Urinary MicroRNA-٩٦ and MicroRNA-١٢٦ as Diagnostic Biomarkers for Bladder Cancer Detection

Thesis

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بسم الله الرحمن الرحيم

وقل اعملوا

فسيرى الله عملكم

ورسوله والمؤمنون

صدق الله العظيم

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LIST OF ABBREVIATION

Abbreviation	Full term
AJCC	American Joint Committee on Cancer
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
BC	Bladder cancer
BCG	Bacillus Calmette-Guerin
CBC	Complete Blood Count
CIS	Carcinoma in situ
CLL	Chronic lymphocytic leukemia
CSS	Cancer specific survival
CT	Computerized tomography
CYFRA ٢١-١	Cytokeratin ١٩ fragment
DNMT	DNA methyl transferases
EGFL٧	Epidermal growth factor (EGF)-like domain ٧
ELISA	Enzyme-linked immunosorbent assay
FDGPET/CT	Fluorodeoxyglucose -positron emission tomography
FISH	Fluorescence in situ hybridization
FOXO٣	Forkhead box O٣
GC	Gemcitabine and cisplatin
GSTM١	Glutathionee-S-transferase genes
HA	Hyaluronic acid
HCC	Hepatocellular carcinoma
INR	International normalized ratio
IVP	Intravenous pyelograms
LOH	Loss of heterozygosity
LRC	Laparoscopic radical cystectomy

Abbreviation	Full term
MIBC	Muscle invasive bladder cancers
MRI	Magnetic resonance imaging
MSA	Microsatellite analysis
MVAC	Methotrexate, vinblastine, adriamycin, and cisplatin
NAC	Neoadjuvant chemotherapy
NATS	N-acetyltransferases
NBI	Narrow-band imaging
NMIBC	Non-muscle invasive bladder cancers
NMP٢٢	Nuclear matrix protein ٢٢
NSCLC	Non-small cell lung cancer
ORC	Open radical cystectomy
OS	Overall survival
PC	Prothrombin concentration
PCR	Polymerrase chain reaction
PDD	Photodynamic diagnosis
PTT	Partial thromboplastin time
PUNLMP	Urothelial neoplasm of low malignant potential
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RC	Radical cystectomy
RISC	RNA-induced silencing complex
RT-PCR	Reverse transcription polymerase chain reaction
SCC	Squamous cell carcinoma
SD	Standard Deviation
TCC	Transitional cell carcinoma
TET	Ten- eleven translocation

Abbreviation	Full term
TGF- β	Transforming growth factor- β
TNM	tumor-node-metastases
TUs	Transcription units
TURBT	Transurethral resection of the bladder tumor
UC	Urotheliall carcinoma
UGT١A٦	UDP-glycosyl transferases
UTR	Untranslated region

Introduction

Bladder cancer (BC) represents a global health problem. It ranks ninth in worldwide cancer incidence ***(Ploeg et al., ٢٠٠٩)***.. It is the fourth most common cancer in men ***(Bray et al., ٢٠١٨).*** A lot of efforts have been devoted in cladder cancer field to find non-invasive, sensitive and specific molecular markers for bladder cancer ***(Cheung et al., ٢٠١٣).***

Numerous urinary markers have been investigated, with the aim of reducing frequency of cystoscopy ***(Yutkin et al., ٢٠١٠)***. Several are commercially available, but none has been adopted into routine standard of care, owing to poor sensitivity and/or expense. These markers may serve as an adjunctive diagnostic test in cases where urine cytology is equivocal ***(Cheung et al., ٢٠١٣)***.

MicroRNAs (miRNAs) are endogenous, non-coding RNA molecules of about ٢٢ nucleotides in length that regulate gene expression. They regulate their targeted messenger RNA (mRNA) by repressing mRNA translation and/or directing mRNA cleavage (***(Brase et al., ٢٠١١)***. A large number of studies have demonstrated that miRNAs are key regulators of a variety of fundamental biological processes such as development, cell proliferation, apoptosis, haematopoiesis and, importantly, tumorigenesis ***(Huang et al., ٢٠١١)***.

