoplastic transformation. This condition can be caused by protein inactivation by mutation of the p53 gene affecting the protein structure (Barzal-Nowosielska *et al*., 2004). Over expression of the p53 suppressor gene is the most common event in bladder cancer. Mutagens from cigarette smoke have been claimed to cause this (Lopez-Beltran *et al*., 1997).

Matrix metalloproteinases (MMPs) are a family of proteolytic enzymes which degrade the extracellular matrix (ECM) or components of the basement membrane. They have essential roles in tumor invasion and metastasis (Kanayama, 2001). In many physiological states or processes, degradation of extracellular matrix is very important and essential, for example, during development, growth, and repair or remodeling of organ tissues (Ravanti and Kahari, 2000). However, excessive degradation of tissues or proteolysis causes several pathological conditions, for example, rheumatoid arthritis, osteoarthritis, autoimmune disorders of skin, and others (Woessner, 1998).

Matrix metalloproteinases are counteracted by the tissue inhibitors of metalloproteinases (TIMPs), which inhibit MMP activity and thereby restricting ECM breakdown. The balance between MMPs and TIMPs plays an important role in maintaining the integrity of healthy tissues. A disturbed balance of MMPs and TIMPs is found in various pathologic conditions, including rheumatoid arthritis, cancer and periodontitis (Verstappen and Von den Hoff, 2006).

Tissue inhibitors of metalloproteinases and MMPs can be produced by many different types of cell and also found in all body fluids, such as saliva,

gingival cerevicular fluid (GCF), serum and urine (Durkan *et al.*, 2003). In bladder cancer, elevated MMP-2 and MMP-9 expression in tumor tissues, correlated with tumor stage, grade or prognosis, were reported in several studies. Moreover, high levels of serum or urine MMP and TIMP were observed in patients with bladder cancer especially in advanced cases (Kanayama, 2001). The MMP-9/TIMP-1ratio in urine is thought to predict the risk of bladder cancer, whereas the TIMP-2 levels in urine from individuals with urothelial carcinomas are significantly decreased (Verstappen and Von den Hoff, 2006).

In Iraq, previous studies focused on the general and local immunological status (Al-Qassab, 1979), correlation with shistosomiasis (Tawfik, 1985), role of cell cycle regulatory proteins (Kadhim, 2004), relation between shistosomiasis and multidrug resistance (Ahmed, 2006), detection of tumor suppressor gene (Ibrahim, 2009). But, the association between bladder cancers with viral infection is not studied yet.

The present study was carried out to focus on the possible role of MMP-2, MMP-9 and their inhibitors during bladder cancer progression and hope that this study will try the first step in detection of these markers in Iraqi patients

Aims of study:-

1- To investigate the prevalence of LMP-1 and HPV16/18 DNA in archival tissue specimens with a range from apparently healthy bladder tissues to invasive bladder tumor using immunohistochemistry (IHC) and *in situ* hybridization technology (ISH).

2- To determine the correlation between viral genome or protein and the development of transitional cell carcinoma of bladder.

3- To assess P35 suppresser gene over expression in transitional cell carcinoma of bladder using IHC and correlation with different clinical parameters like age, gender of patients, viral protein or genome, tumor grade, pattern of growth and presence or absence of muscle invasion.

4- To evaluate MMP-2 and MMP-9 in tissue samples of patients having transitional cell carcinoma of bladder and it's correlation with various clinical parameters mentioned previously.

5- To study the role of TIMP-1 and TIMP-2 through *in situ* hybridization technique in bladder cancer patients.

Chapter Two

Review of Literature

Review of Literature

2.1-Bladder cancer:-

2.1.1- Cancer incidence and prevalence:-

Bladder cancer is a worldwide health problem, with an estimation 375,000 bladder cancer cases annually. It is more common in industrial countries than in developing countries and 77% of the tumors occur in men (Parkin *et al*. 2005).The incidence of bladder cancer has been rising gradually in recent years (Finnish Cancer Registry, 2007). According to the World Health Organization (WHO) in 2000, over 300,000 people were confronted with the disease and more than 40% (130,000) of them die because of bladder cancer every year. Indeed, the incidence of bladder cancer is the eleventh in the list of all cancers in the world (Martin and Aus, 2005).

Transitional cell carcinoma (TCC), is the most common cancer type of the lower urinary tract. More than 57,000 new cases and 12,000 deaths are predicted for the United States in the year 2003, with the incidence of TCC in the United States remaining stable (Jemal *et al*., 2003).

In Iraq, bladder carcinoma is the fifth among the commonest tenth cancers being third in males and eighth in female. Out off 948 cases were registered urinary bladder carcinoma, there were 715 males and 233 females according to the results of Iraqi cancer registry (Iraqi Cancer Board, 2008).

2.1.2- Etiology and risk factors:-

The most common risk Factors which are implicates in bladder cancer are.

A- Genetic predisposition.

A wide variety of genetic abnormalities are seen in bladder cancer, of these mutations involving several genes on chromosome 9 [including p16, p53 and Fibroblast Growth Factor Receptor 3($FGFR_3$)] are the most common

(Kumar *et al*.,2007).

Carcinogenesis of sporadic bladder cancer, although the majority of patients with TCC have no family history of TCC of the urinary tract. No TCC-causing syndromes have been described (Kiemeney and Schoenberg 1996).

B- Cigarette smoking.

Cigarette smoking is perhaps the most prominent risk factor for bladder cancer, estimated to account for around 50-60% of male cases and 20-30% of female cases (Castelao *et al*., 2001).

In 2001 the International Agency for Research on Cancer (IARC) declared smoking a major cause of bladder carcinoma (IARC, 2006). Since then there has been increasing evidence of a strong dose-response relationship between cigarette smoking and bladder cancer (Zeegers *et al.,* 2002).

C- Schistosomiasis.

Schistosomia haematobium, also called urinary schistosomiasis, is the one related to bladder cancer. The disease is common in northeast Africa, southwest Asia, and Madagascar (Vaishnav, 2003). *Schistosomia haematobium* adult mature worms inhabit the mesenteric and pelvic veins of humans, where they mate and reproduce. The female deposit eggs that eventually rupture the venules and discharge into the surrounding tissues (Higashi and Aboul-Enein).

D- Occupation.

Occupational exposure to carcinogens may increase the risk of bladder cancer. Evidence suggests that there are particular occupations that are at higher risk of developing bladder cancer than others. It is estimated that between 4-7% of all bladder cancers were due to occupational factors (Pelucchi *et al*., 2006). People working in the rubber, mining and leather industries, as well as hairdressers, machinists, metal workers, printers, painters, textile workers and truck drivers have been identified as having a higher risk of bladder cancer (Bosetti *et al.,* 2005).

E- Viral infection.

Gazzaniga *et al*.,(1998) have been investigated the presence of Epstein-Barr virus (EBV), Human papillomavirus (HPV), Cytomegalovirus (CMV) and Herpes simplex virus-type 2 (HSV-2), genomes in 35 biopsies of bladder carcinomas by using Polymerase Chain reaction. Sequences of EBV, HPV, CMV and HSV-2 genomes were detected in 34%, 31%, 11% and 9% of tissue samples respectively.

F- Chronic cystitis.

Chronic cystitis in the present of indwelling urinary catheters or infection with *E. coli* is also believed to induce higher rates of transitional cell carcinoma of the bladder (Kumar *et al*., 2007).

G- Radiation.

An increased risk for developing bladder cancer has been associated with radiation therapy to the pelvic for ovarian, uterine cervical and prostate carcinomas (Jung and Messing, 2000).

H- Age and gender.

Age is a major risk factor for bladder cancer, with the majority of cases diagnosed after the age of 50 years. Gender is also an important risk factor, with males three to four times more likely to be diagnosed with bladder cancer than females (Tracey, 2007).

I- Arsenic.

There is evidence of an increased risk for urinary bladder cancer associated with arsenic in drinking water. With arsenic, a dose-response relationship within exposed populations is also evident (Ugnat *et al.*, 2004). Smoking may increase the risk further in people who ingest arsenic (Steinmaus *et al.*, 2003).

J- Diet.

There is some limited evidence that adequate consumption of fruit and vegetables and dairy products have a protective effect against the development of bladder cancer (Castelao *et al*., 2004). However, the evidence for these effects remains inconsistent (Zeegers *et al*., 2001).

K- Others.

There is a some literature that proposes other risk factors for the development of bladder cancer, such as coffee and alcohol consumption, however clear evidence of risk is inconclusive (Donato *et al*., 1997). There is some evidence that increased fluid consumption (including water) may reduce the risk of bladder cancer, however the source of drinking water (if contaminated) may also be an important risk factor for the development of bladder cancer (Pelucchi *et al.*, 2006).

2 .1.3-Molecular biology of transitional cell carcinoma of bladder:-

The carcinogenesis is a multistep process; require the accumulation of multiple (5-9) genetic events in the cell (Bonnez and Reichman, 2000).

Activation of dominantly acting oncogenes such as ras and c-erbB-1 and 2 and transcription factors such as Early2 Factor3($E_2F_{3)}$, have been reported in bladder cancer ,as has the inactivation of tumor suppressor genes such as p53, p21, p16 and the retinoblastoma gene. Activation of many other genes occurs including those coding for enzymes that dissolve the basement membrane, such as the metalloproteinses (stromelysin, collagenase and elastase), lysosomal enzymes such as the cathepsins and others including urinary plasminogen activators, angiogenic factors [e.g. vascular endothelial growth factor (VEGF)] and other peptide growth factors such as the epidermal growth factor and its receptor (Norman *et al*., 2008).

Bladder cancer shows frequent chromosomal alterations. The most frequent gains are in chromosomal regions 1q21-q24, 8q21-q22 and 17q, whereas the most common deletions are in chromosomal regions 11p15-p14, 8pter-p22, 9pter-p21 and 9q (Simon *et al*., 2000). Loss of heterozygosity (LOH) also occurs in bladder cancer, contributing to carcinogenesis through inactivation of tumor-suppressor genes such as p53 (Brandau and Böhle, 2001). The evaluation of product of oncogenes, tumor suppressor genes and cell proliferative activity has recently been proposed as a useful adjunct in tumor pathology.

Moreover others studies have attempted to identify molecular events associated with specific genes that underlie neoplastic progression in the development of bladder cancer. Since the protein products of oncogenes are known to participate directly in cell cycle processes, any alteration of these genes or their proteins can alter their function, leading to uncontrolled cell growth and ultimately to tumor formation. In addition to that, cell-cycle regulatory proteins are important indicators in determining progression through the cell cycle and progression to invasive cancer. Alteration of cell cycle regulation is a key event in determining the biological behavior of bladder cancer (Santos *et al*., 2003).

2.1.4- Markers associated with bladder malignancies:-

Current pathological and clinical parameters such as(tumor, lymph node, metastases) TNM classification provide essential prognostic information yet still have limited ability to predict the true malignant potential of most bladder tumors. In the recent years, investigation of basic mechanisms involved in carcinogenesis and tumor progression by molecular biology has provided a host of tumor markers of potential diagnostic or prognostic value for bladder carcinoma (Tiguert *et al*., 2002). These markers may serve as tools for early and accurate prediction of tumor recurrence and progression, and for development of metastases and prediction of response to therapy. Precise prediction of tumor behavior would facilitate treatment selection of patients

who may benefit from various treatments including surgery and adjuvant therapy. Currently, no single marker is able accurately to predict the clinical course of bladder tumors and thus to serve as a reliable prognostic marker. A combination of prognostic markers could predict which superficial tumors require an aggressive form of therapy. Several urine and serum tests have been developed in the attempt to discover a marker useful for the detection or prognostication of bladder cancer. Despite these numerous research efforts, no diagnostic marker with a specificity and sensitivity comparable to cystoscopy currently exists. Some of the potential tissue markers of bladder cancer are listed in Table (2.1), among the most promising being Ki-67 and p53 (Kausch and Böhle 2002).

Table (2.1): Tissue markers of bladder cancer (Kausch and Böhle, 2002).

Marker	*Biological function*	*Potential prognostic value*
ABO	Blood group antigen	Diagnostic marker, association with progression
Ras,C-myc, c-erbB2	Oncogenes	Prognosticators of disease recurrence (Ras) or survival (c-cerbB2), correlation with tumor grade (c-erbB2, c-MYC) or metastasis (c-erbB2)
Rb, p53	Cell cycle regulators	Correlation with progression and survival (Rb) , marker of progression, or recurrence and survival (p53)
Ki-67, PCNA	Proliferation-associated antigens	Marker of recurrence, progression, and survival (Ki-67), correlation with tumor grade (PCNA)
E-cadherin	Cell adhesion molecule	Association with metastasis, tumor grade, stage, and survival
VEGF	Peptide growth factor	Correlation with tumor stage, grade, and recurrence
EGFR	Growth factor receptor	Correlation with recurrence and progression

2.1.5- Classification of bladder tumors:-

The WHO classification of bladder tumors has four basic criteria stated by Caroll (1995), these are:

- *Pattern of growth*: It is determined by examining histologic sections under hand lens or the low power of the microscope. It is described as papillary, infiltrating, papillary and infiltrating or noninvasive (in situ).
- *Cell type*: Bladder tumor could be classified into four histological types: Transitional, Squamous, Glandular (Adenocarcinoma) and Undifferentiated.
- *Grade of malignancy.*
- *Stage of the tumor.*

2.1.6- Grading of bladder tumors:-

Tumors are arising in the urinary bladder range from small benign papillomas to large invasive cancer, these tumors are classified into a rare benign papilloma, a group of papillary urothelial neoplasms of low malignant potential and two grades of urothelial carcinoma (low and high grade). As illustrated in Table (2.2).

The very rare benign papillomas are 0.2 cm to 1.0 cm frond like structures having a delicate fibrovascular core covered by multilayered, well-differentiated transitional epithelium. Some of these lesions, the covering epithelium appears as normal as the mucosal surface from whence the tumors arose .such lesions are usually solitary. They almost invariably noninvasive and benign and they rarely recur once removed.

Urothelial (transitional) cell carcinomas range from papillary to flat, noninvasive to invasive and low grade to high grade. Low grade carcinomas are always papillary and rarely invasive, but they may recur after removal .whether the regrowth is a true recurrence or a second primary growth is uncertain. Increasing degrees of cellular atypia and anaplasia are encountered in papillary exophytic growths, accompanied by an increase in the size of the

lesion and evidence of invasion of the sub mucosal or muscular layers. High grade cancers can be papillary or occasionally flat, they may cover lager areas of the mucosal surface, invade deeper and have a shaggier necrotic surface than do low grade tumors (Kumar *et al.*, 2007).

Table (2.2): WHO (World Heath Organization), and ISUP (International Society of Urological Pathology) grading systems of transitional cell carcinoma (Epstein *et al*., 1998).

WHO Grade	ISUP Consensus
Papilloma (Grade 0)	Urothelial papilloma
TCC Grade I	Well differentiated urothelial neoplasm of low malignant potential
TCC Grade II	Moderately differentiated urothelial carcinoma of low grade
TCC Grade III	Poorly differentiated urothelial carcinoma of high grade

2.1.7-Staging of bladder tumors:-

The American Joint Committee on Cancer (AJCC) in 2002 had designated staging by TNM classification which is based on three parameters: 'T' describes the extent of the tumor according to invasion depth in the bladder wall 'N' describes the status of regional nodal involvement, while 'M' describes the presence or absence of distant metastases. The individual clinical parameters in the TNM classification system are grouped to determine the appropriate disease stages as it is shown in the following criteria and Table (2.3)

The primary tumor (T) is staged in the following way:

Superficial bladder cancer

- TX: A primary tumor cannot be assessed.
- T0: No primary tumor seen.
- Ta: Superficial cancer is found only in polyps (papillary) on the surface of the inner lining of the bladder.
- Tis: Carcinoma in situ. Tumor is found only in flat lesions on the surface of the inner lining of the bladder.
- T1: Tumor is found in the connective tissue below the lining of the bladder but has not spread to the bladder muscle.

Invasive bladder cancer

- T2a: Tumor has spread to the inner half of the smooth muscle layer (superficial layer) below the lining of the bladder.
- T2b: Tumor has spread to the outer half of the smooth muscle layer (deep layer) of the bladder.
- T3a: Tumor has spread through the muscular wall of the bladder into the fatty tissue layer as identified under a microscope.
- T3b: Tumor has spread through the muscular wall of the bladder into the fatty tissue layer and a mass is visible to the eye.
- T4a: Tumor has spread to the prostate in men and to the uterus or vagina in women.
- T4b: Tumor has spread to the pelvic or abdominal wall.

Lymph node involvement is staged in the following way:

- NX: Lymph nodes in the pelvis cannot be assessed.
- N0: No bladder cancer is found in lymph nodes.
- N1: Bladder cancer is found in one lymph node, 2 cm (0.8 in.) or less in size.
- N2: Bladder cancer is found in one lymph node and is more than 2 cm (0.8 in.) but less than 5 cm(2 in.) in size, or cancer is found in multiple lymph nodes but none are more than 5 cm(2 in.) in size.
- N3: Bladder cancer is found in one or more lymph nodes and is more than 5 cm (2 in.) in size.

The TNM system stages metastasis (M) in the following way:

- MX: Spread of cancer to other organs cannot be evaluated.
- M0: No evidence of bladder cancer exists elsewhere in the body.
- M1: Bladder cancer cells are found somewhere else in the body.

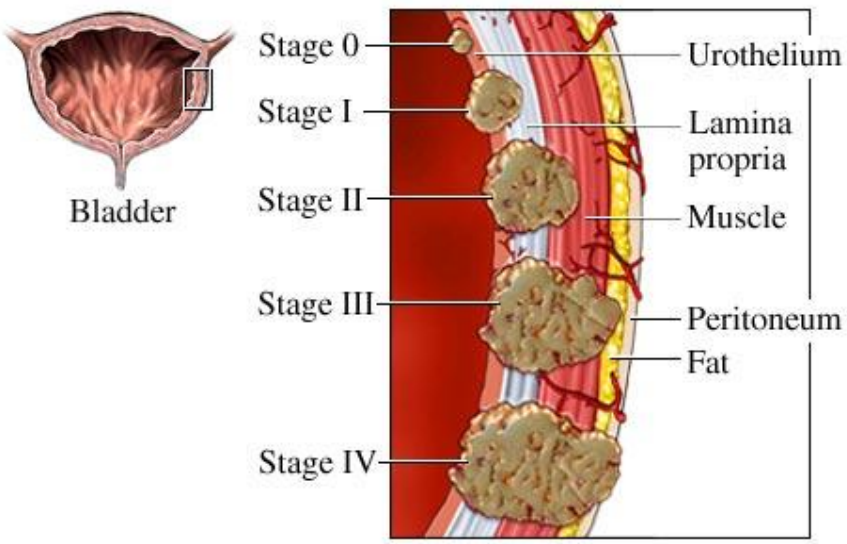

Figure (2.1): *Tumor staging in bladder cancer. Urologic Cancer Unit Moores UCSD Cancer Center, 2002.*

Table (2.3): *Classification of bladder cancer AJCC, 2002. Urologic Cancer Unit Moores UCSD Cancer Center 3855 Health Sciences Drive, La Jolla, CA 92093.*

Stage	TNM Classification
0(a)	TaN0M0
0(is)	TisN0M0
I	T1N0M0
II	T2aN0M0
	T2bN0M0
III	T3aN0M0
	T3bN0M0
	T4aN0M0
IV	T4bN0M0
	Any T, N1, M0
	Any T, N2, M0
	Any T, N3, M0
	Any T, any N, M1

2.2-Epstein Barr Virus (EBV):-

2.2.1- Historical background of EBV Virus:-

Epstein Barr virus (EBV) has been implicated as a cause of malignant transformation in a number of lymphoid and non lymphoid cell types. The first association of EBV with cancer was reported in 1964 and coincided with discovery of the virus by Epstein, Achong and Barr in electron micrographs of cells cultured from patients with endemic Burkitt's lymphoma (Leenman *et al.*, 2004).

Epstein Barr Virus was classified as a group1 carcinogen, an indication that there is the strongest possible evidence linking it to human cancer (WHO-IARC, 1998).

2.2.2- Taxonomy and classification:-

Epstein Barr Virus is grouped as a member of the Herpesviridae family, subfamily gammmaherpesvirinae, genus lymphocryptovirus. The Herpesviridae family contain viruses grouped together based on the architecture of their virion (Roizman, 1990).

Two major types of EBV strains have been recognized, EBV type-1 and type-2, and they differ biologically and in their geographic and ethnic prevalences but have no clear differences in EBV associated clinical diseases (Preiksaitis and Cockfield, 2003).

2.2.3- Structural Characteristics:-

A-Epstein Barr Virus Particles:-

A typical herpesvirion consists of a core containing a linear, double stranded DNA; an icosahedral capsid, approximately 100-110 nm in diameter, containing 162 capsomeres with a hole running down the long axis; an amorphous, sometimes asymmetric material that surrounds the capsid, designated as the tegument; and an envelope containing viral glycoprotein spikes on its surface (Roizman, 1990). The Epstein-Barr virus surface glycoprotein H (gH) is essential for penetration of B cells but also plays a role in attachment of virus to epithelial cells (Molesworth *et al.*,2000).

B-Epstein Barr Virus genome:-

Epstein Barr Virus is one of the eight known human herpesviruses. Its genome is a linear, double stranded DNA, about 170kb in length. Latently infected cells contain the genome as a circular plasmid in the nucleus. The terminal repeat (TR) sequences are present at both ends of the linear form of the genome and these repeats mediate the circularization in the infected cell, An unusually large tandemly repeated DNA sequence in the genome of EBV is known as the major internal repeat (IR1). The IR1 site divides the EBV genome into long and short unique sequences (UL and US). These sequences are filled with closely packed genes (Farrell, 2005).

In addition, the EBV genome contains a viral cytokine, vIL-10, that was pirated from the host genome. This viral cytokine can prevent macrophages and monocytes from activating T-cells are required for EBV-dependent transformation of B-cell (Kumar *et al.,* 2007).

2.2.4-Epstein Barr Virus antigens:-

Epstein Barr Virus antigens are divided into three classes, based on the phase of the viral life cycle in which they are expressed.

1-Latent phase antigens are synthesized by latently infected cells. These include the Epstein–Barr Nuclear Antigens (EBNAs) and the Latent Membrane Protein (LMPs). Their expression reveals that an EBV genome is present only EBNA1, needed to maintain the viral DNA episomes, is invariably expressed; expression of the other latent phase antigens may be regulated in different cells. LMP1 mimics an activated growth factor receptor.

2-Early antigens are non-structural protein whose synthesis is not dependent on viral DNA replication. The expression of early antigens indicates the onset of productive viral replication.

3-Late antigens are the structural component of the viral capsid (viral capsid antigen) and viral envelope (glycoproteins). They are produced abundantly in cells undergoing productive viral infection (Jawetz, 2007).

A- Epstein–Barr Nuclear Antigens-1(EBNA-1).

Epstein Barr Nuclear Antigens-1 was expressed in all actively dividing EBV-infected cells and is responsible for binding the viral episome through its origin of replication (OriP) to the mitotic cellular DNA, assuring replication and transfer of virus genome to all daughter cells (Munz, 2005). EBNA-1 was also involved in the transcriptional control of other latency proteins, a function that is independent of episome maintenance (Altmann *et al*., 2006).

B- Epstein Barr Nuclear Antigens-2 (EBNA-2).

Epstein Barr nuclear antigens-2 is one of the first two viral genes expressed and is a key regulator of viral and cellular gene transcription. EBNA-2 has shown to transactivate EBV-encoded as well as target cell-encoded genes. EBNA-2 is essential for B lymphocyte growth transformation and transactivates expression of the EBNA-1, EBNA-3s, and the LMPs (Robertson *et al.*, 1995).

C- Epstein Barr Nuclear Antigens-3 (EBNA-3).

The EBNA-3 family of proteins, EBNA-3A, EBNA-3B, and EBNA-3C, has now shown to have a function in modulating LMP-1 and LMP-2 transcription by preventing EBNA-2 transactivation of the LMP-1 and LMP-2 promoters (Longnecker and Miller, 1996). EBNA3C has shown to partially overlap the functions of EBNA-2, as it also regulates some viral and cellular genes that are regulated by EBNA-2, including CD21, and LMP-1(Robertson *et al.*, 1995).

D- Epstein Barr Nuclear Antigens -leader protein (EBNA-LP).

Epstein Barr nuclear antigens -leader protein, encoded by the leader of EBNA mRNA (Wang *et al.*, 1987), is one of the least studied EBV latent proteins. EBNA-LP does have a strong role in B lymphocyte growth transactivation, as recombinant Epstein Barr viruses encoding a mutant EBNA-LP have significantly reduced abilities to transform B lymphocytes. It has been postulated that EBNA-LP may be important in regulation EBNA expression or the regulation of virus or cell gene expression mediated by EBNAs (Longnecker and Miller 1996).

E- Latent Membrane Protein-1 (LMP-1).

Latent membrane protein-1 is an integral plasma membrane protein associated to lipid rafts with a pattern of signaling that mimics constitutive CD40 triggering in a ligand-independent manner (Stunz *et al.*, 2004).

Latent membrane protein-1 also induces the expression of cell-surface adhesion molecules and activation antigens, and upregulates anti-apoptotic proteins (e.g. Bcl-2, A20 and Mcl-1) in B cells. In epithelial cells, ectopic LMP-1 expression leads to hyperproliferation, inhibition of differentiation, actin remodeling and invasiveness, along with protection from apoptosis (Brinkmann and Schulz, 2006).

F- Latent Membrane Protein-2 (LMP-2).