Many miRNAs were significantly upregulated or downregulated in bladder urothelial carcinoma compared to matched histologically normal urothelium. Most importantly, different cancer types, stages or differentiation states have unique miRNA expression profiles, suggesting that miRNAs play important roles in the initiation and progression of cancer ***(Han et al., ٢٠١١)***.

Aim of The Work

This study was designed to:

- ✓ Clarify the role of miR-٩٦ and miR-١٢٦ in bladder cancer as non-invasive diagnostic urinary biomarkers and determine their expression in relation to various clinico-pathological parameters in bladder cancer.
- ✓ Determine the relationship between the expression of miR-٩٦ and miR-١٢٦.

Literature Review

Chapter (I): Bladder Cancer

Introduction:

Bladder cancer (BC) is one of the most prevalent urologic malignancies worldwide. It has been known that BC is a heterogeneous disease with a variable natural history; so, new markers are still needed in clinical practice either for better diagnosis and treatment ***(Saginala et al., ٢٠٢٠).***

Currently, no molecular or genetic biomarkers are widely incorporated into routine clinical practice ***(Davis et al., ٢٠٢٠)***. However, better understanding of the molecular alterations in BC will provide the basis for incorporation of molecular and genetic biomarkers into clinical decision making to guide management. Clinical application of such novel molecular and genetic concepts is the fundamental base of introducing the era of precision medicine into patient care ***(Davis et al., ٢٠٢٠).***

Incidence and epidemiology of bladder cancer:

Globally, Bladder cancer is among the top ten most common cancer types. It is considered the second most common malignancy of the genitourinary tract. For the year ٢٠١٩, the American Cancer Society estimates ٨٠،٤٧٠ new bladder cancer cases in the United States, and ١٧،٦٧٠ bladder cancer related deaths. It has been demonstrated that

urinary bladder cancer accounts for >٥٪ of newly diagnosed tumors in European countries ***(Siegel et al., ٢٠١٨).***

It is the fourth most common cancer in men, occurring less frequently in women with respective incidence and mortality rates of ٩,٦ and ٣,٢ per ١٠٠،٠٠٠ in men: about ٤ times those of women globally. The incidence of urinary bladder cancer is increased in developed countries compared with less developed regions, accounting for ٥٪ of all new cancer cases in the United States ***(Bray et al., ٢٠١٨).*** BC prevalence increases with age. About ٩٠٪ of bladder cancer occurs at age ٥٥ years or older; whereas only ١,٨٪ of bladder cancer is developed at age younger than ٤٠ years ***(Cumberbatch & Noon, ٢٠١٩).***

in Egypt, Bladder cancer accounted for around ٧,٢٪ of all new cases of cancer discovered in ٢٠١٨ and was reported to be the second most common malignancy following hepatocellular carcinoma (HCC) in males ***(Ibrahim et al., ٢٠١٤)***. According to the report conducted by the Global Cancer Observatory in ٢٠١٨, Egypt has been ranked among the top twenty countries with the highest incidence rates of bladder cancer. Moreover, Egypt and three countries in the Middle East-North Africa region, namely Lebanon, Turkey and Armenia, have reported the highest mortality rates for bladder cancer ***(Global Cancer Data: GLOBOCAN ٢٠١٨).***

Moreover, a varying pattern of bladder cancer has been observed throughout the years in Egypt. In the past (١٩٢٠- ١٩٩٠), squamous cell carcinoma was the most prevalent bladder due to the high infection rates (٧٠–٨٠٪) with the blood fluke Schistosoma hematobium ***(Khaled, ٢٠١٣).***

However, this profile has been dramatically changed over the past ٢٥ years due to the success in the control of Schistosomiasis through introduction of efficient anti-bilharzial drugs and public awareness campaigns to eradicate schistosomiasis, which subsequently has led to a significant decline in the incidence of squamous cell cancer ***(Antoni et al., ٢٠١٧).*** Furthermore, the high prevalence of tobacco smoking in cigarettes and water pipe smoking increase the risk for developing transitional cell carcinoma (TCC), thus minimizing the differences between its features in Egypt and that in western countries ***(Antoni et al., ٢٠١٧).***

Approximately ٧٠٪ of bladder cancer cases are non-muscle invasive bladder cancers (NMIBC), with roughly ٢٥٪ of those progressing to muscle invasive bladder cancers (MIBC). Patients with NMIBC have very good survival outcomes, with a ٥-year relative survival rate around ٩٠٪, but require long-term clinical management of the disease as they are prone to recurrence. On the other hand, patients with MIBC exhibit a more heterogeneous spread with ٥-year relative survival rates ranging from ٦٣٪ to ١٥٪ ***(Shah et al., ٢٠١١)***.

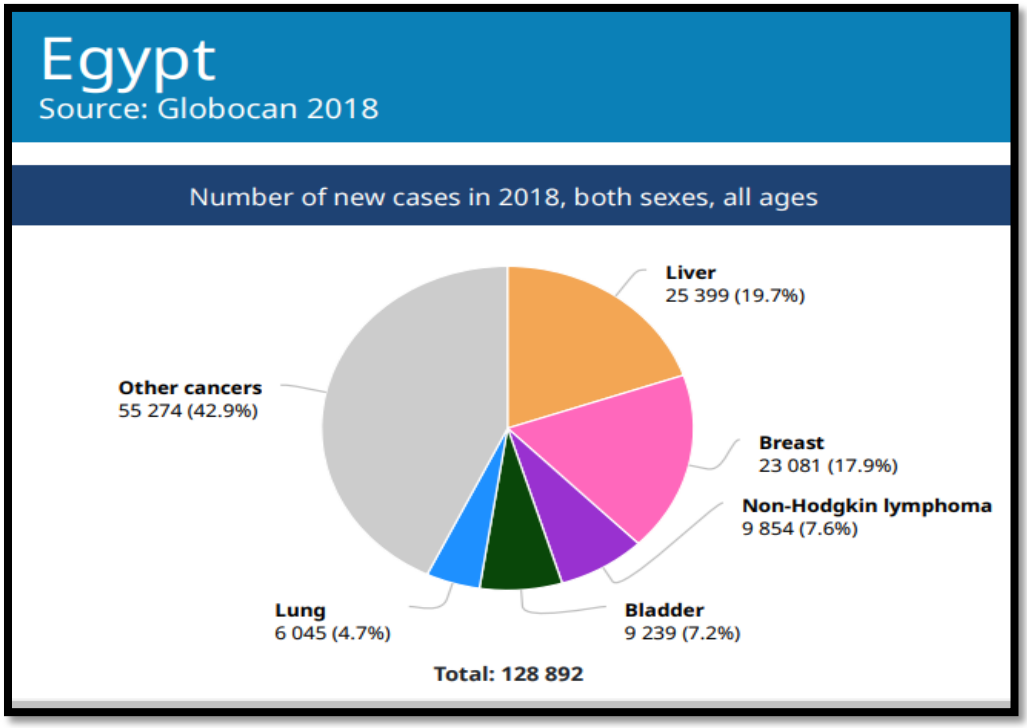


Figure (١): Number of new BC cases in Egypt in both sexes and all ages. ***(Global Cancer Data: GLOBOCAN ٢٠١٨)***

Risk factors of bladder cancer:

Several factors have different impacts on the incidence and pathophysiology of BC. This phenomenon is called etiologic fraction or attributable risk ***(Burger et al., ٢٠١٣)***.These factors include:

➢ Tobacco smoking

Tobacco smoking is considered the most well-established risk factor for BC, accounting for ٥٠-٦٥٪ of male cases and ٢٠-٣٠٪ of female cases ***(van Osch et al., ٢٠١٦)***. The risk of BC development is increased with the number of cigarettes smoked per day and duration of smoking ***(Gallaway et al., ٢٠١٩)***.

It has been stated that there is a significant association for both current and former smokers ***(Gandini et al., ٢٠٠٨)***. Moreover, an immediate decrease in the risk of BC was observed after smoking

cessation. ٤٠٪ risk reduction within ١-٤ years of smoking cessation and ٦٠٪ after ٢٥ years of smoke quitting ***(Grotenhuis et al., ٢٠١٥)***.