Latent Membrane Protein-2 gene encodes two distinct, LMP-2a and b (Longnecker, 2000). LMP-2a and its splice variant LMP-2b is not essential for EBV-induced cell transformation in B lymphocytes, but aid in the transformation (Fukuda and Longnecker, 2007). Migration and invasiveness potential of epithelial cells (Altmann and Hammerschmidt, 2005). Signals from LMP-2a mimic those of the BCR, which promote B lymphocyte survival and proliferation (Brinkmann and Schulz, 2006). Recent studies have pointed to an essential role of LMP-2a in promoting growth transformation and survival in germinal center B cells which lost BCRs due to crippling mutation (Mancao and Hammerschmidt, 2007).

G-Epstein Barr Virus Encoded Small RNA (EBERs).

The small untranslated RNAs EBER-1 and -2 are accumulated at high levels during all forms of latency and regulate apoptosis through different mechanisms. EBER-1 interacts with the interferon-inducible protein kinase R (PKR), and inhibits its activation by double-stranded RNAs, protecting infected cells from IFN-induced apoptosis (Nanbo *et al*., 2002).

Epstein Barr virus encoded small RNA-2 has however a more prominent role in EBV-mediated growth transformation, as viruses lacking the coding sequence for this RNA were significantly less efficient in generating lymphoplastoid cell lines (LCLs) in vitro, and the cell lines generated proliferated at much lower rates, due to reduced autochrine IL-6 production (Yajima *et al*., 2005). These observations have been extended to epithelial cells lines, where EBERs induce the expression of growth factors that promote cell survival (Iwakiri *et al*., 2005).

2.2.5- The life cycle for EBV:-

Epstein Barr Virus is spread through salivary contact and the virus enters through the epithelium that lines the nasopharynx. As illustrated in Figure (2.2) Infection of B-cells is initiated by the binding of the major EBV outer envelope glycoprotein gp 350/220 with the cellular complement receptor type 2 (CR2), also known as CD21.

The major histocompatibility complex (MHC) class II molecule is a cofactor for the infection of B-cells. Infection results in cellular activation and immortalization. The DNA genome of EBV encodes about 100 viral proteins and during viral replication, all these proteins are expressed (Cohen, 2000). After primary infection, the EBV genome becomes circular; forming an episome in B cells, and remains latent in these cells. During

primary infection, as in infectious mononucleosis, the viral antigens expressed by peripheral blood B cells which characterized by the limited expression of a subset of viral gene products, including six nuclear antigens (EBNA-1, -2, -3A, -3B, -3C and -LP) and three integral membrane proteins (LMP-1, -2A and -2B),(Preiksaitis and Cockfield, 2003).

Two small EBV encoded RNAs (EBER-1 and EBER-2) are also found in very high copy numbers in the 33 nuclei of latently infected cells. In most asymptomatic carriers of EBV, the virus is occasionally replicated and infectious virions are then found in oral secretions (Tsurumi *et al.*, 2005).

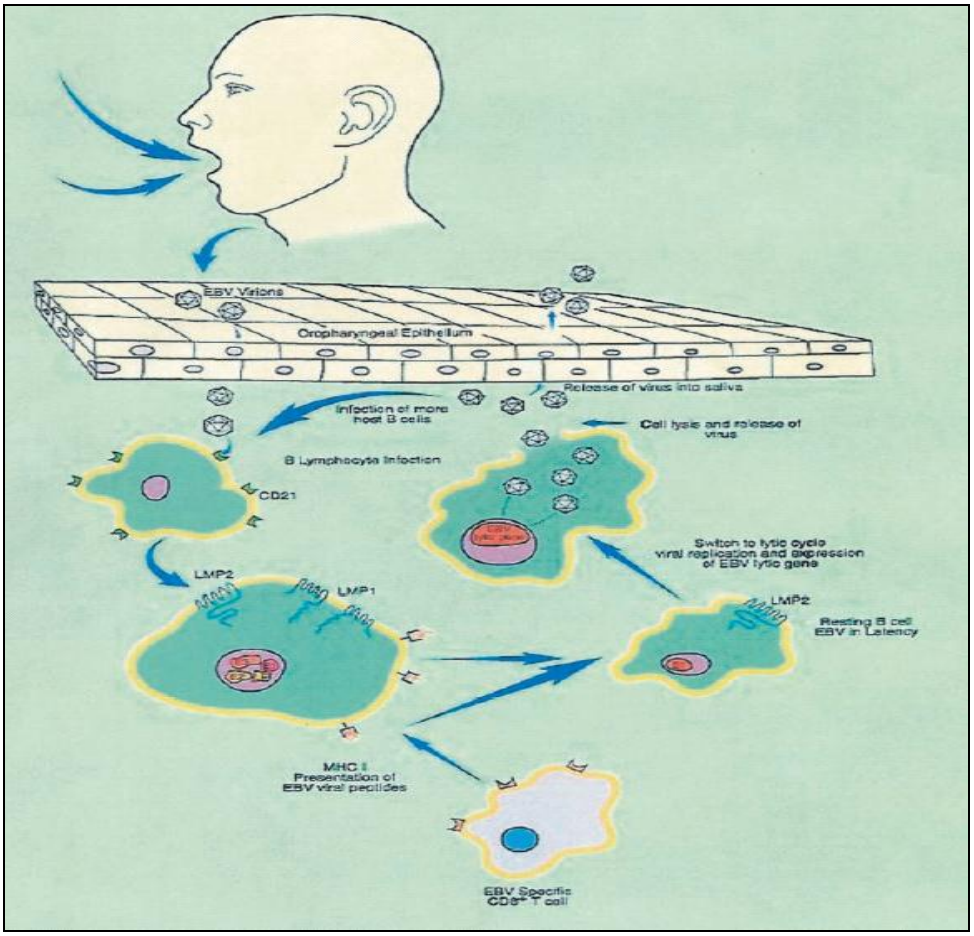

Figure (2.2): The life cycle of EBV (Thompson and Kurzrock, 2004)

2.2.7-Pathogenecity of EBV infection:-

A- Infectious mononucleosis.

Epstein Barr Virus can cause infectious mononucleosis, also known as "glandular fever". Infectious mononucleosis is caused when a person is first exposed to the virus during or after adolescence. Though once deemed "The Kissing Disease" recent research has shown that transmission of Mononucleosis not only occurs from exchanging saliva, but also from contact with the airborne virus. It was predominantly found in the developing world (Bennett, 2008).

B-Burkitt's lymphoma

Epstein Barr virus is associated with over 90% of cases of endemic Burkitt's lymphoma, 30-50% of Hodgkin's lymphomas, and up to 50% of non-Hodgkin's lymphoma in immunosuppressed patients (Leenman *et al.*, 2004). Burkitt's lymphoma commonly affects the jaw bone, forming a huge tumor mass. It responds quickly to chemotherapy treatment, namely cyclophosphamide, but recurrence is common (Weiss, 2002).

C-Nasopharyngeal carcinoma.

This neoplasm merits comment because of the strong epidemiologic links to EBV and the high frequency of this form of cancer in the Chinese, which raises the possibility of viral oncogenesis on a background of genetic susceptibility. Epstein Barr virus infects the host by first replicating in the nasopharyngeal epithelium and then infecting near by tonsillar B lymphocytes (Kumar, 2007).

D-post-transplant lymphoproliferative disorders (PTLD).

Epstein Barr Virus infection after transplantation is a serious problem, because of the risk of developing post-transplant lymphoproliferative disorders (PTLD) (Leblond and Choquet, 2004). Most PTLD cases occur within the first year post-transplant, when the recipient is severely immunocompromised for prevention of rejection and during periods of heavy immunosuppression (Gottschalk *et al.*, 2005), and declines thereafter (Taylor *et al.*, 2005).

2.3- Human papillomavirus (HPV):-

2.3.1- Historical background of HPV Virus:-

Human papillomaviruses are widely distributed throughout the animal Kingdom and cause generation of wart, this was proved in the 19th century. One of the first recorded experiment wart transmission cases in human appeared to have been accidental and was reported in 1845 (Ullman,1923).

The first isolation of these virus particles was performed in 1933 in rabbit papillomatosis. The extract from this lesion was found to contain infectious particles and many of the benign papillomas in rabbits were observed to progress to malignancy (Patrick and Joseph, 2004).

The association between HPV infection and cervical neoplasm was established after the link between genital HPV infections and cervical cancer was first demonstrated in the early 1980s by Harold zur Hausen (De Villiers *et al*, 1981)

In 1999, PCR assay has found HPV DNA in 99.7 % of cervical cancer studies (Venturoli *et al*., 2002).

2.3.2- Taxonomy and classification:-

The papillomaviridae family is a very large virus family currently divided into 16 genera of which five contain members that infect humans (Alpha-, Beta-, Gamma-, Mupa- and Nupapillomavirus).The papillomaviruses are former members of the papovariridae family. Although papillomaviruses and polymaviruses share similarities in morphology, nucleic acid composition and transforming capabilities, differences in genome organization and biology led to their separation into distinct virus families (Jawetz, 2007).

Human Papilloma Virus is named according to the species. In addition to human, other species are affected including cotton tail rabbit, cattalos, dogs, sheep's, elk and mastomys (Chen, 2004).

2.3.3-Structural characteristics:-

A- Human papillomavirus Particles:-

Human Papilloma Virus is a relatively small (55nm diameter) non-enveloped virus. It has an icosahedral capsid composed of 72 capsomers, which contain at least two capsid proteins, L1 and L2 (Hoenil Jo, 2005).

The capsid proteins carry type specific antigenic determinants (Woodman, 2001). The viruses replicate in the nuclei of squamous epithelium cell (Campo, 2002).

The lack of an envelope also rendering these viruses very stable and infectious for years, as well as resistant to heat, organic solvents and to many thereapeutic agent (Nebesio *et al*., 2001).

B- Human papillomavirus genome:-

The virus contains a double-stranded, circular DNA genome containing 7800~7900 base pairs (Hoenil Jo, 2005). This genome, had been recognized in 3 forms, super helical twists(form I); relaxed or open circules (form II) ; and the linearized form (from III) (Cooper, 2000). The viral genome is encased in a capsid layer, consisting of a major structural protein and a minor structural protein (Campo, 2002). All of the potential coding regions, or open reading frams (ORFs). Exist in one of the two DNA strands (Ernard, 2002). This mean that all of the genetic information is located in only one strand (Peh *et al*., 2002).

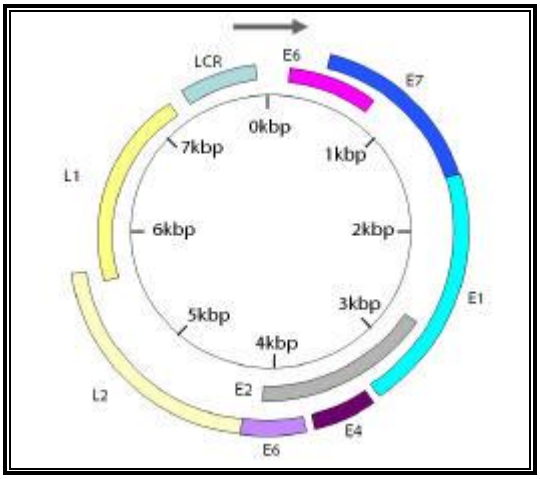

Figure (2.3): Schematic representation of HPV genome (Hoenil Jo, 2005).

The HPV genome can be divided into three regions, the non coding long control region (LCR), or the upper regulatory region (URR), and the early (E) and late (L) gene region (protein encoding). The long control region of 400 to 1,000 bp contains overlapping binding sites for many different transcriptional activators and repressors, including activating protein 1 (AP1), and nuclear factor 1 (NF-I). The LCR regulates transcription from the early and late regions and therefore controls the production of viral proteins and particles. The early region is downstream of the LCR and contains six open reading frames, E1, E2, E4, E5, E6 and E7, is involved in viral replication and oncogenesis as illustrated in Table (2.4). These encode all viral proteins except for the viral capsid proteins, which are encoded in the late region. The L1 and L2 genes in the late region encode the major and minor capsid proteins, both of which are required late in the viral life cycle to encapsulate the virus (Hoenil Jo, 2005).

Table (2.4): Summarizes the major function of each of the proteins encoded by E1, E2, E4, E5, E6 and E7 (Hoenil Jo ,2005).

Early region	Protein functions
E1	Unwinds the DNA strands working with E2 protein Modulate the transcription activity of the E2 protein
E2	Enables E1 protein to bind to the viral origin of replication located within the LCR Encodes a LCR-binding protein that regulates transcription of the early region
E4	Encodes a protein that interacts with cytokeratin Expressed in later stages of infection, when complete virions are being assembled
E5	Augment cellular proliferation and DNA synthesis in a context of cell membrane receptors, such as EGF and PDGF Induces an increase in mitogen-activated protein kinase activity
E6	Binds to p53 and targets it for rapid degradation via a cellular ubiquitin ligase Induces telomerase activation
E7	Binds to the hypophosphorylated Rb proteins and liberate E2F, which results in S phase entry Interacts with inhibitors of cyclin dependent kinases Induces abnormal centrosome duplication resulting in aneuploidy

Late Region

It consists of the viral L1 and L2 gene, which encode viral capsid proteins. The region of HPV genome encoding for L1 protein is highly conserved amony papillomavirus (Nebesio *et al.*, 2001).

Late gene transcription is intimately tied into differentiation program of the host cells, squamous epithelial cells or keratinocytes. High levels of viral DNA synthesis and expression of L1 and L2 gene take place only in the upper layers of skin or squamous epithelia (Fuchs and Pfister, 1997).

2.3.4-The life cycle for HPV:-

Human Papillomavirus infection primarily infected the basal layer if the injured epithelium, The viral genomes are established in the host cell as unintegrated extrachromosomal elements episomes. After infection, the first viral genes to be expressed are E1and E2, which are the replication proteins, these proteins bind to the origin of DNA replication, located on sites within the long coding region. In the basal layer the virus is in a nonproductive stage and is present in low copy number. The virus proliferates were recruiting host factors for viral synthesis and replicating its DNA enough to keep up with the mitosis of basal cells. As the host cells continues their normal life cycle or pattern of maturation a subset of daughter cells detach and migrate from the basal layer. The infected host cells divide and HPV DNA is divided between the daughter cells as they stratify and differentiated. Virus DNA travels with the host cells as they undergo their normal life cycle and mature HPV does not encode a DNA polymerase and hence, it is dependent on host cell differentiation to continue its own life cycle (Leäo *et al.*,2005). The nature of the cell surface receptor used for viral attachment is not known, although heparin sulphate proteoglycans may play a role in initial binding and virus up take. As with other viruses it seems that HPV infection requires the presence of secondary receptor for efficient infection and it has been suggested that this role may be played the α 6integrin (Doorbar, 2006).

The pattern of viral gene expression in these cells is not well defined, but it is generally believed that the viral E1 and E2 proteins are expressed in order to maintain the viral DNA as an episome (Wilson *et al*., 2002) and to facilitate the correct segregation of genomes during cell division (You *et al*., 2004).

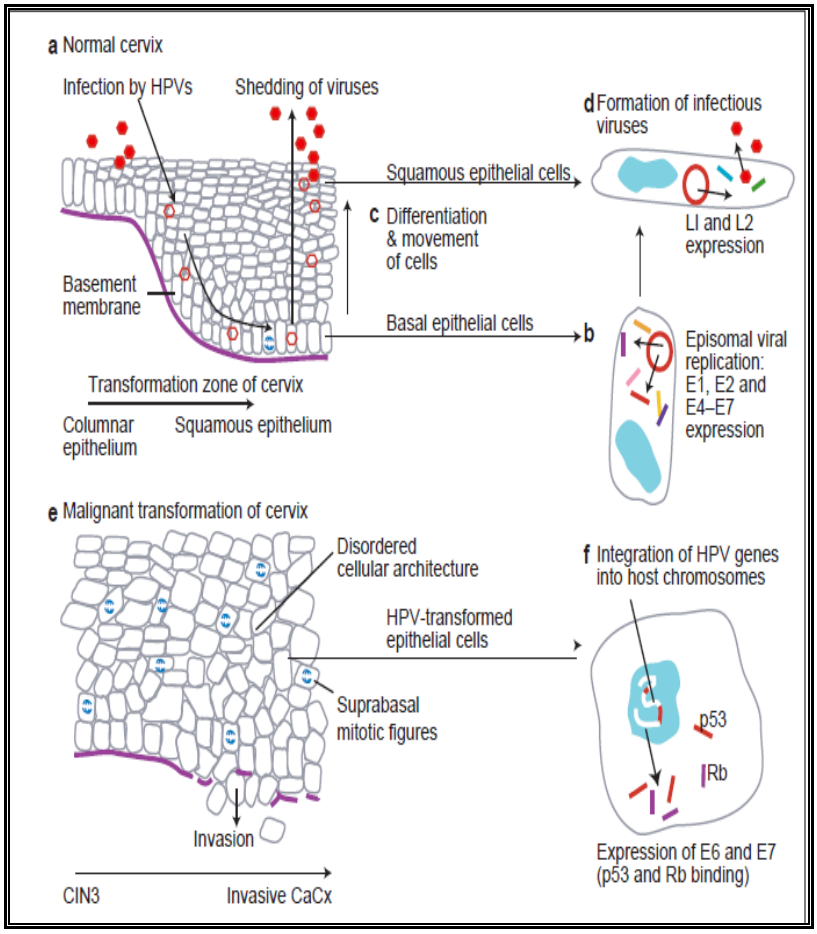

Figure (2.4): Human papillomavirus (HPV) infection and replication in cervical epithelial cells, Expert Reviews in Molecular Medicine ISSN 1462-3994 http://www–ermm.cbcu.cam.ac.uk.

2.3.5-Pathogenecity of HPV infection:-

A-Common warts.

Some "cutaneous" HPV types, such as HPV-1 and HPV-2, cause common skin warts. Common warts are often found on the hands and feet, but can also occur in other areas, such as the elbows or knees. Common warts have a characteristic cauliflower-like surface and are typically slightly raised above the surrounding skin. Cutaneous HPV types do not usually cause genital warts and are not associated with the development of cancer (Lountzis and Rahman, 2008).

B- Genital warts.

Genital or anal warts (condylomata acumminata or venereal warts) are the most easily recognized sign of genital HPV infection. Although a wide variety of HPV types can cause genital warts, types 6 and 11 account for about 90% of all cases (Gearheart *et al*., 2004).

C- Subungual warts .

Subungual or periungual warts form under the finger nail (subungual) around the fingernail or on the cuticle (periungual). They may be more difficult to treat than warts in other locations (Lountzis and Rahman, 2008).

D- Flat warts.

These lesions were named as flat endophytic or inverted condylomas. The cervix uteri are affected with flat rather than acuminate warts, in 30-50 % of cases (Von Krogh *et al*., 2000).

E- Respiratory papillomatosis.

Human Papilloma Virus types 6 and 11 can cause condition known as recurrent respiratory papillomatosis, in which warts form on the larynx. Or other areas of the respiratory tract. These warts can recur frequently may require repetitive surgery, may interfere with breathing, and in extremely rare cases can progress to cancer (Sinal and Woods, 2005).

F- Malignant lesion.

About a dozen HPV types (including types 16, 18, 31 and 45) are called "high-risk" types because they can lead to cervical cancer, as well as anal cancer, vulvar cancer and penile cancer (Parkin, 2006). Several types of HPV, particularly type 16, have been found to be associated with oropharyngeal squamous-cell carcinoma a form of head and neck cancer (D'souza *et al*., 2007).

Human papilloma virus has been more controversially associated with non-melanoma skin malignancies such as basal cell carcinoma, transitional cell carcinoma (TCC) of the urinary bladder, squamous cell and adenosquamous carcinoma of the colon and rectum, squamous cell carcinoma of the fingers, Prostate cancer and ovarian carcinoma (Leäo *et al.*,2005).

Human papilloma virus DNA has been detected in a small proportion of cases of bronchopulmonary carcinoma, and thus HPV infection appears to play a limited role in the tumor genesis of most lung carcinomas (Clavel *et al*., 2000).

In addition to persistent infection with high-risk HPV types ,epidemiological and molecular data suggest that co-factors such as the cigarette smoke carcinogen benzoapyrene (Bap) enhance development of certain HPV-induced cancers (Alam *et al*., 2007).

2.3.6- Diagnostic technologies for viral associated tumor:-

A- Electron microscopy.

Many investigations of the structure of virions or of virus-infected cells involve electron microscopy. Large magnifications are achievable with a transmission electron microscope but the specimen, whether it is a suspension of virions or an ultra thin section of a virus-infected cell must be treated so that details can be visualized. Negative staining techniques generate contrast by using heavy-metal-containing compounds, such as potassium phosphotungstate and ammonium molybdate. In electron micrographs of virions the stains appear as dark areas around the virions (Carter and Venetla, 2007).

B- Immunohistochemistry.

Immunohistochemistry or IHC refers to the process of localizing proteins in cells of a tissue section exploiting the principle of antibodies binding specifically to antigens in biological tissues (Ramos-Vara, 2005). Immunohistochemical staining is widely used in the diagnosis of abnormal cells such as those found in cancerous tumors. Specific molecular markers are characteristic of particular cellular events such as proliferation or cell death (apoptosis). Immunohistochemistry is also widely used in basic research to understand the distribution and localization of biomarkers and differentially expressed proteins in different parts of a biological tissue (O'Malley and Pinder, 2006).

C-In Situ Hybridization (ISH) .

The *In situ* hybridization (ISH) was first described in 1969 (Gall and Pardue, 1969) and used radioactive labels for visualization. Although, this radioactive method reached a high level of sensitivity, it had practical drawbacks such as safety measures and the inability to distinguish more than one nucleic acid target simultaneously. The successful search for alternative, non-radioactive labels in the late (Langer *et al*., 1981).

In situ hybridization is usually applied to histological section or cell smears if performed carefully. *In situ* hybridization can provide not only the exact localization of target sequences but also excellent morphologic details of tissues or cellular contents (Unger, 2000).

In situ hybridization techniques have become important tools to detect nucleic target sequences (Holm, 2000).

D- Polymerase Chain Reaction (PCR).

The Polymerase chain reaction (PCR) firstly invented by Kary Mullis at 1983, is an in vitro technique which allow the amplification of specific deoxyribonucleic acid(DNA) region that lies between two regions of known DNA sequence (Newton and Graham, 2003).

Polymerase chain reaction is commonly used in research and epidemiological studies for ultra sensitive research. It's also used for detection of low levels target DNA in clinical specimens to establish a new etiologic link of a virus with a disease condition (Stoler, 2000). When a sample is likely to contain a low number of copies of a virus nucleic acid the probability of detection can be increased by amplifying virus DNA using a PCR, while RNA can be copied to AGG (Carter and Venetla, 2007).

2.4-Tumor suppressor gene (p53):-

2.4.1- General view.

Tumor suppressor protein was first identified in 1979 as a transformation-related protein and a cellular protein which accumulates in the nuclei of cancer cells (Bai and Wei-Guo, 2006). Human p53 is a nuclear phosphoprotein of MW 53 kDa, encoded by a 20-Kb gene containing 11 exons and 10 introns, which is located on the small arm of chromosome. This gene belongs to a highly conserved gene family containing at least two other members, p63 and p73 (Bai and Wei-Guo, 2006).

2.4.2-The structure of p53:-

The p53 molecule is a tetramer, resembling a hollow skewed cube with node-like vertices of two sizes. Four larger nodes accommodate central core domains, as was demonstrated by fitting of its X-ray structure. (Okorokov *et al*., 2006). Tumor suppressor protein is a polypeptide of ~400 amino acid residues in length 393 amino acid for human p53.

- A Proline rich domain important for the apoptotic activity of p53 : residues 80-94
- A central DNA-binding core domain (DBD). Contains one zinc atom and several Arginine Amino Acid: Residues 100-300.
- A homo-oligomerisation domain (OD): residues 307-355 tetramerization is essential for the activity of p53 *in vivo*.
- A C-terminal involved in downregulation of DNA binding of the central domain: residues 356-393 (Bell *et al*., 2002). As shown in the following Figure (2.5)

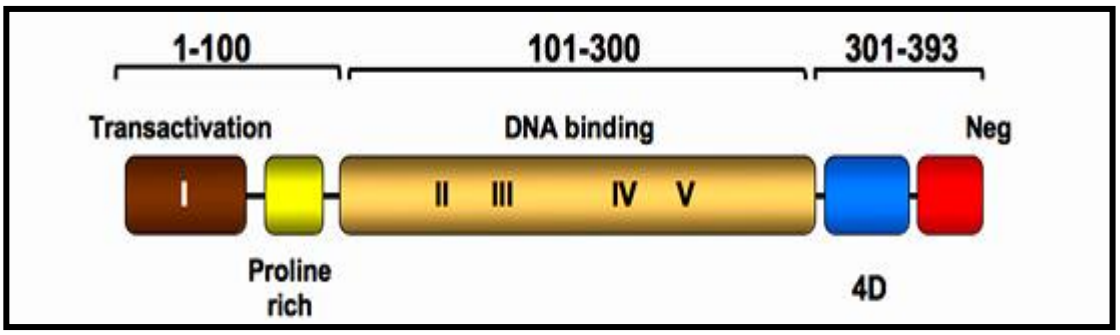

Figure (2-5): P53 molecular structure (Gasco *et al.*2002).

2.4.3- The types of p53:-

A- Wild-type p53 protein. The protein in it's normal appearance is called Wild-type p53 protein the half-life of this wild type protein is very short in range of 15 to 30 minutes (rapid turnover metabolism)and is presented only in very low concentrations in nuclei of normal cells (portefaix *et al*.,2000).