Tobacco smoke contains aromatic amines, such as ٢-naphthylamine, and polycyclic aromatic hydrocarbons known to cause BC. These are excreted via kidneys and exert a carcinogenic effect on the entire urinary system ***(Burger et al., ٢٠١٣)***.

➢ Occupational exposure to chemicals

Occupational exposure is the second most important risk factor for BC. Work-related cases have accounted for ٢٠-٢٥٪ of all BC cases. The substances involved in chemical exposure include benzene derivatives and aryl amines (٢-naphthylamine, o-toluidine, and ٤،٤'-methylenedianiline). These chemicals present in occupations in which dyes, rubbers, textiles, paints, leathers, and chemicals are used ***(Daneshmand et al., ٢٠١٨)***.

After ١٠ years or more of occupational exposure to carcinogenic aromatic amines, the risk of BC is significantly elevated; the mean latency period usually exceeds ٣٠ years ***(Harling et al., ٢٠١٠)***.

The chemicals involved have contributed minimally to the current BC incidence in Western countries because of awareness and strict regulations. Population-based studies have revealed that the occupational factors for BC in men is ٧،١٪, whereas no such attribution has been established in women ***(Ramsden et al., ٢٠١٧)***.

➢ Dietary factors

Many dietary factors have been considered to be related to BC; however, the links remain controversial. Several studies have shown a positive relationship between the western dietary pattern and the risk of BC. Moreover, there are an inverse association between dietary intake of

flavonols and lignans and the risk of BC, especially aggressive tumors ***(Xiao et al., ٢٠١٧)***.

➢ Bladder schistosomiasis and chronic urinary tract infection

For many years, squamous cell carcinoma (SCC) of the urinary bladder has been known to be associated with Schistosoma haematobium infection ***(Ishida & Hsieh, ٢٠١٨),*** although a better control of the disease is decreasing the incidence of squamous carcinoma of the bladder in endemic zones such as Egypt ***(Salem & Mahfouz, ٢٠١٣)***. Haematobium eggs are deposited in the urinary bladder wall inducing a granulomatous host immune response and chronic inflammation with accumulation of eosinophils, lymphocytes and macrophages around the eggs ***(Moghadam & Nowroozi, ٢٠١٩).***

In addition, they release some metabolites, such as estrogen like metabolites and novel compounds that derived directly from ٨ oxo ٢′deoxyguanosine (a product of DNA oxidation), that might act as potential bladder carcinogens ***(Moghadam & Nowroozi, ٢٠١٩).***

➢ Medical conditions

Bladder cancer may occur as a consequence of exposure to ionizing radiation and many pharmaceutical agents ***(Burger et al., ٢٠١٣)***.

long-term use of cyclophosphamide, an alkylating agent mainly applied in lymphoma and leukemia, may increase BC incidence. Also, long-term use of Pioglitazone, an antidiabetic drug of the thiazolidinedione class has a weak relation to BC incidence ***(Cumberbatch & Noon, ٢٠١٩)***.

In addition, it has been found that exposure to radiotherapy for prostate, ovary and testis cancer treatment lead to developing second malignancies of the bladder ***(Cumberbatch & Noon, ٢٠١٩)***.

➢ Genetic factors

There is increasing evidence that there is a correlation between genetic susceptibility factors and family associations and the incidence of BC. It was found that family history of cancer in first-degree relatives was associated with an increased risk of BC; the association being stronger among younger patients ***(Burger et al., ٢٠١٣)***.

Genome-wide association studies (GWAS) of BC identified several susceptibility loci associated with BC risk. Polymorphisms in two carcinogen-metabolizing genes, N-acetyltransferases (NATS) and glutathione-S-transferase genes (GSTM١), have been related to BC risk, and furthermore they have demonstrated, together with UDP-glycosyl transferase (UGT١A٦), to confer additional risk to exposure of carcinogens such as tobacco smoking ***(García-Closas et al., ٢٠٠٥).***

Histopathology of bladder cancer:

➢ **Major pathologic subtypes**:

Bladder cancer is a malignancy of the urinary bladder lining. The most common type of bladder cancer is urothelial carcinoma (UC), formerly known as transitional cell carcinoma (TCC), which develops in the innermost cells **(Yaxley, ٢٠١٦)**.

UC is presented in male more than female cases and occurs at older ages (more than ٦٠ years) than SCC. It accounts for more than ٩٠٪ of bladder malignancies in industrialized countries as USA but for less than ٨٠٪ of cases in Southeast Asia and <٥٠٪ in some areas of Africa ***(Alderson et al., ٢٠٢٠).***

Other types of bladder cancer include squamous cell carcinoma (SCC), which accounts for ٢-٥٪ of the bladder cancer cases. Less common types of bladder cancer include adenocarcinoma (secretory cell carcinoma), small-cell carcinoma, lymphoma, and sarcoma ***(Felix et al., ٢٠٠٨)***.

About ٧٥ % of the patients present with non-muscle-invasive bladder cancer (NMIBC) at the time of diagnosis. It is defined as disease confined to the mucosa (stage Ta and Cancer in situ (CIS)) or submucosa (stage T١). Although these three types of lesions indeed are non-muscle-invasive, T١ and CIS lesions are distinct from Ta lesions since they have a high potential to become invasive (≈٥٠ % of CIS lesions progress if left untreated). Thus, accurate histopathologic assessment and diagnosis is crucial for correct clinical management which differs drastically between Muscle-invasive bladder cancer (MIBC) and NMIBC ***(Bray et al., ٢٠١٨)***.

➢ **Staging:**

The most widely used and universally accepted staging system is the tumor-node-metastases (TNM) system as detailed in Table I ***(Witjes et al., ٢٠٢١).***

Table (١): TNM classification of urinary bladder cancer from The American Joint Committee on Cancer (AJCC) Staging System (٨th edition, ٢٠١٧)

T - Primary Tumor	
Tx	Primary tumor cannot be assessed
T٠	No evidence of primary tumor
Ta	Non-invasive papillary carcinoma

Tis	Carcinoma in situ: “flat tumor”
T١	Tumor invades subepithelial connective tissue
T٢	Tumor invades muscle
T٢a	Tumor invades superficial muscle (inner half)
T٢b	Tumor invades deep muscle (outer half)
T٣	Tumor invades perivesical tissue
T٣a	Microscopically
T٣b	macroscopically (extravesical mass)
T٤	Tumor invades any of the following: prostate stroma, seminal vesicles, uterus, vagina, pelvic wall, abdominal wall
T٤a	Tumor invades prostate stroma, seminal vesicles, uterus, or vagina
T٤b	Tumor invades pelvic wall or abdominal wall
N - Regional Lymph Nodes	
Nx	Regional lymph nodes cannot be assessed
N٠	No regional lymph-node metastasis
N١	Metastasis in a single lymph node in the true pelvis (hypogastric, obturator, external iliac, or presacral)
N٢	Metastasis in multiple lymph nodes in the true pelvis (hypogastric, obturator, external iliac, or presacral)
N٣	Metastasis in common iliac lymph node(s)
M – Distant Metastasis	
M٠	No distant metastasis
M١	Distant metastasis

It should be emphasized that there are efforts to refine the subgrouping of patients in order to better predict the most suitable treatment for a given patient. Five new distinct MIBC subtypes (luminal-papillary, luminal infiltrated, luminal, basal/squamous, and neuronal) have been identified.

These subtypes do not necessarily reflect the histopathological appearance of BC, but are rather associated with specific pathway alterations and other biological features ***(Witjes et al., ٢٠٢١).***