B-Mutated-type p53 protein. Mutant form which induces cell transformation and half life up to 6 hours (Busmanis, 1998). The longer half life of mutated from product increases the concentration of this product in the nucleus (Appella and Anderson, 2001). The mutated protein is known to be resistant to degradation, and this stabilised protein product is often detected using immunohistochemistry (Ishii *et al.*, 2004).

2.4.4-Function of p53:-

1-The tumor suppressor gene p53 plays a central role in the maintenance of normal cell growth and genetic integrity (Chang *et al.*, 2008).

2-The p53 tumour suppressor gene plays an important role in preventing cancer development, and loss of p53 function, or loss of the ability to activate a p53 response, appears to be a prerequisite for malignant progression. In both mice and humans, germ line mutations in *p53* result in a strong predisposition to cancer (Lozano and Zambetti, 2005). Figure (2.6)

3-Tumor suppressor protein stimulates transcription of several genes that mediate cell-cycle arrest and apoptosis (Joerger and Fresht, 2007).

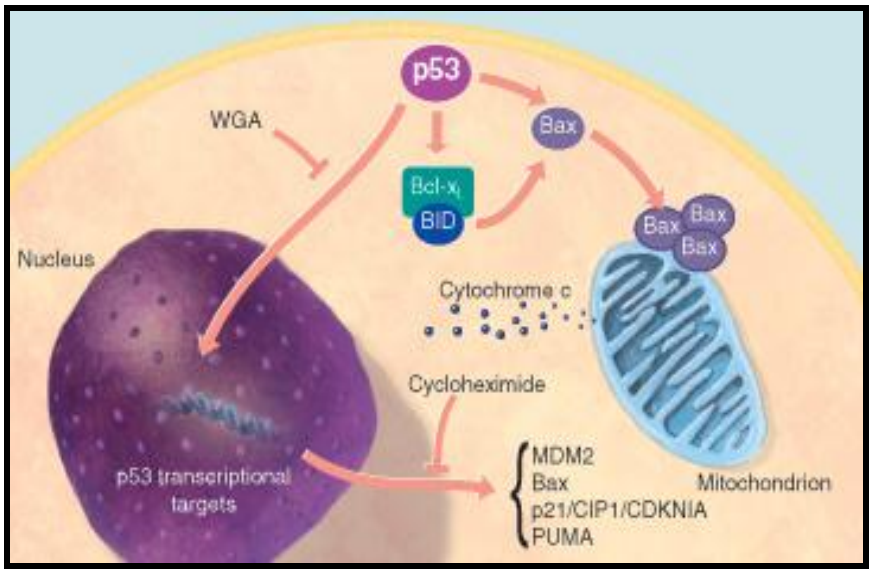

Figure (2.6): p53-dependent apoptosis

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2.4.5- The relationship between p53 and bladder cancer:-

Mutations of the p53 tumor suppressor gene are present in approximately 50% of all human cancers and are the most frequent genetic alteration known in human cancers. Tumor suppressor protein can also be inactivated by mechanisms other than mutation, for example by complexing with viral or cellular proteins such as MDM2. It is clear that the malfunction of this protein plays a central role in the development of cancer. Tumor suppressor protein acts as a transcription factor and is implicated in the regulation of the cell cycle and consequently in growth controls (LaRue *et al*., 2000).

Alterations of the gene p53 are common in bladder cancer, occurring in ~50% of transitional cell carcinoma and are seen more frequently in high-grade invasive tumors. Moreover, loss of p53 function has been associated with progression to invasive disease and decreased survival (Schlichtholz *et al*., 2004).

In bladder cancer, p53 over expression has been associated with a poor prognosis. In general, a good correlation has been observed between over expression of the protein and the presence of mutations. However, the concordance is not perfect suggesting that other mechanisms are also involved in the stabilization of this protein in bladder cancer. The MDM2 protein is known to bind to p53, blocking its transcription activation activity and targeting it for degradation (LaRue *et al*., 2000).

2.4.6- The relationship between p53 and viral infection:-

Tumor suppressor protein acts as a two-edged sword in viral replication. For some DNA viruses, such as polyomaviruses and papillomaviruses, activation of p53 would lead to cell cycle arrest and thus impede viral replication, because viral replication must be supplemented by replicate machineries which are present only in the S-phase cellular environment (Chang *et al.*, 2008).

There are some indications those members of the herpesvirus family, like EBV modulate or mutate p53 for their own survival (Moritani *et al.*, 2002). And Latent membrane protein-1 of EBV also interferes with p53 function (Ishii *et al.*, 2004).

Human papillomavirus-16 and -18. These viruses contain gene products (E6 and E7) that bind wild-type p53 and Rb proteins and eliminate the ability of these proteins to stimulate DNA repair or apoptosis (Williams, 2000). As shown in the following Figure (2.7).

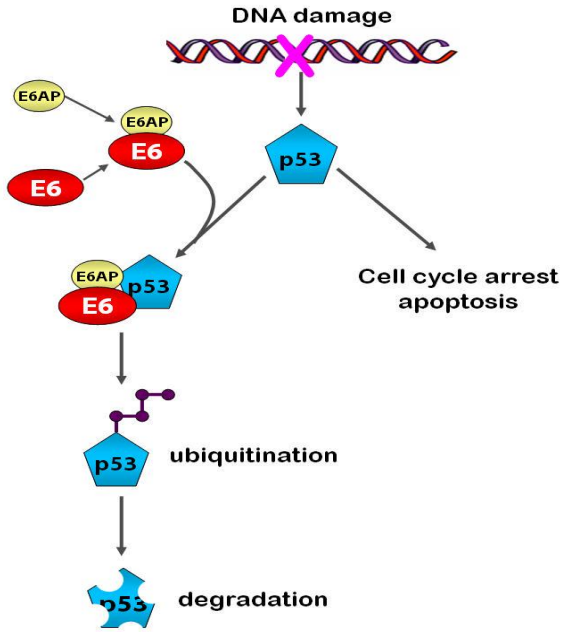


Figure (2.7): DNA damage induces p53 activation, leading to either cell cycle arrest or apoptosis. The HPV E6 binds to E6-AP and redirects it to p53, which results in the E6-APmediated ubiquitination and rapid proteasomal degradation of p53 (Hoenil Jo, 2005).

2.5 -Matrix metalloproteinases(MMPs) :-

2.5.1-Historical background:-

Initially, MMPs were described by Jerome Gross and Charles Lapiere (1962) who observed enzymatic activity (collagen triple helix degradation) during tadpole tail metamorphosis (Gross and Lapiere,1962). Therefore, the enzyme was named interstitial collagenase (MMP-1).

Later it was purified from human skin (1968). Eisen *et al.*, (1968) was recognized it to be synthesized as a zymogen (Harper *et al.*, 1971). More than 25 new members have been discovered (Visse and Nagase 2003).The "cysteine switch" was described in 1990 by Van and Birkedal.

2.5.2- Overview:-

The Matrix Metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases their primary function is degradation of proteins in the extracellular matrix (Duffy *et al.*, 2000) and Bone Marrow(BM) components. In addition, MMPs can modulate many other substrates including ILs and other cytokines, serine proteninases inhibitors (serpins), growth factors and chemokines (Uitto *et al.*,2003).

physiologically these enzymes play a role in normal tissue remodeling events such as embryonic development, angiogenesis, ovulation, mammary gland involution and wound healing (Duffy *et al.*, 2000), but also can process a number of bioactive molecules. They are known to be involved in the cleavage of cell surface receptors, the release of apoptotic ligands (such as the FAS ligand), and chemokine inactivation MMPs are also thought to play a major role on cell behaviors such as cell proliferation, migration (adhesion/dispersion), differentiation, angiogenesis, apoptosis and host defense (Remacle *et al.*,2006).

Abnormal expression appears to contribute to various pathological processes including rheumatoid arthritis and osteoarthritis, pulmonary emphysema and tumor growth, invasion and metastasis (Chambers and Matrisian, 1997). Increased MMP activity has been detected and shown to correlate with invasion and metastatic potential in a wide range of cancer including, ovary, lung, prostate, breast, colorectal and cervical cancer cell (Tutton *et al*., 2003).

2.5.3-Substrate specificity and classification:-

Currently, at least 19 members of this family are known to exist in mammalian system (Duffy *et al.*, 2000) as illustrated in Table (2.5). Matrix Metalloproteinases are grouped mainly based on substrate specify and molecular domain structure to.

1- Interstitial collagenases (MMP-1, MMP-8 and MMP-13).

2- Gelatinases (type IV collagnases; MMP-2 and MMP-9).

3- Stromelysins (MMP-3, MMP-10 and MMP-11).

4- Matrilysins (MMP-7 and MMP-26).

5- Membrane-type (MT)-MMPs (trance membrane type and GPI-anchored elastase) .

6- Other MMPs including metalloelastase, Macrophage elastase (MMP-12), Enamelysin (MMP-20), Epilysin (MMP-28), CA-MMP (MMP-23), MMP-19, MMP-21 and MMP-27 (Nagase *et al.*, 2006).

Several non-matrix substrates have been identified for MMPs in recent years. These include other proteinases, proteinase inhibitors, clotting factors, chemotactic molecules, latent growth factors, growth factor binding proteins, cell surface receptors, and cell-cell and cell-matrix adhesion molecules. The

functions of the MMPs are thus more complex than more ECM degradation. (McCawley and Matrisian, 2001).

All MMPs are synthesized as prepro-enzymes and secreted mostly as inactive proenzymes. They need activation to be fully functional; this activation may be endogenously initiated by serine proteases such as plasmin and urokinase-type plasminogen (uPA), or by some MMPs such as MT-MMPs. In the activation of the MMPs the prodomain with the cysteine-containing sequence is cleaved in a stepwise mannor, disrupting a cysteine - zinc interaction (Visse and Nagase, 2003). Finally protrolytic activity is inhibited by tissue inhibitors known as tissue inhibitors of metalloproteinase (TIMPs), (Duffy *et al.*, 2000).

Table (2.5): Matrix metalloproteinases and their substrates (modified from Nagase, Visse and Murphy 2006).

Enzyme	*MMP Main*	*Substrates*
Collagenase-1	MMP-1	types I, II, III, IV, VII, VIII and X collagen, gelatin, entactin, perlecan, laminin, casein, proMMP-1, -2, -9
Gelatinase-A	MMP-2	gelatin, types I, III, IV, VII, X, and XI collagen, elastin, fibrinogen, laminin, aggregan, vitronectin, decorin, plasminogen
Stromelysin-1, Transin-1	MMP-3	aggregan, laminin, gelatin, fibronectin, types III, IV, V, IX, X, XI and XVIII collagen
Matrilysin-1	MMP-7	fibronectin, laminin, gelatin, aggregan, types I, IV, IX, X, XI and XVIII collagen
Collagenase-2 Neutrophil,collagenase	MMP-8	types I, II, III, VI and X collagen, gelatin, entactin, aggregan, tenascin, proMMP-8
Gelatinase-B	MMP-9	gelatin, types I, IV, VII, X, XI and XVIII collagen, elastin, laminin, fibronectin, vitronectin, proMMP-2, -9
Stomelysin-2, Transin-2	MMP-10	types I, III and IV collagen, gelatin, elastin, proMMP-1,-8,-10
Stromelysin-3 Macrophage,Elastase	MMP-11	fibronectin, laminin, aggregan, gelatin
metalloelastase	MMP-12	elastin, types I and IV collagen, fibronectin, laminin, proteoglycans, fibrinogen
Collagenase-3	MMP-13	types I, II, III, VII, X and XVII collagen, gelatin, entactin, tenascin, aggregan
MT1-MMP	MMP-14	types I, II and III collagen, gelatin, laminin, aggregan, proMMP-2, -13
MT2-MMP	MMP-15	proteoglycans, proMMP-2
MT3-MMP	MMP-16	type III collagen, fibronectin, proMMP-2
MT4-MMP	MMP-17	gelatin, fibrinogen, proMMP-2
Collagenase-4 (Xenopus)	MMP-18	types I, II and III collagen, gelatin
Stomelysin-4	MMP-19	types I and IV collagen, gelatin, laminin, tenascin
Enamelysin	MMP-20	amelogenin, aggregan, laminin
XMMP (Xenopus)	MMP-21	gelatin
CMMP (Chicken)	MMP-22	gelatin, casein
Ca-MMP (Cysteine array)	MMP-23	gelatin
MT5-MMP	MMP-24	fibronectin, gelatin, proMMP-2
MT6-MMP	MMP-25	type IV collagen, gelatin, proMMP-2, 9
Matrilysin-2, Endometase	MMP-26	type IV collagen, gelatin, proMMP-9
CMMP (Gallus)	MMP-27	
Epilysin	MMP-28	casein

2.4.4-Structure of MMPs:-

The MMPs share a common domain structure. The three common domains are the pro-peptide, the catalytic domain and the haemopexin-like C-terminal domain which is linked to the catalytic domain by a flexible hinge region.

A- The pro-peptide.

The MMPs are initially synthesized as inactive zymogens with a pro-peptide domain that must be removed before the enzyme is active. The pro-peptide domain is part of the 'cysteine switch' This contains a conserved cysteine residue which interacts with the zinc in the active site and prevents binding and cleavage of the substrate keeping the enzyme in an inactive form (Pei and Kang, 2000).

B- The catalytic domain.

The catalytic domain forming the active site there is a catalytically important Zn2+ ion, which is bound by three histidine residues found in the conserved sequence HExxHxxGxxH. Hence, this sequence is a zinc-binding motif.

The gelatinases, such as MMP-2, incorporate fibronectin type II modules inserted immediately before in the zinc-binding motif in the catalytic domain (Trexler *et al.*, 2003).

C- The haemopexin-like C-terminal domain.

The C-terminal domain has structural similarities to the serum protein haemopexin. It has a four bladed β-propeller structure. β-propeller structures provide a large flat surface which is thought to be involved in protein-protein interactions. This determines substrate specificity and is the site for interaction with TIMP's (Browner *et al.*, 1995).(Figure 2.8)

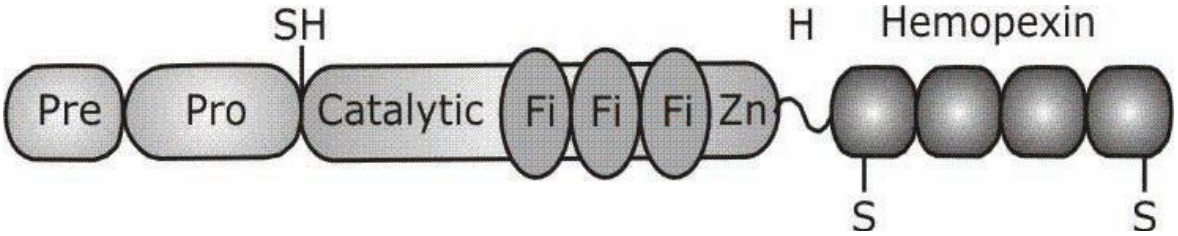


Figure (2.8): Domain structure of the gelatinases. Pre = signal sequence; Pro =propeptide with zinc ligating thiol (SH) group; Fi = fibronectin like collegen; Zn =zinc-binding site; H = hinge region; Hemopexin domain linked by disulfide (S) bond (modified from Sounni *et al*., 2003).

2.4.5-Role of MMPs in Tumor angiogenesis, invasion and metastasis:-

Angiogenesis is necessary for a tumor to grow to a size greater than approximately 2mm in diameter. The process begins with local degradation of the basement membranes that surround capillaries, followed by invasion of the surrounding stroma by the underlying endothelial cells in the direction of angiogenic signal. Endothelial cell migration is accomplished by cell growth at the leading edge of the migrating column. The endothelial cells then organize themselves into three-dimensional structures to form new capillary tubes (Zetter, 1998). As illustrated in Figure (2.9)

Matrix metalloproteinases may promote angiogenesis by at least two different mechanisms by degrading barriers and thereby allowing endothelial cell invasion, and by liberating factors that promote or maintain the angiogenic phenotype (Stetler-Stevensen, 1999). New evidence suggests that

MMPs may also generate or release angiogenesis inhibitors such as angiostatin from the extracellular matrix. The importance of MMPs in angiogenesis is also proved by evidence that endogenous and synthetic inhibitors of MMPs block the process (Hidalgo and Eckhardt,2001), a number of MMPs, such as MMP-3, -7, -9 and -12 can degrade plasminogen, generating the angiogenesis inhibitor angiostating (Duffy *et al.*, 2000). Studies on cancer of different origins such as gastric cancers, Bladder cancers, head and neck saquamous cell cancer, breast cancer(Nomura *et al.*, 1995; Kanayama, 1998; Ruokolainen *et al*., 2004; Leppä *et al*., 2004).

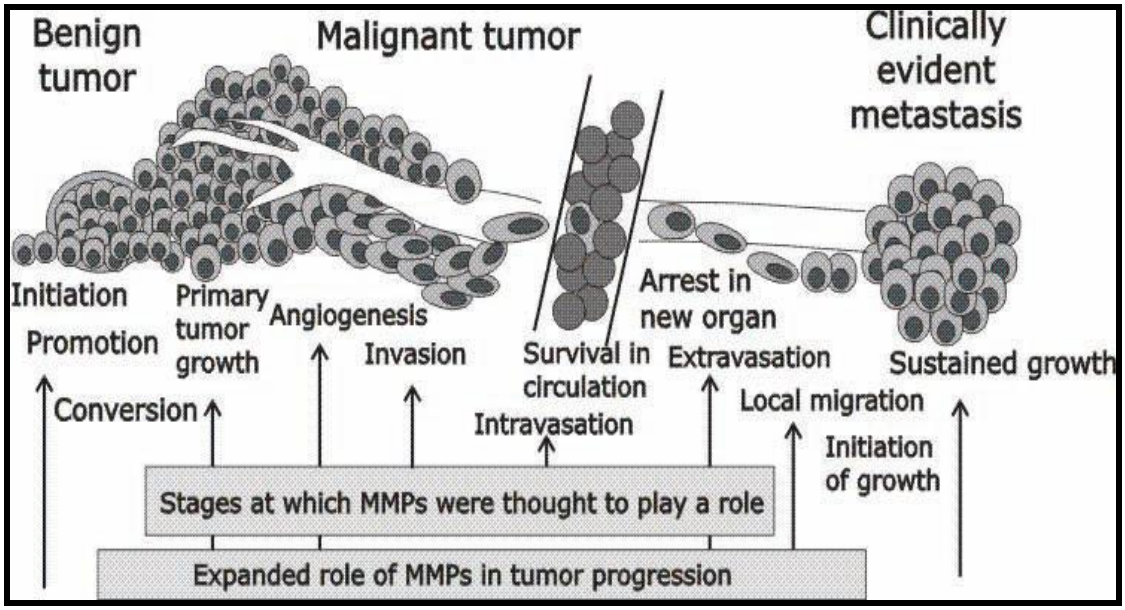


Figure (2.9): MMPs in tumor progression (Nelson *et al*., 2000).

2.6- Tissue Inhibitors of Metalloproteinase (TIMPs):-

2.6.1-Historical background:-

The first TIMP was described in 1975 as a protein, in culture medium of human fibroblasts and in human serum, which was able to inhibit collagenase activity. The molecular weight of this protein was shown to be 28.5 kDa. Since then, 3 new TIMPs have been discovered in different species, and have been designated TIMP-2, -3, and -4, respectively. The molecular weights of TIMP proteins vary between species (Vestappen and Von de Hoff, 2006).

All Four currently known TIMPs are very well-conserved, since they have been identified in humans, other vertebrates, insects, and even in *Caenorhabditis elegans*, a nematode worm that is commonly used as a model organism for genetic and cell biological research (Lambert *et al.*, 2004).

2.6.2-Overview:-

Tissue inhibitors of metalloproteinase (TIMPs) regulate MMP activity controlling the breakdown of extracellular matrix components and thus play an important role in the process of invasion and metastasis. Moreover, there are several new functions, growth control (Gakiopoulou *et al*., 2003). TIMP-1 and TIMP-2 have been identified as potent growth factors for a wide range of cells TIMP-2 also stimulates the proliferation of a wide range of other cells, such as dental pulp fibroblast-like cells and gingival fibroblasts, as well as leukocytes and epithelial cells (Hayakawa *et al*., 1994).

Tissue inhibitors of metalloproteinase seem to play a role in the regulation of apoptosis. The mechanism behind this process remains to be elucidated. Tissue inhibitors of metalloproteinase-1 and TIMP-2 seem to have anti-apoptotic effects, whereas TIMP-3 and TIMP-4 seem to be pro-apoptotic. Furthermore, the direct or indirect effects of TIMPs on apoptosis are cell-type-specific (Li *et al.*, 1999).

2.6.3- Substrate specificity and classification:-

Several different TIMPs (TIMP-1 to TIMP-4) have been identified and each appear to be capable of binding to several MMPs (Gomez *et al.*, 1997), also the four TIMPs are differentially expressed in tissues, and temporally follow the influx of MMPs. The TIMPs are slow, tight-binding inhibitors with low nanomolar inhibition constants (Brew *et al*., 2000).

The four different inhibitors TIMPs have a molecular weight ranging from 21 kD to 30 kD, as shown in Table (2.7).

Table (2.6): Common and unique features of TIMPs (Baker *et al*. 2002).

Criteria	*TIMP-1*	*TIMP-2*	*TIMP-3*	*TIMP-4*
n-glycosylation sites	2	0	1	0
Protein kDa	28	21	24/27	22
Protein localization	soluble	soluble/cell surface	ECM	soluble/cell surface
Protein association	proMMP-9	proMMP-2	proMMP-2/-9	proMMP-2
MMPs poorly inhibited	MT1-, MT2-, MT3- and MT5-MMP, MMP-19	None	None	None

2.6.4-Structure of TIMPs:-

All currently known TIMP proteins contain 6 loops and have a junction between the N- and C-terminal domains. Tissue inhibitors of metalloproteinase are produced in many tissues, although not every tissue expresses all 4 TIMPs. In general, most mesenchymal and epidermal cells are able to produce TIMPs (Rowe *et al.*, 1997). TIMP-1, -2 and -3 are also produced by white blood cells (Bjerkeli *et al*., 2004).

2.6.5-Role of TIMPs in bladder cancer angiogenesis, invasion and metastasis:-

There are several studies that have tried to establish the function of metalloproteinase invasion and the growth of these in bladder and urothelial tumors. Many of these studies have concentrated on exploring the levels of these markers in the urine. The protein levels of MMP-2, MMP-9 and TIMP-2 in urine have recently been suggested as possible non-invasive diagnostic markers for bladder cancer by Eissa *et al*., 2007. They showed that high sensitivity and specificity of MMP zymography, MMP-9/TIMP-1 ratio and MMP-2/TIMP-2 ratio were reached, more favorably so compared to cytology.