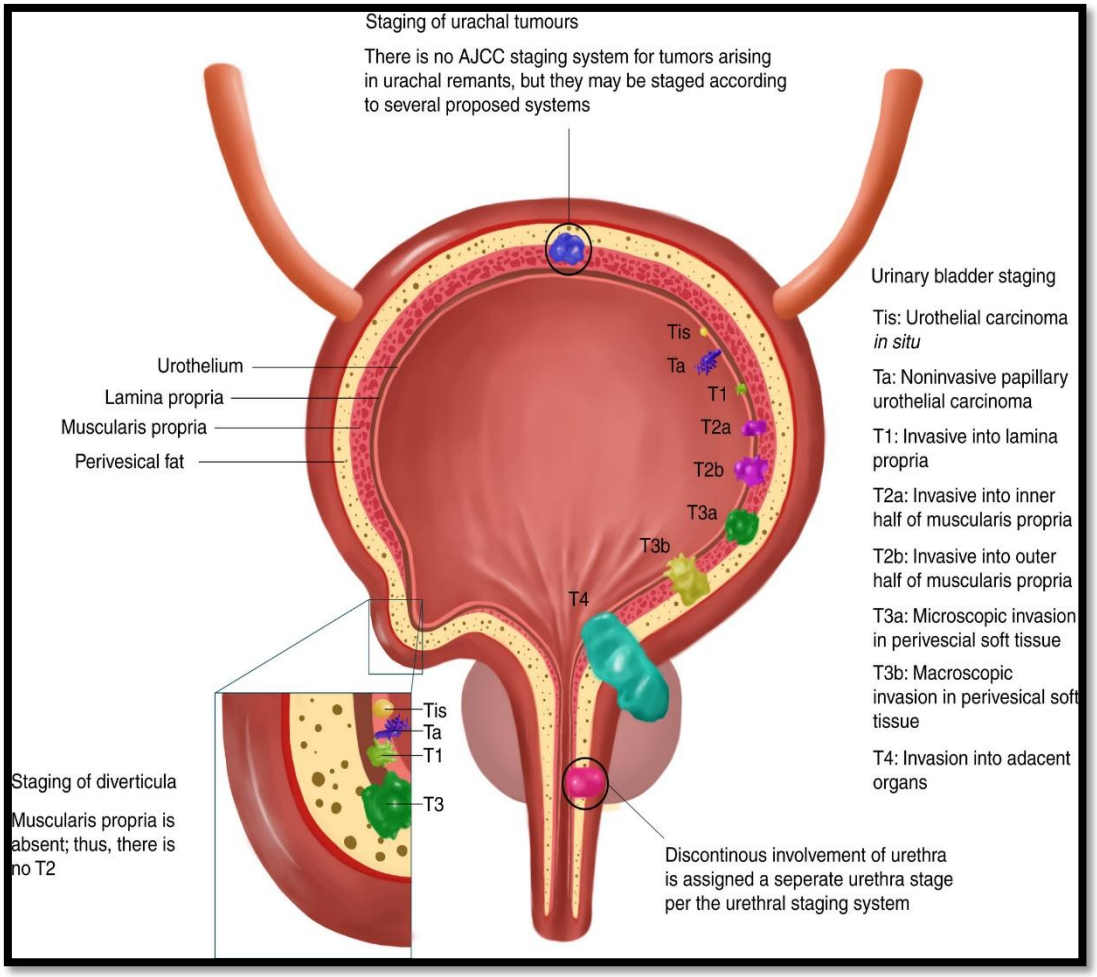


Figure (٢): Illustration of the bladder composition and tumor invasion by stage. Adapted from (Magers et al., ٢٠١٩).

➢ Tumor grading:

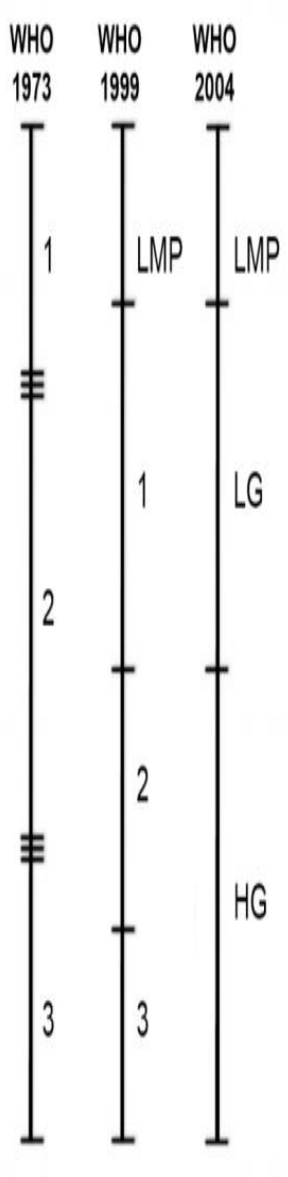
The tumor is also given a histologic grade based on the degree of cellular atypia, growth pattern, and mitotic activity. The heterogeneous behavior of non-muscle invasive bladder tumors is main reason for the importance of grading in clinical decision-making. While the tumor stage is the most important factor for treatment selection, the grade of the

tumor reflects the inherent aggressiveness of the tumor ***(Compérat et al., ٢٠١٨).***