In general, TIMPs inhibit MMPs. TIMPs-2 binds to the active form of MMP-2 and inhibits its proteolytic activity. In fact low doses of TIMP-2 are associated with MTI-MMP-mediated MMP-2 activation. Instead, high TIMP-2 doses inhibit directly both MMP-2 and MT1-MMP-mediated MMP-2 activation (Kurschat *et al*. 1999). As shown in Figure (2.9)

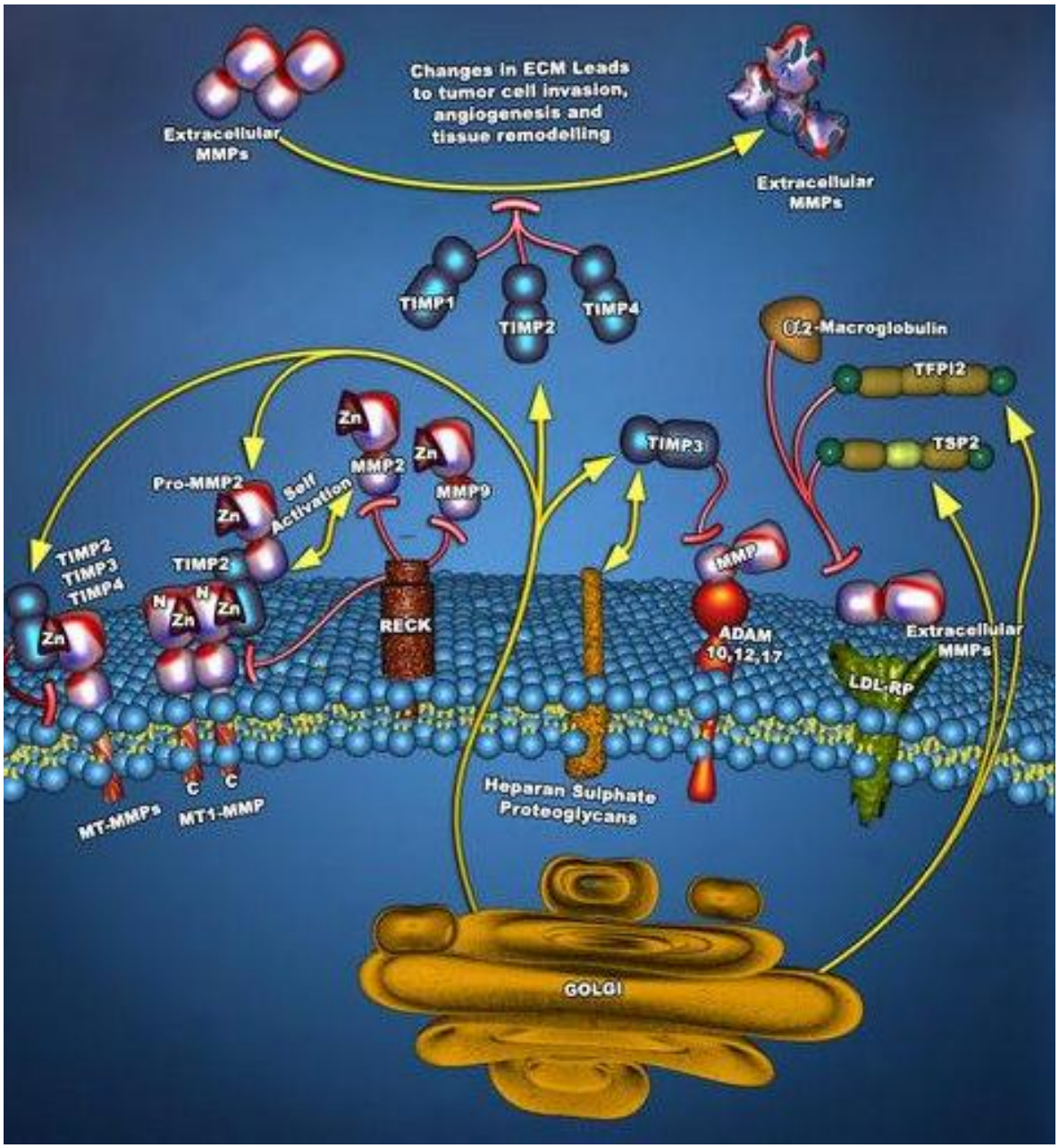


Figure (2.10): TIMPs in tumor progression. TIMP-3 is an ECM, probably bound to heparin sulphate proteoglycans and is a potential inhibitor of the function of some membrane-associated ADAMs (a disintegrin and a metalloproteinase), as well as the matrix-associated ADAM-TS (ADAM-Thrombospondins, notshown). TIMP-2 acts in conjunction with MT1-MMP as a receptor for the pro-form of MMP-2at the cell surface, allowing an efficient activation and focusing of the active form of this soluble proteinase. In some cell types, TIMP-1 and TIMP-2 may have receptor directly linked to intracellular signaling pathways regulating cell behavior , other inhibitors RECK (reversion inducing cysteine rich protein with kazal motifs) is a GPI-anchored glycoprotein that binds and inhibits a number of MMPs. The pan proteinase inhibitor α2-macroglobulin, although very large, has some access to the pericellular space in vascularised tissues and may be involved in MMP endocytosis through the low density lipoprotein receptor – related protein (LDL-RP). The roles of the LDL-RP in MMP-2 removal via a thrombospondin-2 (TSP-2) complex and in direct MMP-9 removal have been described. The tissue factor pathway inhibitor(TFPI-2) has also been described as an MMP binding agent. www.ambion.com/tools/pathway/loadImage.php

Chapter Three

Subjects, Materials and Methods

Subjects, Materials and Methods

3.1- Subjects and specimens:-

Since this study was designed as a retrospective research, the subjects included in this study were represented by their archival formalin-fixed, paraffin embedded tissue blocks with TCC of the bladder. This study was carried out during the period beginning from February 2009 till June 2009. While the pathological samples were collected during the period from March 2008 to June 2009.

3.1.1-Patients study group:-

Fifty formalin-fixed, paraffin embedded blocks tissue were obtained, from transitional cell carcinoma which collected from 35 (males) and 15 (female). Their age ranged from 25 to 70 years. All these patients underwent cystoscopic bladder biopsy. The patient's samples were collected from the archives of histopathology laboratories of Specialized Surgical Hospital in Baghdad.

3.1.2-Healthy control Group:-

Ten bladder autopsies were obtained as apparently normal controls from the Forensic Medicine Institute Archives. They were 5(males) and 5 (female) and their age ranged from 25 to 70 years.

The diagnosis of these tissue blocks were primarily based on the obtained histopathological records of bladder biopsy samples that had accompanied in the hospital laboratory such as age of patients, gender, tumor type, tumor grade and presence or absence of muscle invasion. Confirmatory histopathological re-evaluation of each obtained tissue blocks was done by my supervisor Dr. Alaa Ghani at the department of pathology, Al-Nahrain University.

3.2-Materials:-

3.2.1- Equipments:-

The equipments used during this study are shown in table (3.1).

Table (3.1): List of equipments.

Name of the equipment	*Company and origin*
Biocular light microscope(model CH-A)	Olympus Co., Ltd., Japan
Cold microcentrifuge	Hettichzentrifuge,Sigma-Germany
Disposable knives	Sigma-Germany
Forceps	England
Humidified chamber	Individually hand made
Hot plate	Memmert, Co. Ltd, Germany
Hot air oven (model 11-A)	Memmert, Co. Ltd, Germany
Incubator (model IB-909)	Memmert, Co. Ltd, Germany
Microtome **(**Type AM 325)	Micron-Germany
Ocular grid	Ziess, Germany
Oven	Gallen kamp , England
Photo system(image analyzer)	Bio-RAD, Italy
Refrigerator	Marubeni-japan
Timer with alarm	Junghans, Germany
Water bath (model 1-A)	Memmert, Co. Ltd, Germany

3.2.2-Tools:-

Tools used during this study are shown in table (3.2).

Table (3.2): List of tools.

Name of the tool	*Company and origin*
Aluminium foil	Turkey
Absorbent wipes	Chemical Co., Ltd, Germany
Cover slip	Chance Proper Ltd, Germany
Disposable plastic pipettes	Sterilin, England
Disposable pipette tip	VWR international, USA
Eppendorf tube	VWR international, USA
Flask(250,500,1000 ml)	Oxford. USA
Glass staining jars	Memmert , Co. Ltd, Germany
Latex examination gloves	Beromed-Germany
Microtiter pipettes	Gallen kamp, England
Mask(water resistant)	China
Ordinary slides	Sail brand-China
Positively charged slides	plus Fisher Co., U.S.A**.**
Washing bottles	Sterilin, England

3.2.3-Chemicals:-

The chemicals used during this study are shown in table (3.3).

Table (3.3): List of chemicals.

Name of the chemicals	*Company and origin*
Deionized water or Distilled water	Iraq
DPX (Disteren Plasticizer Xylene)	Sigma-Germany
Eosin Y stain solution	BDH Chemical Ltd., England
Ethanol	BDH pool, England
Formalin	Fluka-Germany
Hematoxyline stain solution	BDH Chemical Ltd., England
Nuclear fast red stain	Sigma - Germany
Phosphate buffered saline (PBS)	Sigma - Germany
Paraffin wax	Fluka - Germany
Xylene	BDH Chemical Ltd., England

3.2.4-Kits:-

3.2.4.1- In situ hybridization kit for detection of HPV -16 DNA, HPV-18 DNA, MMP-2, MMP-9, TIMP-1 and TIMP-2.

A-DNA Probe (Maxim biotech Inc, USA):-

Six DNA probes were used in this study, which include the following:

• **Biotinylated long DNA probe** for HPV Type 16 Cat. No.: IH-60058.

- **Size:** 243bp. This biotin-conjugated probe was produced by PCR primers under PCR conditions using HPV Type 16 DNA and biotin-dUTPs & biotin-d ATPs.
- **Purity:** The biotin-conjugated probe was purified by cartridge and showed a single band on gel.
- **Sequences:** (Alignment on database: Genbank, K02718).

• **Biotinylated long DNA probe** for HPV Type 18 Cat. No.: IH-60059.

- **Size:** 360bp. This biotin-conjugated probe was produced by PCR primers (Maxim's product Catalog # SP-10301) under PCR conditions using HPV Type 18 DNA and biotin-dUTPs & biotin-d ATPs.
- **Purity:** The biotin-conjugated probe was purified by cartridge and showed a single band on gel.
- **Sequences:** (Alignment on database: Genbank, X04773).

• **Biotinylated long DNA probe** for human MMP-2 Cat. No.: IH-60025 (MMP2-6001-B).

- **Size:** 191bp. This biotin-conjugated probe was produced by PCR primers (Maxim's product Catalog # SP-10514) under PCR conditions using human cDNA and biotin-dUTPs & biotin-d ATPs.
- **Purity:** The biotin-conjugated probe was purified by cartridge and showed a single band on gel.
- **Sequences:** (Alignment on database: Genbank, BC002576).

• **Biotinylated long DNA probe** for human MMP-9 Cat. No.: IH-60028 (MMP9-6001-B).

- Size: 216bp. This biotin-conjugated probe was produced by PCR primers (Maxim's product Catalog # SP-10519) under PCR conditions using human cDNA and biotin-dUTPs & biotind ATPs.

- Purity: The biotin-conjugated probe was purified by cartridge and showed a single band on gel.

- Sequences: (Alignment on database: Genbank, NM-004994).

• **Biotinylated long DNA probe** for human TIMP-1 Cat. No.: PB-60166.

- Specific Gene size: 265bp.

-**Genbank Accession Number**: AF366397.

• **Biotinylated long DNA probe** for human TIMP-2 Cat. No.: PB-60167.

- Specific Gene size: 221bp.

- **Genbank Accession Number**: NM003255.

B- In situ hybridization /Detection kit (High-sensitive system):-

Hybridization /Detection system for viral, MMP and TIMP were used purchased from Maxim Biotech /USA cat. Number IH-60001 (IHD-0050), kit contents are detailed in Table (3.4).

Table (3.4): Contents of *In situ* hybridization /Detection kit **(Maxim Biotech Inc. USA).**

Number	*Reagents*
1	Proteinase K
2	DNase and RNase free dilution buffer
3	Hybridization solution
4	Biotinylated housekeeping gene probe
5	Protein block (20X)
6	RNase A (15 µg/ml)
7	Streptavidin-AP conjugate
8	Substrate
9	Detergent wash buffer (20X)
10	Citric buffer, pH 6.0 (100X)

3.2.4.2-Immunohistochemistry Kit for detection of LMP-1 and P53:-

A- Monoclonal Antibodies.

•**Primary antibody:** Mouse monoclonal to EBV Latent membrane protein.
-**Immunogen:** Fusion protein.
-**Isotype:** IgG1.
-**Clone:** CS 1-4.
-**Code No.:** ab 78113.
-**Source:** Cambridge Science Park. England.

•**Primary antibody:** Mouse monoclonal to Anti-Human p53 protein.
-**Immunogen:** Recombinant human Wild-type p53 protein expressed in E. coli.
-**Isotype:** IgG2b.
-**Clone:** DO-7.
-**Code No.:** ab 71101.
-**Source:** Cambridge Science Park. England.

B-The IHC/ Anti Mouse HRP/DAB Detection kit (Cambridge Science Park. England).

Immunohistochemistry Detection system for LMP-1 and p53 was used purchased from Cambridge Science Park. England cat. Number ab 64259, kit contents are detailed in Table (3.5).

Table (3.5): Detection kit contents for immunohistochemistry technique for LMP-1 and p53.

Number	*Reagents*
1	Hydrogen peroxide block
2	Protein block
3	Biotinylated goat anti-mouse IgG (H+L) in PBS with stabilizers (Secondary Ab**)**
4	Sterptavidin peroxidase
5	DAB chromogen
6	DAB substrate

3.3-Methodology:-

3.3.1-Tisssue sectioning and slide preparation:-

1- Using a microtome to cut tissue samples.

2- Serial tissue sections were cut into (4µm) thickness, from each tissue block, 9 sections were collected. Eight sections were mounted on charged slides (to be used for *In situ* hybridization, detecting of HPV-16 DNA, HPV-18 DNA, MMP-2, -9, TIMP-1 and -2 and for IHC, detecting of LMP-1 and p53) and one section was mounted on ordinary slide to be used for Haematoxylin and Eosin stain).

3-New disposable knife was used for each sample to prevent carry-over DNA contaminations from one tissue sample to another.

*3.3.2-processing of Haematoxylin and Eosin Staining (H. & E.): (Harri's Haematoxylin)**

The Haematoxylin and Eosin stain was used for histopathological examination.

1-Deparaffinization was done by leaving sections in oven at 60°C overnight.

2-Then sections were dipped in 2 successive changes of Pre-warmed xylene (55°C) for 5 minutes and Xylene at room temperature 20- 25°C for 2 minutes.

3-The slides were immersed sequentially in the changes of ethanol (99%, 95%, 80% and 70%) for one minute each and in tap water for one minute at room temperature.

4-After the final rinse the slides were allowed to dry completely by incubating them at 37°C for 5 minutes.

5-Stained by Harri's Haematoxylin for 90 second. Washed again with running tap water for 5 minutes and then treated with acid alcohol for 1 minute. Tissue sections then stained with eosin for 15-30 second.

6-Dehydration were done by immersions in ethyl alcohol [70% (2 changes) - 2 minutes each], 80% and 95% one minute each, 2 changes of 100% one minute each cleared in xylene.

7-The slides were then mounted by (DPX) and covered by cover slips (Luna, 1968).

3.3.3-Preparation of reagents as recommended by the manufacturer instruction (Maxim Biotech leaflet, 2009):-

A- Proteinase K (10X).

To reconstitute the proteinase K, the entire contents (2ml) of the DNase and RNase free dilution buffer vial was added to the vial containing the lyophilized proteinase K powder (4mg) and mixed gently. The resulting 10X concentrated proteinase K solution that is not to be used immediately was divided into small aliquots and stored frozen at -20°C. Each 0.1 ml of 10X concentrated solution yields 1 ml of ready-to-use proteinase K solution (1X) sufficient for use on 5-10 slides.

Proteinase K solution (1X) was prepared by addition of 0.9 ml deionized or distilled water to 0.1 ml of 10X concentrated proteinase K.

B-Protein block buffer (1X).

This buffer was prepared by dilution 50 ml of 20X concentrated protein block into 1000 ml of distilled water. The resulting 1X protein block buffer was ready to use and may be stored at 4°C.

*Preparation method at appendix (1).

C- Detergent washing buffer (1X).

This buffer was prepared by dilution 50 ml of 20 X-concentrated detergent wash buffers into 1000 ml distilled water. The resulting 1X detergent wash buffer was ready to use and stored at room temperature.

D- Citric Buffer, PH 6.0

This buffer was prepared by dilution 10 ml of 100X-concentrated protein block buffer into 1000ml distilled water. The resulting 1X citric buffer is ready to use and may be stored at room temperature.

E- The working c DNA probe/hybridization solution.

**MMP-2 and MMP-9* .Probes was prepared at dilution 7% by mixing 7µl from the concentrated probe with 93µl of hybridization solution buffer (supplemented by the manufacturer). Placed at 70°C for 5 minutes and then quench in ice bath directly.

**HPV-16, HPV-18, TIMP-1 and TIMP-2* .Probes was prepared at dilution 8% by mixing 8µl from the concentrated probe with 92µl of hybridization solution buffer (supplemented by the manufacturer). Placed in 95°C at 5 minutes for HPV-16 and -18, and 70°C at 5 minutes for TIMP-1 and TIMP-2, then quench in ice directly.

Note: Determination of working probe concentration was made through a number of standardization protocol because there is no remarked dilutions were mentioned by the instructions of the ISH detection kit.

F- Alcohol preparation.

Absolute ethanol was diluted in distilled water to prepare 95 % and 70 % concentrations of alcohol.

G- Control preparation.

Both positive and negative controls were included for each run of *in situ* hybridization.

-**The positive control Probe:** was obtained by replacing the probe with housekeeping gene probe. This was prepared by placing one drop of housekeeping gene into an eppendorf tube. Then it was denaturated at 95˚C for 5 minutes, and ice-quenched immediately.

-**The positive control tissue:** The positive tissue slide was prepared from tissues that were previously known to contain the target marker. They include uterine cervical carcinoma for HPV-16, HPV-18 and oral lichen planus for MMP-2, MMP-9, TIMP1 and TIMP-2.

-**The negative control**. All reagents were added except the diluted probe was replaced by PBS.

-**The tissue negative control**. Normal bladder tissue these tissues show absence of specific staining.

3.3.4-In situ hybridization procedure:-

The procedure of the ISH assay adopted by this study was carried out in accordance with the manufacturer instruction (Maxim Biotech leaflet, 2009). However, it is worthy to mention that there were certain few modifications away from the manufacturer instructions. Any modification will be mentioned accordingly.

3.3.4.1-ISH procedure for the detection of HPV-16 and -18:-

A- Preparation process of tissue slides.

1. Serial tissue sections were cut 4µm thick and each stickled on one specific slide.
2. The slides were baked in a vertical position at 60°C for overnight.
3. The slides with tissues were immersed sequentially in two changes of xylene for 5 minutes each.

B- Rehydration process.

Rehydration process was done by serial dipping the slides in glass staining jars containing the following solution at room temperature for the indicates times.

1. Immersion in two changes of Absolute ethanol for one minute each.
2. Immersion in ethanol (95%) for one minute.
3. Immersion in ethanol (70%) for one minute.
4. Then Distilled water for 5 minutes to remove residual alcohol.
5. After that, slides were allowed to dry completely by incubating them at 37°C for 5 minutes.

C- Digestion process.

1. Twenty µl of freshly diluted 1X proteinase K solution was applied. Slides were incubated at 37°C for 15 minutes.
2. Then the slides were dehydrated by immersing them sequentially in the following solutions at room temperature for the indicated times: distilled water for 1 minute, 70% ethanol for 1 minute, 95% ethanol for 1 minute and 100% ethanol for 1 minute. Lastly, the slides were dried by incubating them at 37°C for 5 minutes.

D- Hybridization procedure.

1. The addition of 20µl of cDNA probe was added to each section and then slides were covered by cover slips be careful to avoid trapping any air bubbles.
2. Denaturation process of the probe and target DNA was done by placing the cover slipped-slides in pre-warmed oven at 95˚C for 8-10 minutes.
3. Hybridization process was allowed to occur by transferring these slides to a pre-warmed humid hybridization chamber in incubate at 37˚C overnight.

E- Hybridization detection process.

Slides were not allowed to dry out at any time during the hybridization and staining. All reagents used during hybridization and detection were warmed to room temperature.

1-Next day, slides were soaked in pre-warmed protein block at 37°C until the cover slips fall off. One should be careful not to tear the tissue.

2-Then the slides were allowed to remain in the buffer for 3 minutes, at 37°C after the cover slips were removed.

3-One to two drops of streptavidin- alkaline phosphatase conjugate reagent were added to tissue sections. Then Slides were kept in a humid chamber at 37°C for 90 minutes. ***Modification:*** the catalogue recommended incubation time is 20 minutes, but this period was not enough practically. Best outcome was obtained at 90 minutes incubation.

4-Slides were rinsed in detergent wash buffer for 5 minutes and then drained.

5-One to two drops of bromo-chlotro-indolyl phosphate/nitro blue tertrazolium substrate-chromogen solution (BCIP/NBT) were placed on tissue section. Slides were incubated at 37 °C for 30 minutes, or until color development was completed. Color development was monitored by viewing the slides under the microscope. A dark blue colored precipitate will form at the site of the probe in positive cells.

6-Slides were rinsed in distilled water or deionized water for 5-10 minutes.

F- Counter staining process.

1. The immersion of the slides in Nuclear Fast Red* stain for 30 second.
2. Washing process was followed by immersion the slides for 1 minute in distilled water.
3. Sections were dehydrated by ethyl alcohol (95%, once for one minute then, 100% twice for 2 minutes each); cleared by Xylene, then mounted with permanent mounting medium (DPX).

G- Evaluation of Results.

The result was obtained within 2 hours by examining the processed slides under light microscope at power 40X and X100 (X4, X10 objectives and X10 eye pieces respectively), a deposition of a soluble blue purple-product at the sites of hybridization of the probes to their targets is a positive indicator for the presence of the target.

*Preparation method at appendix (2).

3.3.4.2-ISH procedure for the detection of MMP-2, MMP-9, TIMP-1and TIMP-2:-

The procedure of the ISH assay adopted by this study was carried out in accordance with the manufacturer instruction (Maxim Biotech leaflet, 2009). Preparation Process of tissue slides, rehydration process and digestion process. A similar steps used in the procedure for HPV-16 and -18.

- Hybridization procedure.

1.Hybridization was performed by placing 10µl of probe onto each tissue section, and then slides were covered by cover slips with avoidance of trapping any air bubbles.

2. Slides were placed in an oven at 70˚C for 8-10 minutes.

3. Slides were placed in humidity chamber and incubated at 37˚C over night to allow hybridization of the probe with the target nucleic acid.

- Hybridization detection process.

In the next day, slides were soaked in (1X) detergent wash at 37˚C until the cover slips fell off.

1. One to two drops (10µl) of RNase A solution were added to each tissue sections and slides were placed in humidity chamber and incubated at 37˚C for 30 minutes.
2. Slides were placed in pre-warmed protein block buffer for 3 minutes at 37˚C. Then slides were drained and blotted.
3. One to two drops (10µl) of streptavidin- alkaline phosphatase conjugate were placed on each tissue section. Slides were placed in humidity chamber, and then incubated for 90 minutes at 37˚C.

4. Slides were rinsed in detergent wash buffer for 5 minutes; they were then drained and bloated.

   One to two drops (10µl) of the substrate- chromogen solution (BCIP/NBT) were placed on each tissue sections. Slides were incubated at 37˚C for 30 minutes, or until the color development was optimal.
5. Slides were rinsed in 2-3 changes of distilled water.
6. One drop (10µl) of NFR was added for 30 seconds as a counter stain.
7. Slides were washed with distilled water.
8. Dehydration of tissue sections was done by serial dipping of slides in glass staining jars containing the following: ethyl alcohol (95%, once for one minute then, 100% twice for 2 minutes each); cleared by Xylene, then mounted with permanent mounting medium (DPX). Then covered with cover slips and left to dry overnight.

In Situ hybridization for MMP-2, MMP-9, TIMP-1 and TIMP-2 was given percentage scores, based on number of cells staining, ISH positive graded as low, intermediate and high. Positive cells were counted in ten different fields for each samples and the average of positive cells of the ten fields was determined assigning cases to one of the three following score categories (Blancato *et al*., 2004).

1. Score-1(low) = 1-25%.
2. Score-2(Intermediate) = 26-50%.
3. Score-3(High) >50%.

3.3.5-Immunohistochemistry procedure for LMP-1 and P35 protein:-

The procedure of the IHC assay adopted by this study was carried out in accordance with the manufacturer instruction Cambridge Science Park. England

A- Deparaffinization and rehydration.

1-Serial thin sectioning of (4µm) thickness was done for each paraffin-embedded tissue block and sticking of each section on charged slides.

2-Paraffin section were deparaffinized in oven at 60°C overnight, and then dipping in glass staining jar containing the following

- Pre-warmed (55°C) xylene for 5 minutes and Xylene (at room temperature 20- 25°C) for 5 minutes.
- Three changes of ethanol (100%, 95% and 70%) for 1minute each and in de-ionized water for 5minute, at room temperature.
- The slides were washed in phosphate buffered saline (PH 7.6) for 5 minutes.

B- Antigen retrieval.

Unmasking antigenic epitopes by retrieval methods is of paramount importance for successful IHC staining and detection of target protein.

1- Immersion slides in citrate buffer pH: 6 at 95-99°C for 15 minutes.

2- Cooling the slides at room temperature (about 25°C) for 20 minutes.

3- Four washes in deionized water or distilled water and then allowing the tissue on slide to be dried for 5 minutes.

C- Quenching endogenous peroxidase.

Enough drops of hydrogen peroxide block were added to slides and incubated at 37°C for 10 minutes. Then socked 2 times in phosphate buffer (5 minutes for each).

D- Protein block.

Enough drops of protein block were added to slides and incubated at 37°C for 5 minutes. Then washed 2 times in phosphate buffer (5 minutes for each).

E- Primary Antibody.

Enough drops of primary antibody were applied to cover slides and incubated for 1hours in humidity chamber at 37°C [Primary Antibody was prepared at dilution 1-50 by mixing 1µl from the concentrated protein with 49µl of PBS]. After that the slides were washed with PBS and socked 2 times in PBS for 5 minutes for each one, then excess liquid was tapped off from the slides and any fluid remain around the slides was wiped with tissue paper.

F- Secondary Antibody.

Enough drops of secondary antibody (link antibody yellow drops) reagent were added and incubated for 10 minutes at room temperature. After that, the slides were washed carefully with PBS and then socked 2 times in phosphate buffer (5 minute for each) finally drained and blotted gently.

G- Streptavidin peroxidase.

Streptavidine-HRP antibodies (red drops) was applied on tissue and incubated for 10 minutes at room temperature. After that, the slides were washed carefully with PBS and then socked 2 times in phosphate buffer (5 minute for each) finally drained and blotted gently.

H- Substrate chromogen solution.

Add 20µl DAB chromogen to 1ml of DAB substrate, mix by swirling and apply to tissues (this process was done in dark room), then the slides were incubated for 10 minutes within humidity chamber at 37°C. Then slides washed carefully with PBS for 5 minutes and then drained and blotted gently.

I- Hematoxylin counter stain.

One hindered microliter of counter stain hematoxylin was placed onto the section and incubated for 1 minute at room temperature. Slides were washed with tap water and dehydrated by placing them in Ethanol then xylene in the following order (70% ethanol, 95% ethanol, and absolute ethanol) for one minute each. Then Xylene for 5 minutes.

j- Mounting medium and cover slipping.

Using DPX, a drop or more was placed onto the xylene-wetted slides and the section was quickly covered with a cover slip. Slides were let to dry. Then slides were examined by pathologist by light microscope at X400 magnification. Immunoassaying was scored according (Kraggerud *et al.*, 1997). [Score 1, less than 10 %, Score 2, more than 10 % and less than 50% and Score 3, more than 50 %].

K-Quality control

In each run of immunhistochmestry, two control slides were employed. First one is known to be strongly positive for the target Ag. (Gastric adenocarcinoma for LMP-1 and oral squamous cell carcinoma for p53), while the second; the negative control was used by adding 20 µl of PBS instead of diluted probe. The two control tissue sections were necessary to keep fidelity in terms of specificity and sensitivity.

3.4-Statistical analysis:-

Statistical analysis were conducted to describe different variables and parameters in this research and to describe relationships with each other as well.

1- t- test was used to find out the significant of differences between the age of patients and health control group.

2- Chi-square and Fischer exact test was used to find out the effect of different patients criteria on the reading of each marker of *in situ* hybridization and immunohistochemistry.

Chapter Four

Results

Results

4.1-Clinical findings:-

4.1.1-Distribution of patients with transitional cell carcinoma of the bladder and the healthy control group according to age strata:-

The mean age of the patients with transitional cell carcinoma of the bladder was 57.30 years when comparing with healthy control group was 54.70 years as shows in Table (4.1), there was no significant differences (P>0.05) noticed between both groups.

In the present study it was observed that transitional cell carcinoma of the bladder percentage was increased with the increasing age (Table 4.2).

Table (4.1): Distribution of mean age (years) among the studied groups (TCC of the bladder patients and healthy control group)according to age strata.

studied groups	N	Mean	Std. Deviation	Mini.	Maxi.	Student (t-test)	
						P-value	Sig.
Healthy Control group	10	54.70	10.55	38	70	0.512	Non Sig. (P>0.05)
Transitional cell carcinoma of the bladder	50	57.30	11.53	25	70		
Total							

Table (4.2): Distribution of transitional cell carcinoma of the bladder patients according to their age strata.

Age stratum (Year)	No.	%	Comparison of Significance	
			Chi^2- value	Sig.
25-39	4	8 %	0.00	Highly Sig. (P<0.01)
40-54	11	22 %		
55-70	35	70 %		
Total	50	100 %		

4.1.2-Distribution of patients with transitional cell carcinoma of the bladder according to their gender:-

The percentage of males was 70 % (35 out of 50) which more than females 30% (15 out of 50) in transitional cell carcinoma of the bladder. The statistical analysis showed significant differences (P<0.05) between both of them, but no-significant differences was detected when comparing with the healthy control group (P>0.05). Table (4.3).

Table (4.3): Distribution of transitional cell carcinoma of the bladder patients according to their gender.

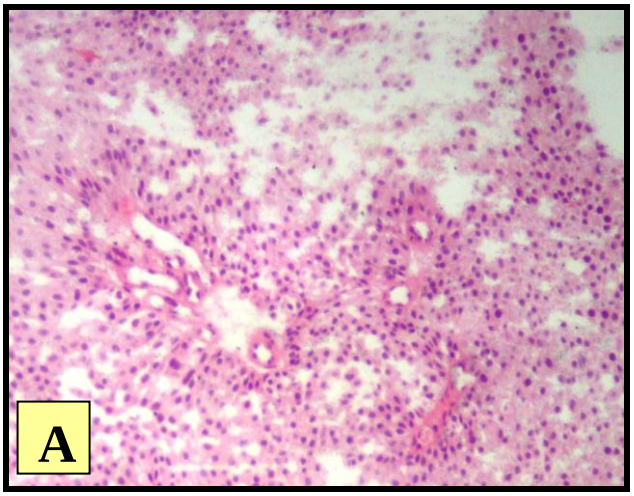
Studied groups		Gender		Comparison of significant	
		Male	Female	Chi^2-value	Sig.
TCC	N %	35 (70 %)	15 (30 %)	0.22	Non- Sig. (P>0.05)
Healthy Control group	N %	5 (50 %)	5 (50 %)		
Total	N %	40 (66.7 %)	20 (33.3 %)		

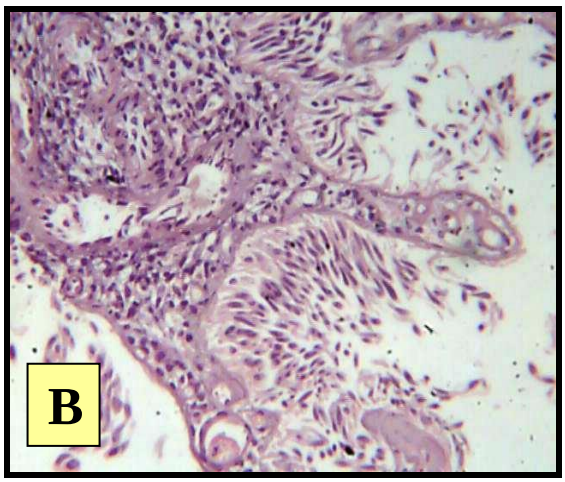
4.1.3-Distribution of patients with transitional cell carcinoma of the bladder according to the tumor grade/differentiation:-

According to the histopathological results of the patient's tumor, it found that 4 patients (8%) had well-differentiation or grade I and 31(62%) had moderately-differentiation or grade II while poorly-differentiation or grade III, included 15 patients (30%) and the moderately-differentiation group was the most common (grade II). However statistical significant differences were found among them ($p<0.01$) as shows in Table (4.4) and Figure (4.1).

Table (4.4): Distribution of transitional cell carcinoma of the bladder patients according to their grade/differentiation.

Transitional cell carcinoma grade/ differentiated	N	%	Comparison of Significance	
			Chi^2-value	Sig.
Well-differentiated	4	8 %	0.00	Highly Sig. (P<0.01)
Moderately-differentiated	31	62 %		
Poorly-differentiated	15	30 %		
Total	50	100 %		





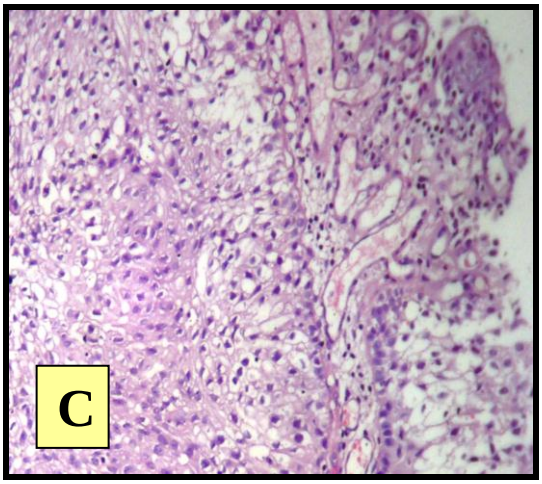


Figure (4.1): Sections from tissue with transitional cell carcinoma of the bladder staining by Hematoxylin and Eosin ((Magnification power, 10X). (A) Well-differentiated. (B) Moderately-differentiated. (C) Poorly-differentiated.

4.1.4-Distribution of patients with transitional cell carcinoma of the bladder according to the Pattern of growth and presence or absence of muscle invasion cancer:-

The pattern of growth for the total number of transitional cell carcinoma of the bladder patients is illustrated in Table (4.5), which revealed that 28 patients out of 50 (56%) had papillary type and 22 out of 50 (44%) had solid type. Statistical differences were observed between the two patterns. On the other hand invasion of muscle tissue showed that 26 patients out of 50 (52%) was invasive, where as 24 out of 50 (48%) was non invasive. However there is statistical significant differences noticed between both of them (P<0.01).

Table (4.5): Distribution of patients with transitional cell carcinoma of the bladder according to the Pattern of growth and muscle invasion of cancer.

Pathological criteria		Results		Comparison of significant	
		N	%	Chi^2-value	Sig.
Pattern of growth	Papillary	28	56 %	0.00	Highly Sig. (P<0.01)
	Solid	22	44 %		
Muscle invasion	Invasive	26	52%	0.00	Highly Sig. (P<0.01)
	Non invasive	24	48 %		

4.2-Viral associated tumor:-

4.2.1-Detection of LMP-1 in patients with transitional cell carcinoma of the bladder by using immunohistochemistry technique:-

It was the first time that LMP-1 had been detected by the IHC technique in bladder cancers in Iraqi patients. This molecular technique is very sensitive for detection of any protein. The results shown in Table (4.6) and Figure (4.2) demonstrated that 22 patients out of 50 (44 %) of bladder carcinoma cases were positive for LMP-1, while 28 patients out of 50 (56 %) were negative. on the other hand 8 cases (36.4 %) were of low score and 7 cases (31.8 %) were of Intermediate score while 7 cases (31.8 %) were of high score.

A statistically significant differences was found between the patients with transitional cell carcinoma of the bladder and healthy control group ($p<0.01$).

Table (4.6): The percentage of LMP-1 IHC-detection tests in the studied groups (Healthy control group and transitional cell carcinoma of the bladder).

LMP-1 IHC tests results		Healthy Control group	Transitional cell carcinoma of the bladder	Comparison of Significance	
				P-value	Sig.
Positive	Low	0	8	0.00	Highly Sig. (P<0.01)
	Intermediate	0	7		
	High	0	7		
	Total	0	22 (44%)		
Negative	N %	10	28 (56 %)		
Total	N %	10 (100 %)	50 (100 %)		

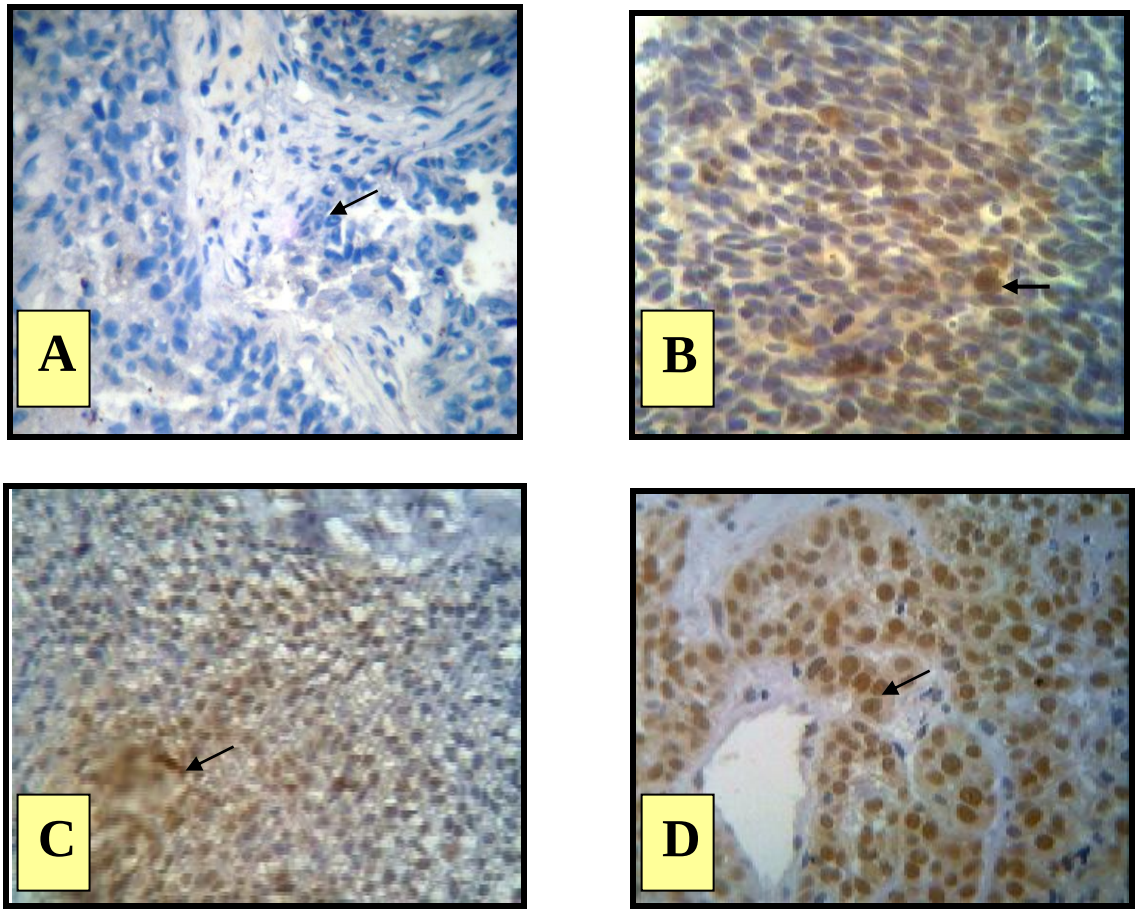


Figure (4.2): Immunohistochemical staining of LMP-1 in bladder tumor section stained by DAB chromogen (brown) and counter stained with heamatoxylin (Magnification power, 400), A- Moderately differentiated TCC, LMP-1 negative expression B- Moderately differentiated TCC, low LMP-1 positive expression, C- Moderately differentiated TCC, intermediate LMP-1 positive expression, D- Poorly differentiated, High LMP- 1 positive expression.

4.2.2-Relation between positive and negative immunohistichemical expression of LMP-1 with age in cases of transitional cell carcinoma of the bladder:-

The age of patients with positive LMP-1 results had ranged between 25 to 70 years as shown in Table (4.7). In the 25-39 years-age group with transitional cell carcinoma of the bladder was 4.5% (1 case out of 22), in 40-54 years-age group with transitional cell carcinoma of the bladder was 9.1% (2 cases out of 22) while in the other age groups was 86.4 % (19 cases out of 22). However there was no statistical significant differences noticed among them (P>0.05).

Table (4.7): Distribution of patients with LMP-1 positive and negative in different age groups.

LMP-1 IHC tests results		Age stratum (Year)			Total	Comparison of Significance	
		25-39	40-54	55-70		Chi^2-value	Sig.
Positive LMP-1	N	1	2	19	22	0.07	Non Sig. (P>0.05)
	%	4.5 %	9.1 %	86.4 %	100 %		
Negative LMP-1	N	3	9	16	28		
	%	10.8 %	32.1 %	57.1 %	100 %		

4.2.3-Distribution of transitional cell carcinoma of the bladder with LMP-1 positive and negative according to the gender and tumor grade/differentiation:-

The distribution of gender had revealed that higher percentage of LMP-1 positive cases was detected in males 72.7% (16 out of 22) other than females 27.3% (6 out of 22). However there was no statistical significant differences noticed between both of them (P>0.05) when comparing these results with their LMP-1 negative detection.

Table (4.8) showed that most our LMP-1 positive and negative transitional cell carcinoma of the bladder cases occurred within grade II and grade III. However the difference failed to reach the level of statistical significances (P>0.05).

Table (4.8): The distribution of LMP-1 IHC results according to the gender and tumor grade/differentiation in patients with TCC of the bladder.

LMP-1 IHC tests results		Gender		Grade			Comparison of significant	
		Male	Female	I	II	III	Chi^2-value	Sig.
Positive LMP-1	N	16	6	2	11	9	0.07 ✯ 0.28	Non Sig. (P>0.05)
	%	72.7%	27.3%	9.1%	50%	40.9%		
Negative LMP-1	N	19	9	2	20	6		
	%	67.9%	32.1%	7.2%	71.4%	21.4%		
Total	N	35	15	4	31	15		
	%	70%	30%	8%	62%	30%		

✯**For gender**

4.2.4-Distribution of transitional cell carcinoma of the bladder with LMP-1 positive and negative cases according to the pattern of growth and muscle invasion:-

The results of IHC were shown in Table (4.9) which demonstrated that 9 positive cases out of 22 (40.9%) of transitional cell carcinoma of the bladder cases were papillary type while 13 positive cases out of 22 of transitional cell carcinoma of the bladder were solid type. On the other hand divided the positive sample according to invasion revealed that 13 cases (59.1%) was invasive, while 9 cases (40.9%) non-invasive. Statistically, these results expressed non-significant differences (P>0.05).

Table (4.9): The distribution of LMP-1 IHC results in relation to pattern of growth and muscle invasive in patients with transitional cell carcinoma of the bladder.

Pathological criteria		pattern of growth		Comparison of significant		Muscle invasion		Comparison of significant	
		papillary	solid	Chi^2-value	Sig.	Invasive	Non invasive	Chi^2-value	Sig.
Positive LMP-1	N %	9 (40.9%)	13 (59.1%)	0.05	Non Sig. (P>0.05)	13 (59.1%)	9 (40.9%)	0.16	Non Sig. (P>0.05)
Negative LMP-1	N %	19 (67.9%)	9 (32.1%)			11 (39.3%)	17 (60.7%)		
Total	N %	28 (56%)	22 (44%)			24 (48%)	26 (52%)		

4.2.5-Detection of HPV16 and 18 DNA in transitional cell carcinoma of the bladder by using in situ hybridization technique:-

The results of ISH which demonstrated that 18 out of 50 (36%) with transitional cell carcinoma of the bladder cases were positive for HPV-16 DNA while 7 out of 50 (14%) with transitional cell carcinoma of the bladder cases were positive for HPV-18 DNA. Human papillomavirus16 and 18 DNA was not detected in healthy control group. However the statistical analysis of the distribution of positive results which demonstrated that significant differences as shown in (Table 4.10 and Figure 4.3).

Table (4.10): The percentage of HPV16 and 18 DNA ISH-detection tests in the studied groups.

HPV DNA-ISH reaction results		Studied groups			Comparison of Significance	
			Transitional cell carcinoma of the bladder	Healthy Control	p-value	Sig.
Type 16HPV	Positive	N %	18 (36 %)	0	0.00	Highly Sig. (P<0.01)
	Negative	N %	32 (64 %)	10		
	Total	N %	50 (100%)	10 (100%)		
Type 18 HPV	Positive	N %	7 (14 %)	0	0.00	Highly Sig. (P<0.01)
	Negative	N %	43 (86 %)	10		
	Total	N %	50 (100 %)	10 (100%)		

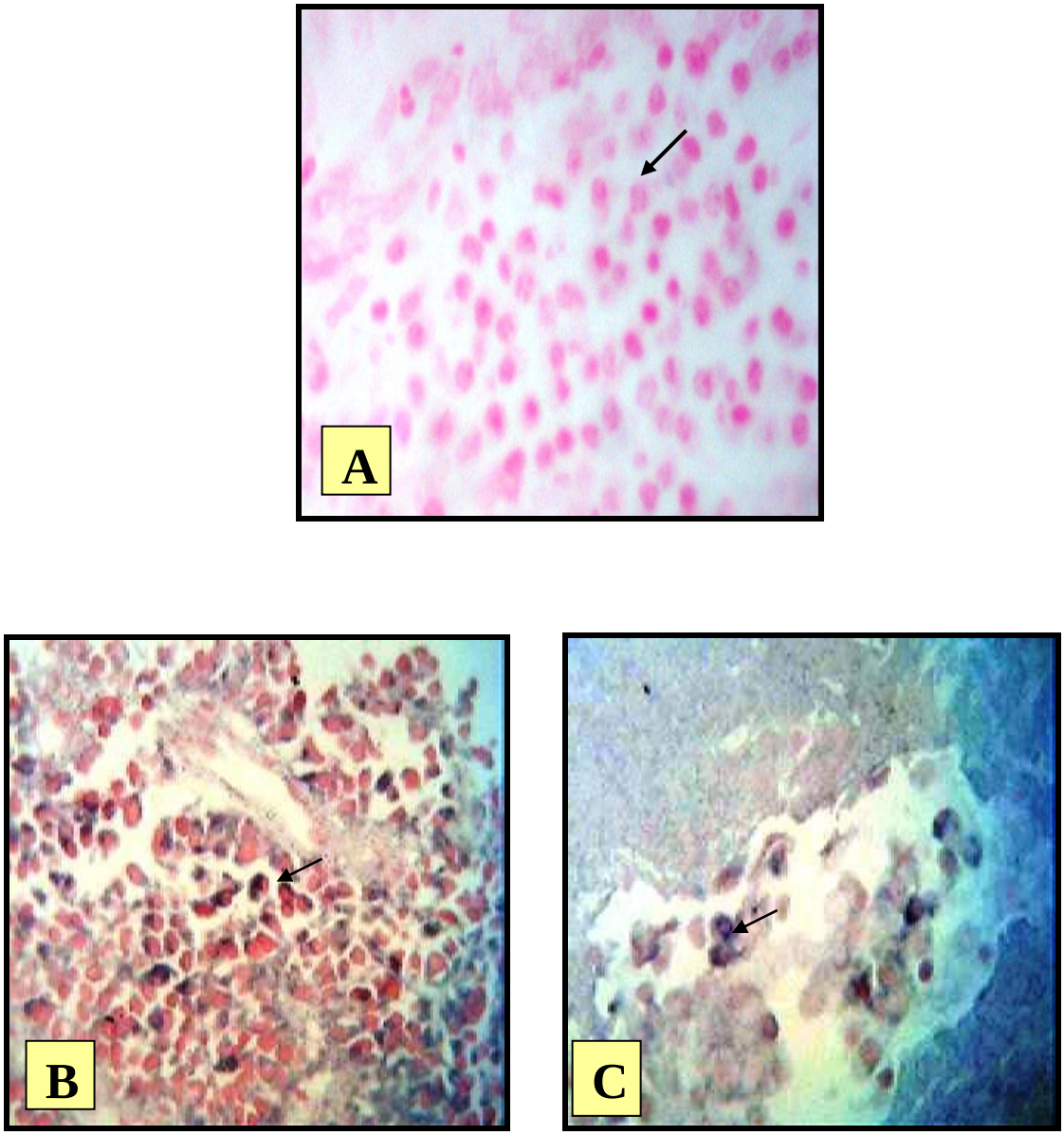


Figure (4.3): ***In situ*** **hybridization for detection HPV 16 and HPV 18 DNA in bladder tumor section, stained by BCIP/NBT-chromogen and counter stained with nuclear fast red (NFR) as dark-blue of the nuclei in positive cases (Magnification power, 400). A- Moderately differentiated TCC, HPV-DNA negative expression, B- Moderately differentiated TCC, HPV-16 DNA positive expression, C- Moderately differentiated TCC, HPV-18 DNA positive expression.**

4.2.6-Relation between positive and negative ISH expression of HPV16 and 18 DNA with age in cases of transitional cell carcinoma of the bladder:-

Fifty patients with transitional cell carcinoma of the bladder were presented in this study (Table 4.11). The age group of those patients mostly affected with TCC of bladder was the age group of 55-70 years (77.8%), (57.1%) for HPV16 and 18 respectively, followed by the age group of 40-54 years (11.1%), (28.6%) respectively, and the lastly affected group was the 25-45 years(11.1%), (14.3%) respectively. Statistical comparison of these age groups was revealed no significant differences among them (P>0.05).

Table (4.11): Distribution of patients with HPV16 and 18-DNA positive and negative in different age groups.

HPV-DNA ISH tests results		Age stratum (Year)			Total	Comparison of Significance	
		25-39	40-54	55-70		Chi^2-value	Sig.
Positive HPV-16 DNA	N %	2 (11.1%)	2 (11.1%)	14 (77.8%)	18 (100%)	0.35	Non Sig. (P>0.05)
Negative HPV-16 DNA	N %	2 (6.3%)	9 (28.1%)	21 (65.6%)	32 (100%)		
Positive HPV- 18 DNA	N %	1 (14.3%)	2 (28.6%)	4 (57.1%)	7 (100%)	0.68	Non Sig. (P>0.05)
Negative HPV-18 DNA	N %	3 (7%)	9 (20.9%)	31 (72.1%)	43 (100%)		

4.2.7-Distribution of transitional cell carcinoma of the bladder with HPV16 and 18 DNA positive and negative according to the gender and tumor grade/differentiation:-

The relationship between HPV16 and 18 DNA and gender demonstrated that the males 12(66.7%), 6(85.7%) was higher than females 6(33.3%), 1(14.3%) respectively, but there was no statistically significant recorded. Regarding the tumor grade. The percentage of positive ISH reaction in the well differentiated group was 11.1% (2 cases) and no case in HPV18, with moderate differentiated group showed positive ISH reaction the percentage of these tissues was 50% (9 cases) and 57.1% (4 cases) respectively. Lastly, the percentage of these tissues that had poor differentiation and showed positive ISH reaction was 38.9% (7cases) and 42.9% (3 cases) respectively. Statistical analysis showed no significant differences among them (P>0.05).Table (4.12).

Table (4.12): The distribution of HPV-DNA ISH results according to the gender and tumor grade/differentiation in patients with transitional cell carcinoma of the bladder.

HPV –DNA ISH tests results		Gender		Grade			Comparison of significant	
		Male	Female	I	II	III	Chi^2-value	Sig.
Positive HPV16 DNA	N %	12 (66.7%)	6 (33.3%)	2 (11.1%)	9 (50%)	7 38.9%)	0.70 ✯ 0.42	Non Sig. (P>0.05)
Negative HPV 16 DNA	N %	23 (71.9%)	9 (28.1%)	2 (6.3%)	22 (68.8%)	8 (25%)		
Total	N %	35 (70%)	15 (30%)	4 (8%)	31 (62%)	15 30%)		
Positive HPV18 DNA	N %	6 (85.7%)	1 (14.3%)	0	4 (57.1%)	3 (42.9%)	0.32 ✯ 0.56	Non Sig. (P>0.05)
Negative HPV18 DNA	N %	29 (67.4%)	14 (32.6%)	4 (9.3%)	27 (62.8%)	12 (27.9%)		
Total	N %	35 (70%)	15 (30%)	4 (8%)	31 (62%)	15 (30%)		

✯**For gender**

4.2.8-Distribution of transitional cell carcinoma of the bladder with HPV 16 and 18 DNA positive and negative according to the pattern of growth and muscle invasion:-

Positive results were recorded among patients in the papillary type which present in 8 and 3 cases for HPV16 and 18 respectively while in the solid type were present in 10 and 4 cases respectively. Moreover, muscle invasive were seen in 11 cases (61.1%) and 3 cases (42.9%), while non-invasive were seen in 7cases (38.9%) and 4 cases (57.1%). Statistically the distribution of HPV16 and 18 DNA according to the pattern of growth and muscle invasion showed no significant differences among them (P>0.05) as shown in Table (4.13).

Table (4.13): The distribution of HPV16 and 18 DNA ISH results in relation to pattern of growth and muscle invasive in patients with transitional cell carcinoma of the bladder.

HPV –DNA ISH tests results		pattern of growth		muscle invasion		Comparison of significant	
		papillary	solid	invasive	Non invasive	Chi^2-value	Sig.
Positive HPV 16 DNA	N %	8 (44.4%)	10 (55.6%)	11 (61.1%)	7 (38.9%)	0.21 ✯ 0.16	Non Sig. (P>0.05)
Negative HPV 16 DNA	N %	20 (62.5%)	12 (37.5%)	13 (40.6%)	19 (59.4%)		
Total	N %	28 (56%)	22 (44%)	24 (48%)	26 (52%)		
Positive HPV 18 DNA	N %	3 (42.9%)	4 (57.1%)	3 (42.9%)	4 (57.1)	0.45 ✯ 0.76	Non Sig. (P>0.05)
Negative HPV 18 DNA	N %	25 (58.1%)	18 (41.9%)	21 (48.8%)	22 (51.2%)		
Total	N %	28 (56%)	22 (44%)	24 (48%)	26 (52%)		

✯**For pattern of growth**

4.3-P53 Over expression:-

4.3.1-Detection of P53 over expression in transitional cell carcinoma of the bladder by using immunohistochemistry technique:-

Immunohistochemistry staining for p53 protein showed that 23 (46%) patients were positive cases and 27 (54%) patients were negative cases in transitional cell carcinoma of the bladder patients. Most of the positive cases were of low score 10 (43.4%), while 9 (39.1%) intermediate and 4 (17.3%) high. No P53 over expression was detected in any of the cases of healthy control group. The difference between the percentage of p53 protein in each of specimens are statistically highly significant (P<0.01) as illustrated in (Table 4.14 and Figure 4.4) but no significant correlation between each score and any patients was observed.

Table (4.14): The percentage of the P53 over expression tests results in the studied groups.

Mutated P53 over expression tests results		Healthy Control group	Transitional cell carcinoma of the bladder	Comparison of Significance	
				p-value	Sig.
Positive p53 IHC	Low	0	10	0.00	Highly Sig. (P<0.01)
	Intermediate	0	9		
	High	0	4		
	Total	0	23 (46 %)		
Negative p53 IHC	N %	10 (100%)	27 (54%)		
Total	N %	10 (100 %)	50 (100%)		

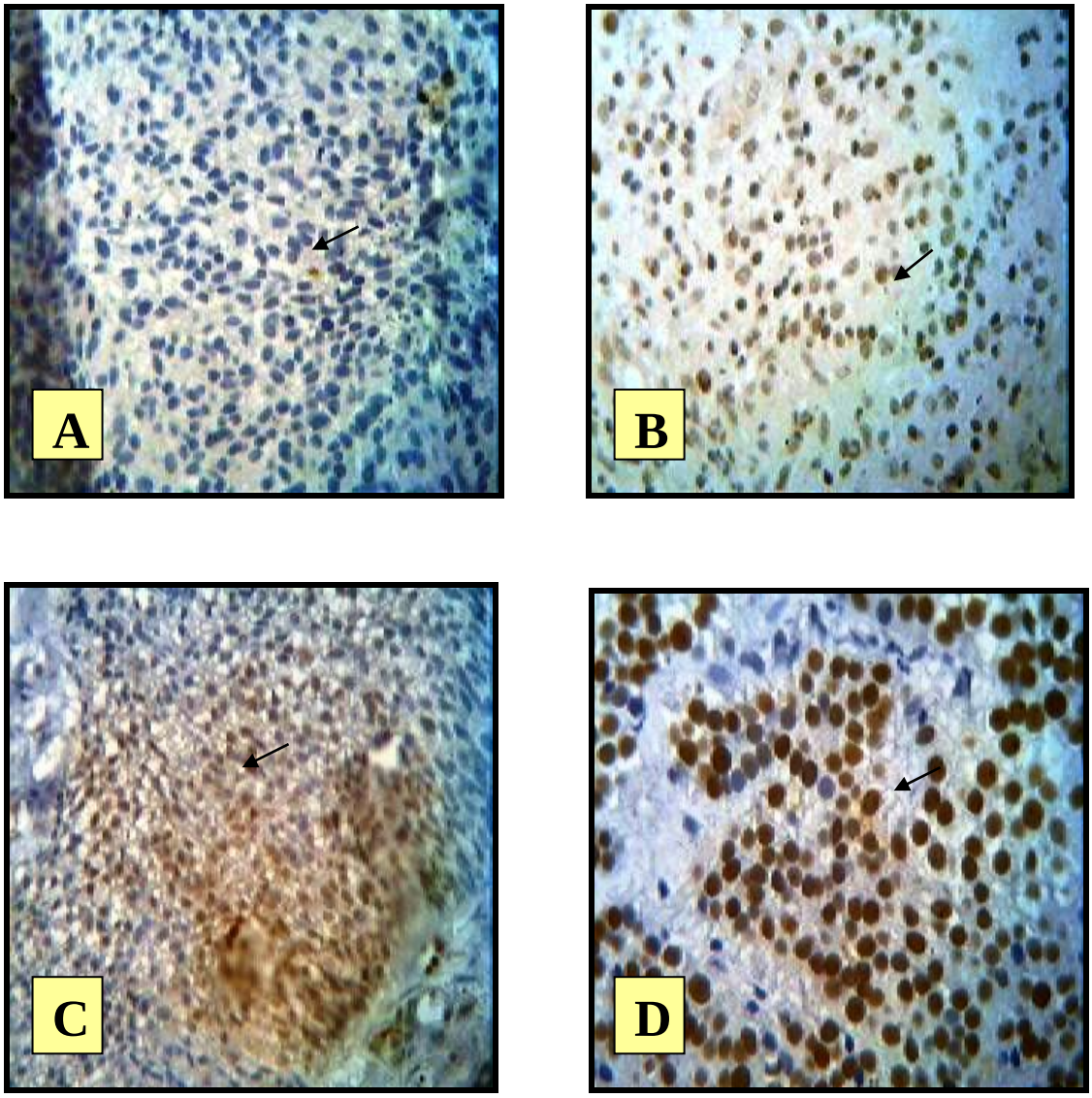


Figure (4.4): Immunostaining for p53 protein stained by DAB chromogen and counter stained with heamatoxylin showing radish brown granules in nuclei of transitional cell carcinoma of the bladder patients in positive cases (Magnification power, 400). A- Moderately differentiated TCC, negative expression, B- Moderately differentiated TCC, low P53 positive expression, C- Poorly differentiated TCC, intermediate P53 positive expression, D- Poorly differentiated TCC, high P53 positive expression.

4.3.2- Age distribution in patients with transitional cell carcinoma of the bladder and P53 over expression:-

Immunohistochemistry for P53 protein detection was carried out on all samples, and as shown in Table (4.15). One case (4.3%) showed positive P53 expression in age group (25-39) years, where six cases (26.1%) were recorded positive in age group (40-54) years. while age group (55-70) showed positive in 16 cases (69.6%). However there is no statistical significant differences noticed among them (P>0.05).

Table (4.15): The distribution of P53 over expression test results according to age group (Year) of the patients with transitional cell carcinoma of the bladder.

Mutated P53 Over expression tests results		Age stratum (Year)			Total	Comparison of Significance	
		25-39	40-54	55-70		Chi^2-value	Sig.
Positive p53 IHC	N	1	6	16	23	0.59	Non Sig. (P>0.05)
	%	4.3 %	26.1 %	69.6 %	46 %		
Negative p53 IHC	N	3	5	19	27		
	%	11.1 %	18.5 %	70.4 %	54 %		

4.3.3-Gender and grade distribution in patients with transitional cell carcinoma of the bladder and P53 over expression:-

Table (4.16) demonstrated the frequency of P53 IHC over expression in transitional cell carcinoma of the bladder patients. Seventeen out of 23 (73.9%) cases were males and 6 out of 23 (26.1%) were females in P53 positive cases ,while in P53 negative cases 18 out of 23 (66.7%) were males and 9 (33.3%) were females. In relation to tumor grade distribution, all our P53 positive and negative transitional cell carcinoma of the bladder cases occurred within grade II and III, of these two patients had well differentiation, 14 had moderately differentiation and 7 had poorly differentiation bladder cancer. The result of frequency distribution of p53 score showed no significant correlation between each score and degree of tumor differentiation (P>0.05). However positive expression was seen in relation with loss of differentiation.

Table (4.16): The distribution of P53 over expression tests results according to gender and tumor grade/ differentiation of transitional cell carcinoma of the bladder.

Mutated P53 over expression tests results		Transitional cell carcinoma of the bladder/ tumor grade			Gender		Comparison of Significance	
		Well	Moderately	Poor	Male	Female	Chi^2-value	Sig.
Positive p53 IHC	N %	2 (8.6 %)	14 (60.8 %)	7 (30.4%)	17 (73.9%)	6 (26.11)	0.98*	Non Sig. (P>0.05)
Negative p53 IHC	N %	2 (7.4%)	17 (63%)	8 (29.6%)	18 (66.7%)	9 (33.3%)	0.57	

***For grade**

4.3.4-pattern of growth and muscle invasion distribution in patients with transitional cell carcinoma of the bladder and P53 over expression:-

Tumor suppressor protein positive expression was detected in 14 papillary and 9 solid types. Concerning the muscle invasion, p53 positive expression was demonstrated in 14 muscle and 9 non-muscle invaded cases. No significant correlation was found between p53 expression and pattern of growth as shown in (Table 4.17 and 4.18).

Table (4.17): The distribution of patients with transitional cell carcinoma of the bladder of P53 positive and negative results according pattern of growth.

Mutated P53 over expression tests results		pattern of growth		Comparison of significant	
		papillary	solid	Chi^2-value	Sig.
Positive p53	N %	14 (60.9%)	9 (39.1%)	0.52	Non Sig. (P>0.05)
Negative p53	N %	14 (51.9%)	13 (48.1%)		
Total	N %	28 (56%)	22 (44%)		

Table (4.18): The distribution of patients with transitional cell carcinoma of the bladder of P53 positive and negative results according muscle invasion.

Mutated P53 over expression tests results		Muscle invasion		Comparison of significant	
		Invasive	Non invasive	Chi^2-value	Sig.
Positive p53	N %	14 (60.9%)	9 (39.1%)	0.09	Non Sig. (P>0.05)
Negative p53	N %	10 (37%)	17 (63%)		
Total	N %	24 (48%)	26 (52%)		

4.3.5-Association between P53 over expression and LMP-1 in patients with transitional cell carcinoma of the bladder:-

The correlation between P53 over expression and prevalence of LMP-1 in patients with transitional cell carcinoma of the bladder were investigated, as causal factors for bladder carcinomas. The results of p53 expression and LMP-1 were presented in Table (4.19). Which revealed that P53-IHC reaction was found in most cases that showed negative LMP-1 IHC reaction cases. Statistically there was no significant association between P53 over expression and LMP-1 in patients with transitional cell carcinoma of the bladder.

Table (4.19): The relationship between the mutated P53 over expression tests results and LMP-1 tests results.

LMP-1 IHC reaction results			P53-Immuno-histochemical assay		Comparison of Significance	
			Positive	Negative	Chi^2-value	Sig.
LMP-1	Positive	N %	11(47.8%)	11(40.7%)	0.61	Non Sig. (P>0.05)
	Negative	N %	12 (52.2%)	16 (59.3%)		
	Total	N %	23 (100%)	27(100%)		

4.3.6-Association between P53 over expression and HPV16 and 18 DNA in patients with transitional cell carcinoma of the bladder:-

Table (4.20) demonstrated P53 expression in transitional cell carcinoma of the bladder against genome of HPV DNA 16 and 18. Fourteen of the 18 HPV16 positive transitional cell carcinoma of the bladder (60.9%), assessed by ISH, and gave positive p53 immunostaining. These results showed a strong association between over expression of P53 protein and TCC with HPV-16 while no association occur between over expression of P53 protein and TCC with HPV-18.

Table (4.20): The relationship between the mutated P53 over expression tests results and HPV16 and 18-DNA tests results.

HPV DNA-ISH reaction results			P53-Immuno-histochemical assay		Comparison of Significance	
			Positive	Negative	Chi^2-value	Sig.
Type 16 HPV	Positive	N %	14(60.9%)	4(14. 8%)	0.00	Highly Sig. (P<0.01)
	Negative	N %	9(39.1%)	23(85.2%)		
	Total	N	23	27		
		%	100%	100%		
Type 18 HPV	Positive	N %	5 (21.7%)	2 (7.4%)	0.14	Non Sig. (P>0.05)
	Negative	N %	18 (78.3%)	25 (92.6%)		
	Total	N	23	27		
		%	100 %	100 %		

4.4-Matrixmetalloproteinases and their inhibitors:-

4.4.1-MMP-2 and MMP-9 In situ hybridization expression in transitional cell carcinoma of the bladder:-

The results showed in Table (4.21) and Figure (4.5),(4.6) which were demonstrated that 32 cases (64%) of bladder carcinoma cases were positive for both of them, while 18 cases (36%) cases were negative for both of them. On the other hand statistical analysis were demonstrated a highly significant differences in MMP-2 and MMP-9 expression among patients with transitional cell carcinoma of the bladder when compared with healthy control group.

Table (4.21): The expression of MMP-2 and MMP-9 in patients with transitional cell carcinoma of the bladder.

Result of MMP expression			MMP-2 Expression	MMP-9 Expression	Comparison of Significance	
					p-value	Sig.
Patients	Positive	Low	10	19	0.01	Highly Sig. (P<0.01)
		Intermediate	17	10		
		High	5	3		
		Total	32 (64 %)	32 (64 %)		
	Negative	N %	18(36%)	18(36%)		
	Total	N %	50 (100%)	50 (100%)		
control	Positive	N %	0	0		
	Negative	N %	10(100%)	10 (100%)		

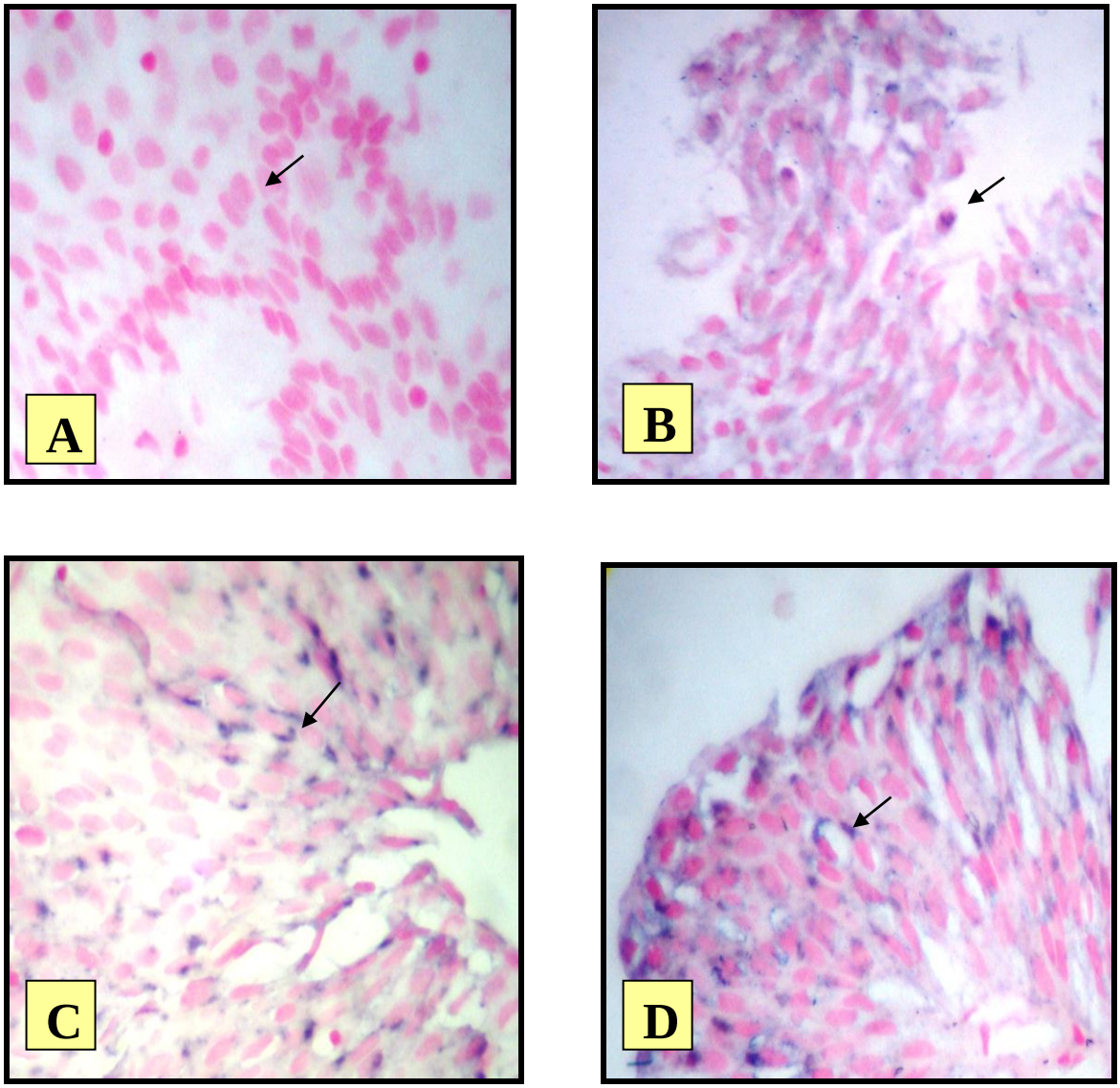


Figure (4.5): *In situ* hybridization for MMP-2 of patient with transitional cell carcinoma of the bladder, stained by BCIP/NBT-chromogen and counter stained with NFR as dark-bluish purple in positive cases (magnification power, 400), A- Moderately differentiated TCC, negative expression, B- Well differentiated TCC, low MMP-2 positive expression, C- Moderately differentiated TCC, intermediate MMP-2 positive expression, D- Well differentiated TCC, high MMP-2 positive expression,

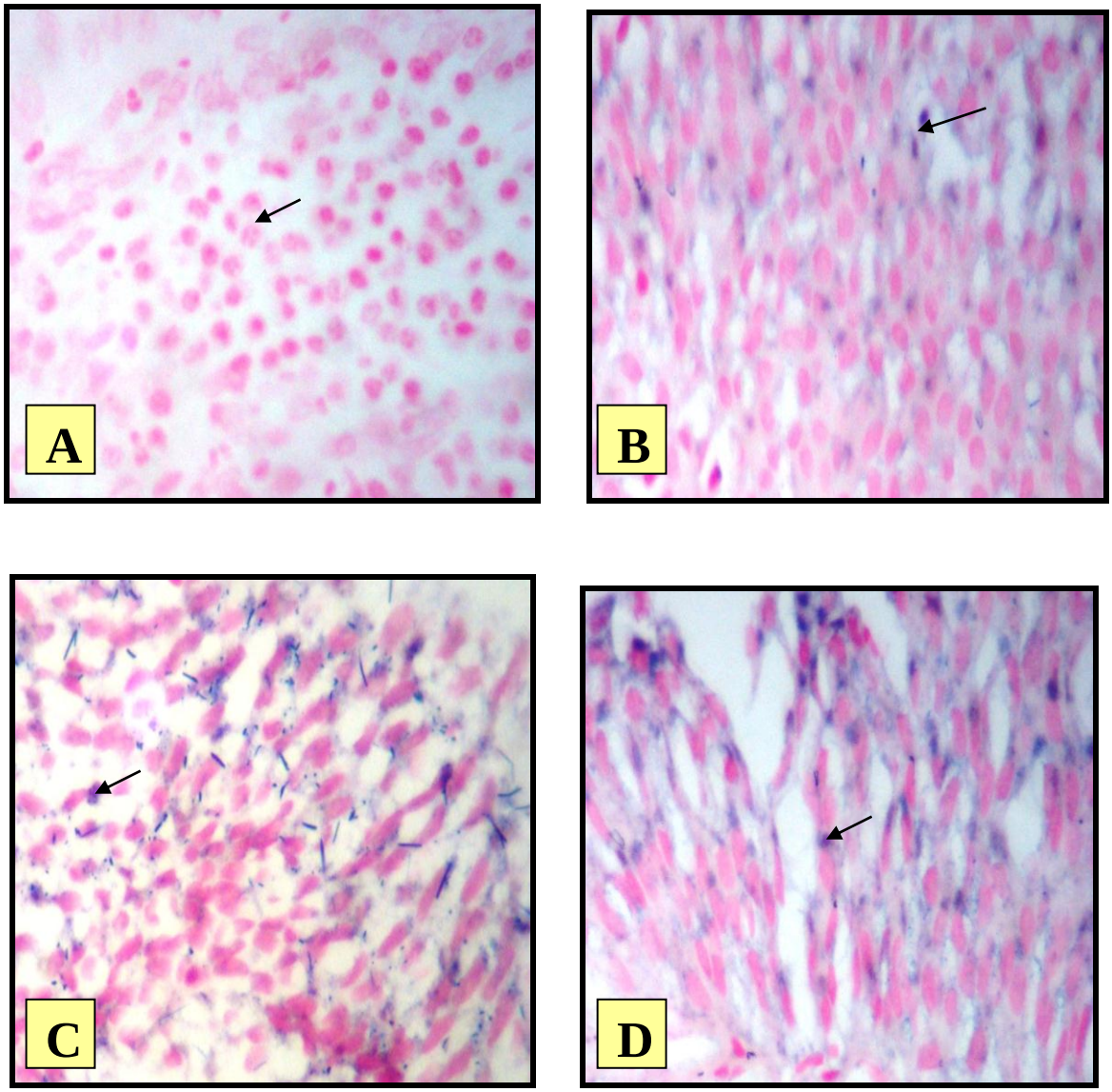


Figure (4.6): *In situ* hybridization for MMP-9 of patient with transitional cell carcinoma of the bladder, stained by BCIP/NBT-chromogen and counter stained with NFR as dark-bluish purple in positive cases (magnification power, 400), A- Moderately differentiated TCC, negative expression, B- Moderately differentiated TCC, low MMP-9 positive expression, C- Moderately differentiated TCC, intermediate MMP-9 positive expression, D- Moderately differentiated TCC, high MMP-9 positive expression .

4.4.2- Correlation between MMP-2 and MMP-9 expression with clinical parameters:-

Table (4.22) and (4.23) demonstrated the correlation between expression of MMP-2 score and MMP-9 score, with different variables. Results showed that there were no significant differences between *in situ* hybridization expression of both MMP-2 and MMP-9 with age, gender, grade, pattern of growth, and muscle invasive. The results of frequency distribution of positive and negative MMP-2 and -9 showed significant correlations between MMP-2 expression and pattern of growth while no correlation occur with MMP-9 based on Chi-square test of analysis (Table 4.24 and 4.25).

Table (4.22): Correlation of MMP-2 scores and related with clinical parameters.

Variables		MMP-2 scores			Comparison of Significance	
		Low	Intermediate	High	Chi^2-value	Sig.
Age	25-39	1(10%)	0	1(20%)	0.48	Non Sig. (P>0.05)
	40-54	1(10%)	3 (17.6%)	1(20%)		
	55-70	8(80%)	14 (82.4%)	3(60%)		
Gender	Male	8(80%)	11(64.7%)	2(40%)	0.33	Non Sig. (P>0.05)
	Female	2(20%)	6 (35.3%)	3(60%)		
Tumor grade	I	0	1 (5.6%)	0	0.13	Non Sig. (P>0.05)
	II	7 (70%)	11(64.7%)	1(20%)		
	III	3 (30%)	5 (29.4%)	4(80%)		
Pattern of growth	Papillary	5(50%)	8 (47.1%)	1(20%)	0.07	Non Sig. (P>0.05)
	Solid	5(50%)	9 (52.9%)	4(80%)		
Muscle invasion	Invasive	4(40%)	10 (58.8%)	4(80%)	0.19	Non Sig. (P>0.05)
	Non invasive	6(60%)	7 (41.2%)	1(20%)		

Table (4.23): Correlation of MMP-9 scores and related with clinical parameters.

Variables		MMP-9 scores			Comparison of Significance	
		Low	Intermediate	High	Chi^2-value	Sig.
Age	25-39	0	1(10%)	1(33.3%)	0.37	Non Sig. (P>0.05)
	40-54	4(21.1%)	1(10%)	1(33.3%)		
	55-70	15(78.9%)	8(80%)	1(33.3%)		
Gender	Male	14(73.7%)	9(90%)	2(66.7%)	0.28	Non Sig. (P>0.05)
	Female	5(26.3%)	1(10%)	1(33.3%)		
Tumor grade	I	3(15.8%)	0	0	0.50	Non Sig. (P>0.05)
	II	12(63.3%)	6(60%)	1(33.3%)		
	III	4(21.1%)	4(40%)	2(66.7%)		
Pattern of growth	Papillary	11(57.9%)	5(50%)	1(33.3%)	0.80	Non Sig. (P>0.05)
	Solid	8(42.1%)	5(50%	2(66.7%)		
Muscle invasion	Invasive	10(52.6%)	3(30%)	2(66.7%)	0.59	Non Sig. (P>0.05)
	Non invasive	9(47.4%)	7(70%)	1(33.3%)		

Table (4.24): *In situ* hybridization expression of positive and negative MMP-2 and related with clinicpathological profile of patients with TCC .

Variables		MMP-2 positive	MMP-2 negative	Comparison of Significance	
				Chi^2-value	Sig.
Age	25-39	2 (6.3%)	2(11.1%)	0.24	Non Sig. (P>0.05)
	40-54	5(15.6%)	6(33.3%)		
	55-70	25(78.1%)	10(55.6%)		
Gender	Male	21(65.6%)	14(77.8)	0.36	Non Sig. (P>0.05)
	Female	11(34.4%)	4(22.2%)		
Tumor grade	I	1(3.1%)	3(16.7%)	0.10	Non Sig. (P>0.05)
	II	19(59.4%)	12(66.7%)		
	III	12(37.55%)	3(16.7%)		
Pattern of growth	Papillary	14(43.8%)	14(77.8%)	0.02	Sig. (P<0.05)
	Solid	18(56.2%)	4(22.2%)		
Muscle invasion	Invasive	18(56.2%)	6(33.3%)	0.11	Non Sig. (P>0.05)
	Non invasive	14(43.8%)	12(66.7%)		

Table (4.25): *In situ* hybridization expression of positive and negative MMP-9 and related with clinicpathological profile of patients with TCC .

Variables		MMP-9 positive	MMP-9 negative	Comparison of Significance	
				Chi^2-value	Sig.
Age	25-39	2(6.2%)	2(11.1%)	0.58	Non Sig. (P>0.05)
	40-54	6(18.8%)	5(27.8%)		
	55-70	24(75%)	11(61.1%)		
Gender	Male	25 (78.1%)	10(55.6%)	0.95	Non Sig. (P>0.05)
	Female	7(71.4%)	8(44.4%)		
Tumor grade	I	3(9.4%)	1(5.6%)	0.83	Non Sig. (P>0.05)
	II	19(59.4%)	12(66.7%)		
	III	10(31.3%)	5(27.8%)		
Pattern of growth	Papillary	17(53.1%)	11(61.1%)	0.58	Non Sig. (P>0.05)
	Solid	15(46.9%)	7(38.9%)		
Muscle invasion	Invasive	15(56.9%)	9(50%)	0.83	Non Sig. (P>0.05)
	Non invasive	17(53.1%)	9(50%)		

4.4.3-Association between MMP-2 and MMP-9 expression and viral protein LMP-1 and genome HPV16 and 18 in patients with transitional cell carcinoma of the bladder:-

The association between the expression of ISH of marker (MMP-2 and MMP-9) and the viral protein and genome was shown in Table (4.26). The results demonstrated that no significant association between MMP-2 ISH expression and LMP-1, HPV-16 and HPV-18. However on the contrary there was highly positive cases of MMP-2 and MMP-9 ISH expression occurred within negative viral protein and genome but also non significant.

Table (4.26): Expression of MMP-2 and MMP-9 and related with viruses.

Result of TIMP Expression		MMP-2 Expression		MMP-9 Expression		Comparison of Significance	
		Positive	Negative	Positive	Negative	Chi^2-value	Sig.
LMP-1	Positive	14 (43.8%)	8 (44.4%)	16 (50%)	6 (33.3%)	0.96* 0.25	Non Sig. (P>0.05)
	Negative	18 (56.2%)	10 (55.6%)	16 (50%)	12 (66.7%)		
HPV-16	Positive	10 (31.2%)	8 (44.4%)	12 (37.5%)	6 (33.3%)	0.35* 0.76	
	Negative	22 (68.8%)	10 (55.6%)	20 (62.5%)	12 (66.7%)		
HPV-18	Positive	5 (15.6%)	2 (11.1%)	6 (18.8%)	1 (5.6%)	0.65* 0.19	
	Negative	27 (84.4%)	16 (88.9%)	26 (81.3%)	17 (94.4%)		

***For MMP-2.**

4.4.4-TIMP-1 and TIMP-2 In situ hybridization expression in transitional cell carcinoma of the bladder:-

Formalin-fixed paraffin-embedded obtained blocks from 50 patients with transitional cell carcinoma of the bladder were investigated for determining TIMP-1 and TIMP-2 cytoplasmic expression based on *in situ* hybridization technique. There was a high expressivity of TIMP-1(66%) and TIMP-2 (62%) among patients with transitional cell carcinoma of the bladder (Table 4.27, Figure 4.7 and 4.8).

Table (4.27): The expression of TIMP-1 and TIMP-2 in patients with transitional cell carcinoma of the bladder.

Result of TIMP Expression			TIMP-1 Expression	TIMP-2 Expression	Comparison of Significance	
					Chi^2-value	Sig.
Patients	Positive	Low	19	17	0.00	Highly Sig. (P<0.01)
		Intermediate	9	10		
		High	5	4		
		Total	33 (66%)	31(62%)		
	Negative	N %	17 (34%)	19(38%)		
	Total	N %	50 (100%)	50 (100%)		
Control	Positive	N %	0	0		
	Negative	N %	10 (100%)	10 (100%)		

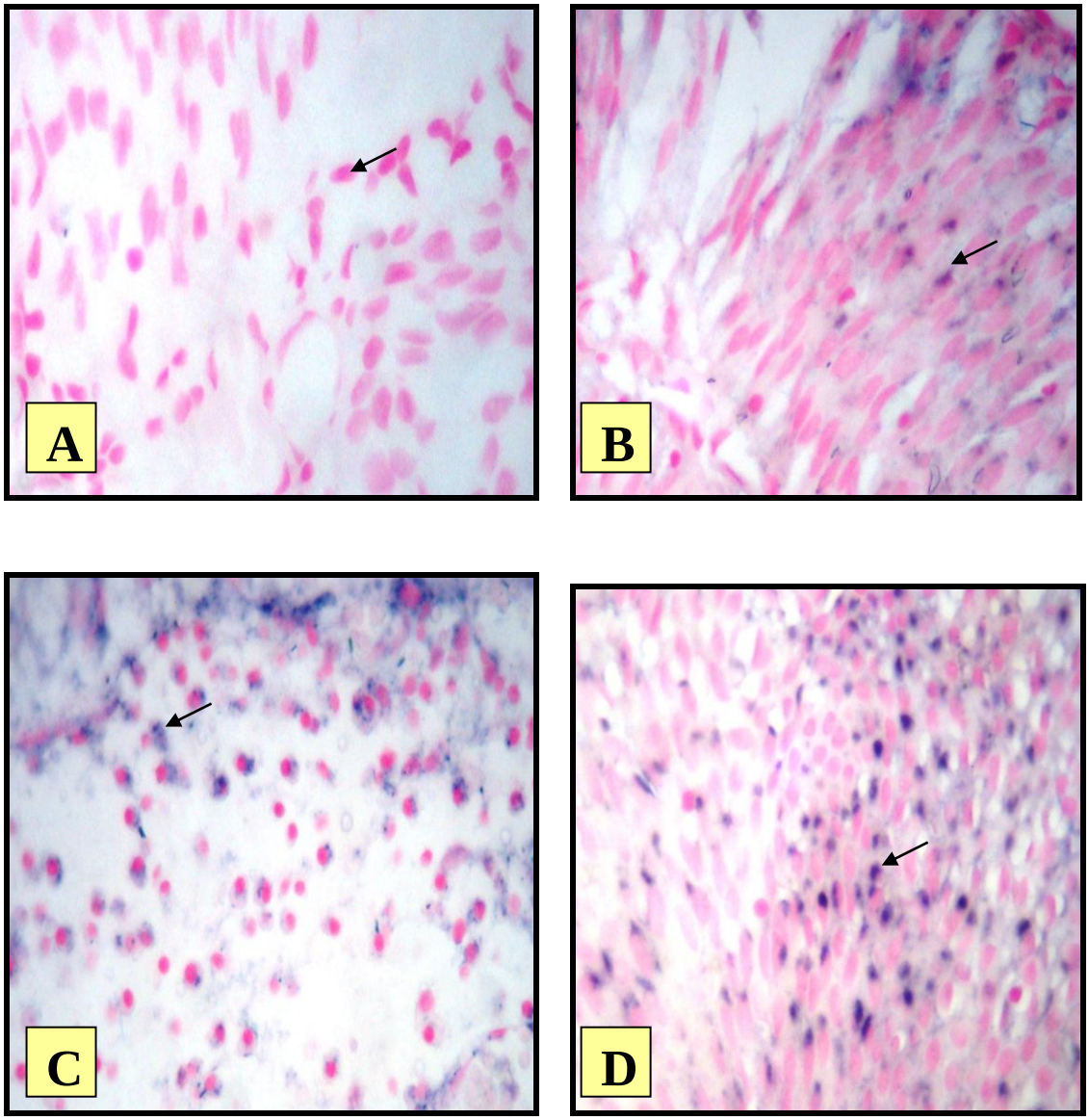


Figure (4.7): Expression of TIMP-1 in patient with transitional cell carcinoma of the bladder by ISH, stained by BCIP/NBT-chromogen and counter stained with NFR as dark-bluish purple in positive cases (Magnification power, 400), A-Well differentiated TCC, negative expression, B- Well differentiated TCC, low TIMP-1 positive expression, C- Moderately differentiated TCC, intermediate TIMP-1 positive expression, D- Moderately differentiated TCC, high TIMP-1 positive expression.

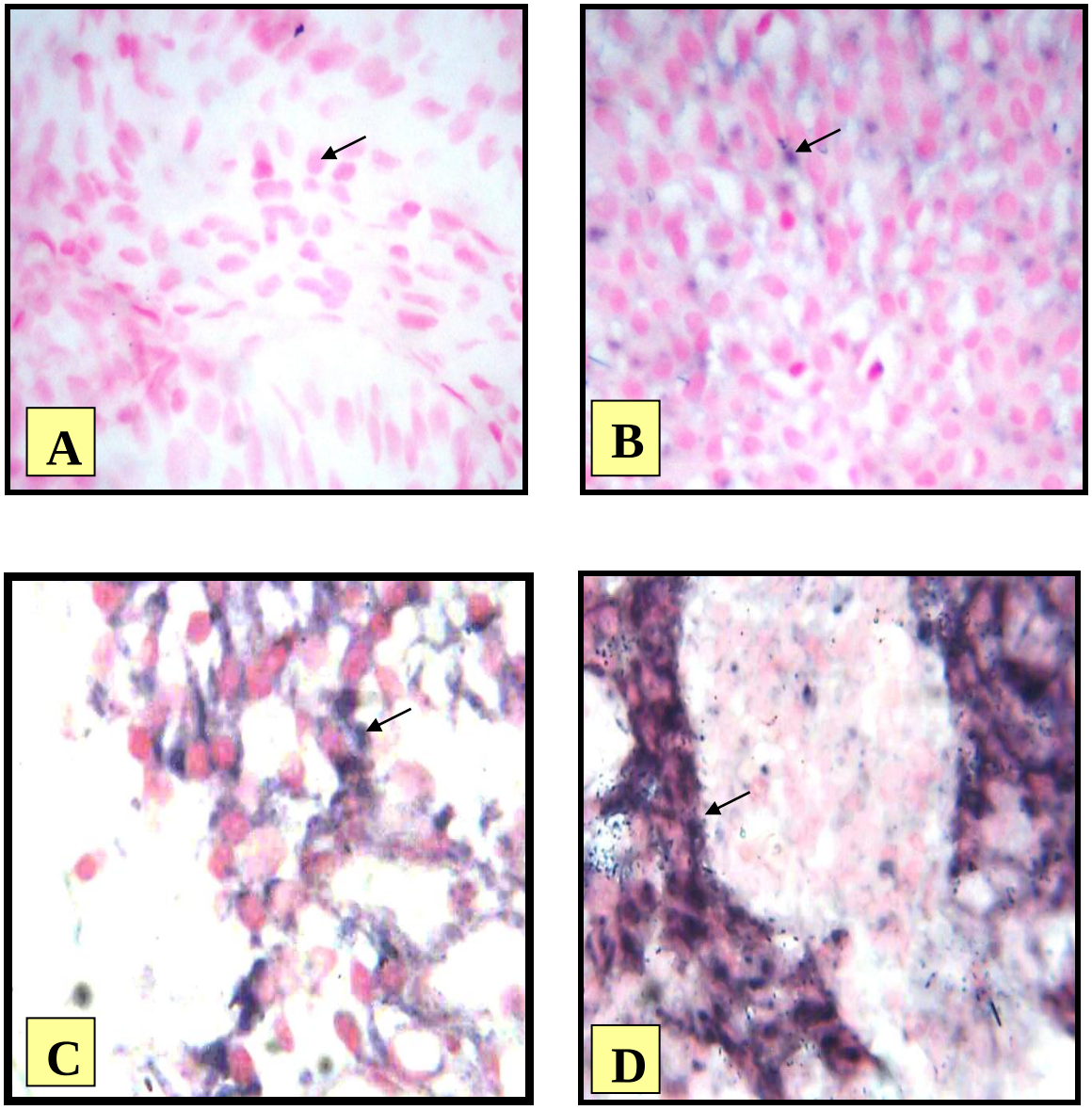


Figure (4.8): Expression of TIMP-2 in patient with transitional cell carcinoma of the bladder by ISH, stained by BCIP/NBT-chromogen and counter stained with NFR as dark-bluish purple in positive cases (Magnification power, 400), A- Moderately differentiated TCC, negative expression, B- Poorly differentiated TCC, low TIMP-2 positive expression, C- Moderately differentiated TCC, intermediate TIMP-2 positive expression, D- Moderately differentiated TCC, high TIMP-2 positive expression.

4.4.5-Correlation between TIMP-1 and TIMP-2 expression with clinical parameters:-

A correlation was made between every two markers according to the clinical parameter of 50 patients with transitional cell carcinoma of the bladder. The correlation was shown in Table (4.28) and (4.29) and revealed that no significant correlation with age(p=0.59), (p=0.69), gender (p=0.10),(p-0.99), pattern of growth (p=0.17), (p=0.05) and muscle invasive (p=0.84),(p=0.91) respectively was recorded, while with grade distribution significant correlation was recorded (p=0.03),(p=0.00) for both of them. According to positive and negative cases significant correlation occur between TIMP-1 and pattern of growth while no correlation between TIMP-2 and pattern of growth (Table 4.30 and 4.31).

Table (4.28): Correlation of TIMP-1 scores and related with clinical parameters.

Variables		TIMP-1			Comparison of Significance	
		Low	Intermediate	High	Chi^2-value	Sig.
Age	25-39	3(15.8)	1(11.1%)	0	0.59	Non Sig. (P>0.05)
	40-54	3(15.8%)	3(33.3%)	1(20%)		
	55-70	13(68.4%)	5(55.6%)	4(80%)		
Gender	Male	10(52.6%)	6(66.7%)	2(66.7%)	0.10	Non Sig. (P>0.05)
	Female	9(47.4%)	3(33.3%)	5(100%)		
Tumor grade	I	0	0	2(40%)	0.02	Sig. (P<0.05)
	II	12(63.3%)	4(44.4%)	2(40%)		
	III	7(36.8%)	5(55.5%)	1(20%)		
Pattern of growth	Papillary	8(42.1%)	4(44.4%)	3(60%)	0.17	Non Sig. (P>0.05)
	Solid	11(57.9%)	5(55.6%)	2(40%)		
Muscle invasion	Invasive	9(46.4%)	5(55.6%)	3(60%)	0.84	Non Sig. (P>0.05)
	Non invasive	10(52.6%)	4(44.4%)	2(40%)		

(4.29): Correlation of TIMP-2 scores and related with clinical parameters.

Variables		TIMP-2			Comparison of Significance	
		Low	Intermediate	High	Chi^2-value	Sig.
Age	25-39	3(17.6)	0	0	0.69	Non Sig. (P>0.05)
	40-54	4(35.5%)	2(20%)	1(25%)		
	55-70	10(58.8%)	8(80%)	3(75%)		
Gander	Male	12(70.6%)	7(70%)	3(75%)	0.99	Non Sig. (P>0.05)
	Female	5(29.4%)	3(30%)	1(25%)		
Tumor grade	I	0	0	4(100%)	0. 00	Sig. (P>0.05)
	II	11(64.7%)	6(60%)	0		
	III	6(35. 3%)	4(40%)	0		
Pattern of growth	Papillary	8(47.1%)	3(30%)	4(100%)	0.05	Non Sig. (P>0.05)
	Solid	9(52.9%)	7(70%)	0		
Muscle invasion	Invasive	7(41.2%)	5(50%)	2(50%)	0.91	Non Sig. (P>0.05)
	Non invasive	10(58.8%)	5(50%)	2(50%)		

Table (4.30): *In situ* hybridization expression of positive and negative TIMP-1 and related with clinicpathological profile of patients with TCC .

Variables		TIMP-1 positive	TIMP-1 Negative	Comparison of Significance	
				Chi^2-value	Sig.
Age	25-39	4(12.1%)	0	0.32	Non Sig. (P>0.05)
	40-54	7(21.2%)	4(23.5%)		
	55-70	22(66.7%)	13(76.5%)		
Gender	Male	21(63.6%)	14(82.4%)	0.17	Non Sig. (P>0.05)
	Female	12(36.4%)	3(17.6%)		
Tumor grade	I	2(6.1%)	2(11.8%)	0.12	Non Sig. (P>0.05)
	II	18(54.5%	13(76.5%)		
	III	13(39.4%)	2(11.8%)		
Pattern of growth	Papillary	15(45.5%)	13(76.5%)	0.03	Sig. (P<0.05)
	Solid	18(54.5%)	4(23.5%)		
Muscle invasion	Invasive	17(51.5%)	17(51.5%)	0.48	Non Sig. (P>0.05)
	Non invasive	16(48.5%)	16(48.5%)		

Table (4.31): *In situ* hybridization expression of positive and negative TIMP-2 and related with clinicpathological profile of patients with TCC.

Variables		TIMP-2 Positive	TIMP-2 Negative	Comparison of Significance	
				Chi^2-value	Sig.
Age	25-39	3(9.7%)	1(5.3%)	0.86	Non Sig. (P>0.05)
	40-54	7(22.6%)	4(21.1%)		
	55-70	21(67.7%)	14(73.7%)		
Gender	Male	22(71%)	13(68.4%)	0.84	Non Sig. (P>0.05)
	Female	9(29%)	6(31.6%)		
Tumor grade	I	4(12.9%)	0	0.19	Non Sig. (P>0.05)
	II	17(54.8%)	14(73.7%)		
	III	10(32.3%)	5(26.3%)		
Pattern of growth	Papillary	13(68.4%)	13(48.4%)	0.16	Non Sig. (P>0.05)
	Solid	6(31.6%)	6(31.6%)	0.60	Non Sig. (P>0.05)

Chapter Five

Discussion

Discussion

5.1-Transitional cell carcinoma of the bladder in relation to different studied parameters:-

Concerning age distribution the present study it is observed that most of the patients with bladder carcinoma have the age range of (55-70) years and that such carcinoma is uncommon in other age strata as shown in Table (4.2), these result are in accordance with the studies of other researchers who found that (Al-Haidary, 2006; Ibrahem, 2009).

The results of present study was in agreements with other studies from USA by Kumar *et al*., (2007), who reported that transitional cell carcinoma of the bladder usually develop between the age of 50 and 70 years. The rising incidence with age may be explained by the accumulation of somatic mutations associated with the emergence of malignant neoplasms. In addition, the observed impairment in the immune system in such ages, due to senescent decline in the immune surveillance, might lead to accumulation of cellular DNA mutation that could be regarded as an additional significant factor in the development of such malignancies (Burns and Leventhal, 2000). Also the thymus function is known to decline with age. The thymus reaches its maximal size at puberty and then atrophies, with a significant decrease in both cortical and medullary cells and an increase in the total fat content of the organ. Whereas the average weight of the thymus is 30 grams in human infants, its age-dependent involution leaves an organ with an average weight of only 3 grams in the elderly (Kindt *et al.*, 2007). Also may be related with decline in the number of NK, which play an important role in early natural surveillance against cancer and infectious disease, a progressive age-related shift in the circulating lymphocyte population from conventional T cells to NK cells (Ravaglia *et al*., 2000).

Bladder cancer is nearly three times more common in men than women (Martin and Aus, 2005). In present study found that transitional cell carcinoma of the bladder occurs most commonly in males (70%) than females (30%) as shown in Table (4.3), this is in agreements with other findings which referred that (Cohen *et al*., 2000; Kadhim, 2004; Kumar *et al*., 2007).

Women have joined a male workplace and have changed habits that have exposed them to both industrial and environmental carcinogens (such as cigarette smoking) from which they previously had been excluded. It is possible that genetic, hormonal, anatomical (e.g. relative urinary retention in older men because of prostatic enlargement), or other factors may explain this puzzling trend (Ferlay *et al*., 2002).

Regarding the histological grade as shown in Table (4.4). The results were in agreement with other studies which showed high frequency of moderately differentiated transitional cell carcinoma of the bladder (Ahmed, 2006; Al-Haidary, 2006; Ibrahem, 2009). These results also could be explained due to limited sample size.

According to type of growth the present study demonstrated that most TCC of the bladder occurred within papillary type as shown in Table (4.5). this may be related to higher percent of bladder cancer was detected in papillary type. Regarding the results of presence or absence of muscle invasion which revealed that most bladder carcinoma cases was recorded within muscle invasive.

5.2-Detection of LMP-1 in transitional cell carcinoma of the bladder and associated with clinical parameters:-

Epstein-Barr virus-DNA and EBV-gene expressions have been shown in all malignant cells and therefore it is considered to have a pathogenic role (Abe *et al*., 2008).

In this study, investigated the association between LMP-1 and bladder cancer by IHC, which is the first study in Iraq of this kind. The results which demonstrated that LMP-1 was observed in 44% of patients with TCC of the bladder as shown in (Table 4.6 and Figure 4.4).

This result was in agreement with the finding of Gazzaniga *et al*., (1998) which detected the EBV genome in 34% of whole bulk tissue samples using PCR and Chuang and Liao (2004) who demonstrated EBV-encoded RNA within both carcinoma cell and infiltrating lymphocytes in 21% only infiltrating lymphocytes in 7% and only carcinoma cells in 3% of bladder cancers obtained from Taiwanese population. Abe *et al*., (2008) reported that EBER-expressing lymphocytes were detected in the bladder carcinomas in 26 out of 39 cases (66.7%) while all normal urinary bladder specimens showed negative results.

In this study LMP-1 was detected by IHC technique, the result revealed that the prevalence of LMP-1 was found to be higher in males (72.7%) than females (27.3%) as recorded in Table (4.8), this may be related to the higher incidence rate of transitional cell carcinoma of the bladder in males than females.

Regarding comparison of LMP-1 expression results obtained according to age, grade, pattern of growth and muscle invasion with transitional cell carcinoma of the bladder in the present study showed no statistically

significant differences. The absence of EBV infection in normal bladder and low levels of infection in grade I could indicate that EBV protein occurs late in bladder oncogenesis. It may be that this virus could act as a cofactor for development of transitional cell carcinoma of the bladder.

5.3-Detection of HPV-16 and HPV-18 in transitional cell carcinoma (TCC) of the bladder and associated with different parameters:-

High-risk human papillomvirus (HPV) usually HPV type 16 (HPV-16) and HPV-18 had been theorized that integration of HPV DNA into the human genome (Hoenil Jo, 2005).

Current study demonstrated that the prevalence of HPV-16 and -18 DNA was found in 32% and 14% respectively of patients with transitional cell carcinoma of the bladder (Table 4.10 and Figure 4.3). This result was in agreement with the findings of El Mawla *et al*.(2001) which reported that, bladder carcinogenesis is probably related to bacterial and Human papilloma virus and Agliano *et al*., (1994) Who study 46 transitional carcinomas and 10 non-neoplastic normal urinary samples, HPV16 and HPV18 genomes were detected in 23 of 46 (50%) bladder carcinomas and none of 10 (0%) non-neoplastic urinary samples. The results of this study was also consistent with Badawi *et al*., (2008) who reported that HPV-16 and HPV-18 DNA was detected in 44.1% of bladder cancer cases versus 11.1% of cystitis cases. There were a significant association of transitional cell carcinoma with HPV-16 in 69.2% and 61.1% by serology and bladder tissue biopsies, respectively. This results was also consistent with Aggarwal *et al*., (2009) who reported that high risk HPV was found in 14 of 33 (42%) bladder carcinomas.

However these results was disagreement with Lu *et al.*, (1997) who demonstrated that no correlation between HPV 16 and 18 and transitional cell carcinoma of the bladder when examined thirty-one samples of formalin-fixed paraffin-embedded bladder carcinomas (4 adenocarcinomas, 5 squamous cell and 22 transitional cell carcinomas) using non-isotopic ISH with biotin-labelled DNA probes of HPV 16 and 18 subtypes.

The percent of HPV-16 was higher than HPV-18. Human papilloma virus-16 is the most prevalent genotype in cervical carcinoma, and is also the most frequently detected HPV type in oropharyngeal and tonsillor squamous cell carcinoma (Licitra, 2006). It is found up to 90% of HPV-positive cases (Weinberger *et al*., 2006).

Human papilloma virus-16 may be latent for a long time in the episomal form in the oral mucosa and that it may be responsible for the initiation and development of tumor growth as a result of a multicarcinogenic interaction together with some other carcinogens and co-carcinogens (Yaltirik *et al.*, 2001). The results of present study was in agreement with Simoneau *et al*., (1999) who investigated a 187 newly diagnosed superficial papillary bladder tumors for the presence of L1-HPV DNA by the polymerase chain reaction method and hybridization with specific probes for HPV 6, 11, 16, 18, 33. HPV DNA was detected in 16 (8.5%) of the 187 specimens tested, although in a low copy number compared with SiHa cervical cancer cells used as control. HPV type 16 was observed in eight tumors while HPV type 6 and type 11 were each observed in three tumors. Two tumor specimens contained two types of HPV: one tumor hybridized with type 6 and 16 and the other with type 11 and 18.

This results was disagree with Chan *et al*., (1997) who reported that HPV type 18 was found in 60% and 30% of cases of inverted papilloma and papillary transitional cell carcinoma of the bladder, respectively. These tumors were rarely associated with HPV types 6, 11, 16, 31, and 33.

This results was also inagreement with the finding of Barghi *et al.*, (2005) who detected on HPV DNA by PCR in 21 (35.6%) samples, HPV18 was the most common type of virus with the incidence rate of 17/21(81%). This could be frankly related to the criteria of PCR as the most sensitive technique for DNA amplification than *in situ* hybridization for detection of viral DNA.

In this study higher percentage of the HPV-16 and -18 DNA positive tumors occurred in male and there was no significant association between HPV-16 and -18 presence and gender. Previous researcher has found either no association between HPV-16 and -18 presence and gender (Gillison *et al*., 2000; Zang, 2004; Khashman, 2008). Regarding comparison of HPV16 and 18 DNA expression results according to grade, pattern of growth and muscle invasion of patient with transitional cell carcinoma of the bladder revealed that no significant correlation among them. Moonen *et al*., (2007) who reported that the prevalence of all-type and high-risk HPV infection in malignancies of the bladder was 15.2% and 8.1%, respectively. In high-grade tumours this prevalence was 18.2% and 10.6%, respectively. In grade 1, 2 and 3 tumours the infection rate of high-risk HPV types was 0%, 3.3%, and 10.6%. These results was comparable to these of Khashman (2008) who reported that grade II are the most common type of grade in patients with positive HPV.

Studies from the general population showed a variable incidence of high risk HPV DNA which ranged from 2.5% to 81%, with HPV-16 DNA occurring more frequently. HPV was detected in both papillary and invasive cancers (Lopez-Beltran and Escudero, 1997). The present results are consist with Yu *et al*., (1999) who detected on high risk human papillomaviruses in papillary transitional cell carcinoma of urinary bladder 36.5% of 52 cases were HPV-positive.Such variation in research out come could be anticipated on the following bases: difference in the number of the investigated cases, type of molecular technique and sensitivity and specifity of the utilized reagents.

5.4-Detection of p53 protein in TCC of the bladder:-

In the present study, mutant-type p53 was detected by IHC technique (Table 4.14 and Figure 4.4). The results which revealed that 46% of the TCC of the bladder were positive.

The incidence of positive p53 immunostaining is comparable to those of Esrig *et al*., (1994), Ye *et al*., (1998) and Ibrahem (2009) which reported positive immunostaining in 42.2%, 40% and 46% respectively. However, it is higher than that reported by schlichtholz *et al*., (2004) and Ahmed (2006) who detected nuclear over expression in 26% and 35.4% respectively. Higher rates (57.5%) had been reported in other study (Al-Qaysi, 2002).

This study showed that were no significant correlation between p53 over expression and patient's age, gender and this result is nearly compatible with the result of (Al-Qaysi, 2002; Al-Haidary, 2006). Most p53 positive cases occur within papillary and invasive but statistically no significant correlation among them which was in agreement with the study of Zekri *et al*., (2001) reported that p53 tumor suppressor gene are the most common genetic change detected in human cancer as well as in papillary and invasive bladder cancer. No significant association between p53 over expression and tumor grade was also confirmed in this study as shown in Table (4.16). This results was in agreement with Al-Haidary (2006) demonstrated that p53 scores highly significant correlation with tumor stage but no correlation was found with tumor grade. Esrig *et al.*, (1994) which referred that p53 was identified more frequently in grade IV tumors than grade II or III tumors, but this association was not significant while significant result seen in muscle invasion. The results different with Kadhim (2004) and Ahmed (2006) that showed significant correlation with tumor grade and muscle invasion.

Regarding correlation between p53 over expression and viral protein or genome which revealed that p53 over expression had no significant with LMP-1 as shown in Table (4-19). This result was in agreement with Baylin and Herman (2000) observed that EBV- encoded EBNA-5 protein (alternatively designated as EBNA-LP) forms a molecular complex with both p53 and retinoblastoma proteins (Rb) and it is conceivable now that such binding may lead to an accelerated degradation of either or both these tumor suppressor protein. This observation also supported by other researchers who found that BZLF-1 protein bind to P53 protein and inactivated P53 (Zhang *et al*, 1994).

The association between the p53 protein and HPV 16 and 18 DNA. It has demonstrated significant correlation between p53 protein and HPV-16 while not significant association between p53 over expression and HPV 18 in TCC of the bladder as shown in Table (4.20).

The present study are consist with Chen *et al*., (2000) who reported that among 75 patients with bladder transitional cell carcinoma; 9.3% were positive for low-risk HPV types and 34.7% for high-risk HPV types. This could indicate the oncogenic potential of HPV can be related to products of two early viral genes, E6 and E7 together they interact with a variety of growth regulating proteins encoded by proto-oncogenes and tumor suppressor genes. The E7 protein binds to the retinoblastoma protein and displaces the E2F transcription factors that are normally sequestered by RB, promoting progression through the cell cycle. Interestingly, E7 protein from high risk HPV types has a higher affinity for RB than does E7 from low risk HPV types. The E7 also inactivates the CDKIs CDKN1A/p21 and CDKN1B/p27. E7 protein from HPV types (types 16, 18 and 31) also bind and presumably activate cyclins E and A. The E6 protein had complementary effects. It binds to and mediates the degradation of p53 and Bax a pro-apoptotic member of the Bcl2 family (Kumar *et al*., 2007).

Similar study carried out by Khaled *et al*., (2001). Who reported that HPV was detected 46% of Egyptian bladder carcinomas (23/50 cases). positivity was 47.8% for squamous cell carcinoma and 36.4% for transitional cell carcinoma. There was a possible viral-bilharzial association as 52.8% of Bilharzial cases, whereas only 12.5% of non Bilharzial cases. Tumor suppressor protein found in 19/41 (46.3%) cases and there was a concordance between HPV and p53 in 58.5% of cases. The current study was revealed that no association between p53 over expression and HPV-18.

The differences between the results of the previously mentioned studies and even with the results of present study could be related to many factors, like type of the tumor whether squamous or transitional, grade and stage of the tumor, the methodology and affinity of the antibody, the duration of incubation, the sensitivity of detection system and lack of standardized technique because these factors also affect the expression of p53.

5.5-Cytoplasmic expression of MMP-2 and MMP-9 in TCC of the bladder:-

The current study had demonstrated that MMP-2 and MMP-9 were over expressed in transitional cell carcinoma of the bladder as shown in (Table 4.21, Figure 4.5 and 4.6). These results might possibly reflect the association between cellular expression of MMP-2 and MMP-9 and bladder tumorgenesis. This was in agreement with the findings of (Grignon *et al*., 1996; Papathoma *et al*., 2000; Kanayama, 2001), since they found over expression of this enzyme in transitional cell carcinoma of the bladder. In comparison with other studies both enzyme are increased in malignant tissues compared to their benign counterparts (Iurlaro *et al*., 1999). This is raises the question why MMPs expression was rare in benign tumor, know that benign tumor have no metastasis and no invasion, so that no need for additional degradation of ECM and finally no need for exaggerated MMPs expression.

In fact, analysis of both primary and metastatic tumors demonstrated increased MMPs at the metastatic site had pointed out their role in tumor migration and spread (Sutinen *et al*., 1998).

In this study, the results showed no correlation between MMP-2 expression and age, gender, also any correlation with tumor grade and muscle invasion not observed (Table 4.22 and 4.24). The results was in agreement with Grignon *et al*., (1996) who did not find any association between the expression of MMP-2 immunoreaction protein in bladder cancer tissue or the grade or stage in TCC of the bladder. However, this result was also in agreement with the results of Kanayama *et al.* (1998) who reported that MMP-2 contributes to the invasive properties of bladder carcinoma. Moreover, in some studies where gelatine zymography was used, the expression of activated MMP-2 was higher in invasive tumor tissue (Kanda *et al.*, 2000). Other studies have also shown a correlation to grade and stage of the cancer of the cancer (Miyata *et al.*, 2004).

Concerning MMP-9 expression, positivity did not correlate to the age, gander of the patients, pattern of growth, grade of the tumor (Table 4.23 and 4.25). This result was in agreement with the findings of (Ozemir *et al*., 1999) who pointed out that no correlation between MMP-9 over expression and tumor grade was recorded. Mohammed *et al*., (2000) measured the level of MMP-9 in serum by western blot technique and revealed that serum level of MMP-9 showed highly significant elevation compared to healthy normal subjects but this elevation did not correlate with age, gender or even grade of the disease.

Durkan *et al*., (2003) showed no correlation to grade was found but instead, the MMP-9 levels measured by enzyme-linked immunoassay (ELISA) correlated to stage when measuring MMP-9 protein in urine samples of bladder cancer patients. It had been found that patients with no relapse had

a higher urine MMP-9 protein level than patients with relapses, the difference being statistically significant (Monier *et al.*, 2002).

MMP-9 is quite well examined in bladder cancer whether using tissue samples or serum or urine detection, it seems that high or elevated expression of MMP-9 enzyme correlates with clinical stage or histological grade of the tumor (Eissa *et al*., 2003) and (Guan *et al.*, 2003).

5.6-Correlation between cellular expression of MMP-2 and MMP-9 and viral protein and genome:-

In this present study most LMP-1 positive occur within MMP-9 but this association not significant and may be related to LMP-1, which is expressed in NPC, has two essential signaling domains within the carboxyl terminus, termed C-terminal activation regions 1 (CTAR-1) and CTAR-2. Either signaling domain can activate the MMP-9 promoter and induce MMP-9 activity; however, LMP-1 deletion mutants lacking either CTAR-1 or CTAR-2 had a decreased ability to induce MMP-9 expression. The deletion of both activation regions completely abolished the induction of MMP-9 activity, while the cotransfection of both the CTAR-1 and CTAR-2 deletion mutants restored MMP-9 activity to levels produced by wild-type LMP-1.The NF-κB and activator protein 1 (AP-1) binding sites in the MMP-9 promoter were essential for the activation of MMP-9 gene expression by both CTAR-1 and CTAR-2. The induction of MMP-9 expression by LMP-1 and both CTAR-1 and CTAR-2 (Takeshita *et al*., 1999).

Regarding expression of MMP-2 and MMP-9 in related to HPV16 and 18 genome, the current study did not found any relationship between MMP-2 and MMP-9 expression and HPV16 and 18 genome Table (4.26). This result was in agreement with findings of Talvensaar *et al.*, (1999) and Song *et al*.,(2006) who evaluated the expression of MMP-2 in cervical cancer and suggest that MMP-2 expression were not correlated with HPV load, and this result was

inagreement with findings of Malina *et al*., (2004) who evaluated the expression of MMP-2 in the neoplastic tissues of patients with low-grade cervical intraepithelial neoplasias (CIN1) and high-grade cervical intraepithelial neoplasias (CIN 3) and suggested that MMP-2 expression might be involved in the neoplastic changes of the uterian cervical saquamous epithelium. Behren *et al*., (2005) reported that Papillomavirus E2 Protein Induces Expression of the Matrix Metalloproteinase-9 via the Extracellular Signal-Regulated Kinase/Activator Protein-1 Signaling Pathway.

Indeed, previous experiments with mice transgenic for HPV16 and additionally devoid of MMP-9 showed only a reduction of 50% in squamous cell carcinoma incidence in comparison with HPV16/MMP-9-proficient mice (Van Kempen *et al*, 2002).

The transcription of the MMP-9 and MMP-2 genes is, however, differently controlled. Whereas the promoter of MMP-9 contains cis-elements [activator protein-1 (AP-1) and nuclear factor-κB (NF-κB)] that can be regulated through mitogen-activated protein kinases (MAPK) (Simon *et al*., 2001). The MAPKs are a family of enzymes that transduce signals via several phosphorylation steps into the nucleus (Edmunds and Mahadevan, 2004). The three best characterized members of the MAPK are the stress-activated c-Jun NH2-terminal kinase, the p38 kinase, and the extracellular signal-regulated kinase (ERK). Growth factors lead to an activation of ERK by upstream regulator proteins, such as Raf, which itself can be activated by Ras, a key mediator of cell proliferation (Roux and Blenis, 2004). Therefore, activation of ERK and entry into the nucleus can often be found in cancer and has been linked to cervical and other epithelial neoplasia (Branca *et al*., 2004).

Therefore, activation of the ERK/AP-1 signal pathway by E2 proteins of papillomaviruses or by LMP-1 of EBV may not only contribute to the tumorigenic potential of these viruses but also could constitute in papillomaviruses an autocrine stimulation mechanism important for the very early steps following successful infection of the target cell (Behren *et al*., 2005). Epithelial stem cells in the hair follicle represent the primary target cells, which express immediately after infection high levels of viral transcripts encoding E6, E7 and E2 (Schmitt *et al*., 1996). While the promoter of MMP-2 shares no such conserved cis-elements (Westermarck and Kahari, 1999). Studies carried out by Yoshida *et al*., (2008), reported that H-ras contribute to cellular transformation in cooperation with high-risk E7 expression also found that the induction of Matrix metalloproteinases (MMPs) by E7 and H-ras was involved in invasiveness.

Katori *et al*., (2006) reported that on patients with inverted papilloma (IP) Found significant increase of MMP-2 and MMP-9 with human papilloma virus. HPV16/18 positive IP compared to HPV16/18 negative IP ($p<0.05$).

5.7-Cytoplasmic expression of TIMP-1 and TIMP-2 in TCC of the bladder:-

Transitional cell carcinoma of the bladder cases in this study found that there was significant increase (66%) and (62%) in TIMP-1 and TIMP-2 expression (Table 4.27, Figure 4.7 and 4.8), this was in agreement with (Grignon *et al*., 1996; Durkan *et al*., 2003). found over expression of these enzyme in patients with TCC of the bladder. However, this up regulation was transient and thus may reflect one of the subsequent acute host responses to the remodeling stimuli to balance the local tissue degradation process (Moran *et al*., 2005). The results of present study had demonstrated that no significant differences between TIMP-1 and TIMP-2 expression with age of patients, gender, pattern of growth and muscle invasion, since the limited number of investigated cases did not reveal significant differences, while significant correlation was found with grade as shown in Table (4.28 and 4.29).

Tissue inhibitor of matrix metalloproteinase's role in the prognosis of bladder carcinoma seems to be controversial. Two tissue experiments, of which one was done by reverse transcriptase polymerase chain reaction (rPCR) and the other by immunohistochemistry, both showed that high expression of TIMP-2 correlated with advancerd stage and grade (Gakiopoulou *et al.*, 2003). Patients with high expression of the gene for TIMP-2 in the primary tumor had an unfavorable prognosis when compared to patients with low TIMP-2 expression in the tumor (Kanayama *et al.*, 1998).

Miyata *et al*., (2004) found that there were no statistical correlations between TIMP-2 and stage or tumor grade.

Staack *et al*., (2006) found that plasma concentrations of TIMP-2 showed no correlation with grade. The levels in the group of non-metastasized bladder cancer patients were significantly lower when compared to levels in the control group.

5.8-Relationship between MMP-2, MMP-9 and TIMP-1,TIMP-2:-

Tissue inhibitor of matrix metalloproteinase-1 binds preferably to MMP-9 (Wilhelm *et al.*, 1989), and TIMP-2 to MMP-2 (Stetler *et al*., 1989). Tissue inhibitor of matrix metalloproteinase-1 and TIMP-2 are also capable of binding tightly to the latent form of gelatinases (Wilhelm *et al.*, 1989).

The current study had demonstrated that high expression of MMP-2, MMP-9, TIMP-1 and TIMP-2 among patients with transitional cell carcinoma. Indeed, it is often a feature of malignant disease in which MMP levels are increased that TIMP levels are also increased. This may reflect an attempt to control the increasing degradative potential, or it may indicate that TIMPs are multifunctional molecules. It has been determined that TIMP-1 has a high homology with growth factor activity known as erythroid potentiating activity. In addition, there seems to be a requirement for TIMP-2 binding to allow activation of gelatinase A by MT1-MMP (Nelson *et al*., 2000).

Tissue inhibitor of matrix metalloproteinase-1 can be co-expressed with MMPs even some studies have showed a reciprocal regulation of their expression, which may depend on endogenously expressed (growth) factor and cytokines (Gomez *et al*., 1997). Interlukin-1 and migrating cells under inflammatory process stimulate fibroblasts to produce both collagnases and its inhibitors (Murphy *et al*., 1983). TNF-α stimulates the expression of several MMPs and thus contributes to tissue degradation in inflammatory conditions (Shimizu *et al*., 2005). Thus, in general changes in cytokine expression may affect the MMP/TIMP balance.

The imbalance of MMP-2 and its inhibitor TIMP-2 had previously been shown to be an indicator of recurrence in urothelial carcinoma. Patients with high levels of MMP-2/TIMP-2 ratio measured from the serum of urothelial cancer had a higher risk for recurrence than patients with low levels of this ratio (Gohji *et al*., 1998).

Monier *et al.*, (2002) reported that reduced TIMP-2 and increased levels of active MMP-2 in urine were correlated to grade and stage in bladder cancer. Stetler-Stevenson *et al.*, (2008) have demonstrated a cell surface signaling receptor for a member of the TIMP family. They suggest that TIMP-2 regulates cellular responses to growth factors.

Chapter Six

Conclusions and Recommendations

Conclusions

1-The commonest age group for transitional cell carcinoma of the bladder was 55-70 years and the ratio of male patients to their female counterparts was found to be 2.3:1.

2-Distribution of transitional cell carcinoma of the bladder according to the grade revealed that most of cases occurred within grade II and III.

3-The papillary type and muscle invasion was the commonest in patients with transitional cell carcinoma of the bladder and found significant correlation among them.

4-Amonge the TCC of the bladder 22 cases (44%) were found to be LMP-1 positive, where as 28 cases (56%) were LMP-1 negative.

5-Expression of HPV-16 DNA in TCC of the bladder by ISH was more frequency (36%), while low frequency of HPV-18 (14%) was found in TCC of the bladder.

6-The overall expression of mutated P53 protein in bladder cancer cases in this study was (46%), most positive cases occur within high grade However, there was no significant correlation between p53 expression and grade.

7-Strong correlation between HPV-16 and p53 immunostaining result, while no correlation between p53 immunostaining result and HPV-18 and LMP-1.

8-Significant expression of both MMP-2 and MMP-9 *in situ* hybridization seem to have potential role in TCC of the bladder.

9-High expression of TIMP-1 and TIMP-2 noticed in patient with transitional cell carcinoma of the bladder.

Recommendation

1- Further studies with large sample size, follow up long-run starting from the collection of the samples from the patients directly and supplemented with a complete history of the patients associated with clinical examination and questionnaire like medical history, geographical area.

2- Studying the role of other viruses like CMV and HSV-2 in the development of bladder carcinoma.

3- Studying the role of other tumor suppressor genes like p16, p21 in deregulation of cell cycle pathway in EBV and HPV positive bladder carcinoma.

4- Further studies to evaluate the role of anti-p53 antibodies in patient's serum, which can serve as diagnostic and prognostic markers in bladder cancer.

5- Additional studies to identify the molecular biomarkers that play essential role in oncogenesis and disease progression in bladder cancer, such as epidermal growth factor receptor and fibroblast growth factor receptor.

6- Detection of level of expression of MMP-2 and MMP-9 in urine and serum of patient with Transitional cell carcinoma of the bladder.

7- Detection of level of expression of TIMP-1 and TIMP-2 before and after treatment.

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Appendix

Appendix

Appendix (1): Hematoxylin-Eosin Stain (H. &E.)

(Harri's Haematoxylin) (Luna, 1968).

Hematoxylin crystals…….. 5 gm

Alcohol, absolute…… 50 ml

Potassium Alum……..100 gm

Distilled water…… 1000 ml

Mercuric oxide (red)…… 2.5gm

Hematoxylin had dissolved in alcohol; the alum in the water by the aid of heat. Then removed from heat and the two solutions mixed and; boiled for less than 1 minute with stirring (bringing to boiling should be rapid as soon as possible). Then; removed from heat and, the mercuric oxide added slowly. Re-heated to a simmer until it becomes dark purple, removed from heat and, the vessel plunged into a basin of cold water till cooled. Two-4ml of glacial acetic acid per 100ml added (to increase the precision of nuclear stain). Filtered before use.

Eosin…as counter stain for Hematoxylin

1% stock alcoholic Eosin:

Eosin Y, water soluble…….. 1gm
Distilled Water……..20ml
Dissolved, and add……
Alcohol, 95%……80ml

Working Eosin solution:

Eosin stock solution…….1 part
Alcohol, 80%……..3 parts
0.5ml/100ml of glacial acetic acid was added just before use with stirring. No filtration needs.

Appendix (2): Nuclear Fast Red (NFR)
Product Number N 8002/ Sigma.

Preparation:

25gm of aluminum sulphate was dissolved in 500ml of distilled water then, (0.5gm) of nuclear fast red powder was added to be dissolved with aid of simmer heat. After cooled, filtered, and a few grains of thymol were added as a preservative.

- Aluminum sulphate…….. Fluka/ Germany
- NFR powder…… Sigma/ Germany
- Thymol ……. Fluka/ Germany

الخلاصة

تضمنت الدراسة الاسترجاعية خمسين مريضاً مصاباً بسرطان خلايا المثانة الانتقالي ، وقد تم مقارنتهم بعشرة اشخاص اصحاء متوافقين من حيث العمر والجنس.

تمت عملية جمع النماذج من ارشيف الانسجة المرضية لمختبرات مستشفى الجراحات التخصصي وارشيف معهد الطب العدلي في بغداد. عملية جمع النماذج امتدت للفترة من شباط ٢٠٠٩ ولغاية حزيران ٢٠٠٩. والعينات تعود للفترة مابين اذار ٢٠٠٨ – حزيران ٢٠٠٩.

اجريت عملية التقطيع النسيجي بسمك اربعة مايكروميتر لكل عينة محفوظة بالفورمالين ومطمورة بشمع البرافين لمجموعة المرضى والسيطرة وبواقع مقطع واحد لصبغة الهيماتوكسيلين والايوسين، بعدها تم اعادة فحصها، وستة مقاطع استخدمت للكشف عن الفايروس الحليمي البشري ذو الاخطار العالي-١٦ و-١٨ والتعبير MMP-٢ و MMP-9 ومثبطاتها TIMP-1 , TIMP-2 باستخدام تقنية التهجين الموضعي ذات الحساسية العالية. ومقطعين للصبغة المناعية الكيميائية النسيجية للكشف عن بروتين الغشاء المستتر نوع -١ والبروتين المثبط للورم P53.

١- اوضحت نتائج الدراسة الحالية ان عمر مرضى سرطان خلايا المثانة الانتقالي تراوح من ٢٥-٧٠ سنة وبمتوسط ٥٧.٣٠ سنة، اعلى نسبة مئوية كانت ضمن الفئة العمرية ٥٥-٧٠ سنة. نسبة الذكور الى الاناث كانت ٣.٢:١.

٢- شكلت عينات سرطان خلايا المثانة الانتقالي متوسطة التمايز النسبة الاعلى (٦٢%) اما جيدة التمايز فشكلت النسبة الاقل(٨%).

٣- اظهر سرطان خلايا المثانة الانتقالي ذو النوع الحليمي(٥٦ %) اعلى نسبة من النوع الصلد (٤٤ %) كما شكل انتهاك الورم للطبقة العضلية النسبة الاكثر (٥٢ %) من بين حالات سرطانات خلايا المثانة الانتقالي.

٤- ضمن مجموعة سرطانات خلايا المثانة الانتقالي ٢٢ عينة محفوظة بالفورمالين ومطمورة بشمع البرافين اظهرت نتائج موجبة لفايروس ابشتاين- باراذ بلغت النسبة ٤٤% .بينما ١٨ عينة و٧ عينات اظهرت نتائج موجبة للفايروس الحليمي البشري ذو الاخطار العالي-١٦ و-١٨ على التوالي وشكلت النسبة ٣٦% و١٤% على التوالي.

٥- البروتين المثبط للورم شكل نسبة ٤٦% في المرضى المصابين بسرطان خلايا المثانة الانتقالي.

٦- باستخدام تقنية التهجين الموضعي ذات الحساسية العالية تم الكشف عن التعبير MMP-٢ و ٩ MMP-داخل سايتوبلازم الخلايا السرطانية اذ كانت النسبة (٦٤%) لكل عينة.

٧- سجلت الدراسة الحالية زيادة واضحة في مستوى TIMP-1 وTIMP-2 بين المرضى المصابين بسرطان خلايا المثانة الانتقالي وكانت هذة الزيادة ذات دلالة احصائية.

٨- ضمن مجموعة السيطرة لم تسجل اي نتيجة موجبة لبروتين الغشاء المستتر نوع -١ و الفايروس الحليمي البشري نوع -١٦، -١٨ ، البروتين المثبط للورمP53, التعبير MMP-٢ ، MMP-٩ ومثبطاتها TIMP-1, TIMP-2.

الدراسة الجزيئية المقارنة عن فايـروس الابشتاين ـ بار والفايروس الحليمي البشـري في المرضى المصابين بسرطان المثانة

اطروحة مقدمة إلى
كلية الطب ـ جامعة بغداد
كجزء من متطلبات نيل درجة الدكتوراه فلسفة في علم الأحياء المجهرية الطبية

من قبل

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١٤٣١/ ربيع الاول

٢٠١٠/ اذار