The most frequently used grading system is the WHO/ISUP ٢٠٠٤/٢٠١٦ (International Society of Urological Pathology) grading system first proposed in ١٩٩٨, and updated in ٢٠٠٤ and ٢٠١٦. The WHO/ISUP system introduced the papillary urothelial neoplasm of low malignant potential (PUNLMP) category also used in the WHO ١٩٩٩ system, but differs from the ١٩٧٣ and ١٩٩٩ systems by separating tumors into only two categories; low grade (LG) and high grade (HG), with "high grade" largely equating to those of grade ٢ and ٣ in the WHO ١٩٩٩ system ***(Soukup et al., ٢٠١٧)***. Comparisons of the ١٩٧٣ and ٢٠٠٤ classification systems are shown in table (٢).

Table (٢): Bladder cancer grading
WHO ١٩٧٣ grading
Urothelial papilloma
Grade ١: (G١) Well differentiated

Grade ٢: (G٢) Moderately differentiated
Grade ٣: (G٣) Poorly differentiated
WHO ١٩٩٩ grading
Urothelial papilloma
Papillary urothelial neoplasm of low malignant potential (PUNLMP)
Low-grade urothelial carcinoma, Grade ١
High-grade urothelial carcinoma, Grade ٢
WHO ٢٠٠٤/٢٠١٦ grading
Urothelial papilloma
Papillary urothelial neoplasm of low malignant potential (PUNLMP)
Low-grade papillary urothelial carcinoma
High-grade papillary urothelial carcinoma



Diagnostic evaluation of BC:

١. Clinical picture

The main clinical presentation in bladder cancer is intermittent or persistent hematuria. ٧٨,٣٪ of patients present with macroscopic and ١٣,٧٪ of them present with microscopic hematuria ***(Ramirez et al., ٢٠١٦).*** Patients with macroscopic hematuria usually suffered from advanced pathological stage. Unfortunately, many patients with microscopic hematuria suffer from inadequate evaluation especially in the absence of active BC screening ***(Elias et al., ٢٠١٠).***

The second most common presentation in BC patients is that of a symptom complex of bladder irritability and urinary frequency, urgency and dysuria. These symptoms are usually associated with diffuse

carcinoma in situ (CIS) or invasive bladder cancer ***(Anastasiadis & de Reijke, ٢٠١٢)***.

Other uncommon symptoms include flank pain caused by ureteral obstruction, lower extremity edema and a palpable pelvic mass. Very rarely, patients present with symptoms of advanced disease, such as loss of weight and abdominal or bone pain from distant metastases. However, these symptoms almost occur with microscopic or macroscopic hematuria ***(Anastasiadis & de Reijke, ٢٠١٢)***.

Physical examination includes rectal and vaginal bimanual palpation. A palpable pelvic mass can be found in patients with locally advanced tumors. In addition, bimanual examination under anesthesia should be carried out before and after transurethral resection of the bladder tumor (TURB), to assess whether there is a palpable mass or if the tumor is fixed to the pelvic wall ***(Özbir et al., ٢٠١٤)***.

There is discrepancy between bimanual clinical examination and pathological (pT) stage after cystectomy (١١٪ clinical overstaging and ٣١٪ clinical understaging), so some caution is suggested with the interpretation of bimanual examination ***(Özbir et al., ٢٠١٤)***.

٢. Investigations:

A. Cytology: Urine cytology is an essential modality for the detection of urothelial neoplasia. It has long been known that urine cytology is accurate in the diagnosis of high-grade urothelial carcinoma. However, it carries a much lower diagnostic yield for low-grade urothelial neoplastic lesions ***(Brimo et al., ٢٠٠٩).*** Many studies have evaluated the accuracy of urine cytology in the detection of bladder cancer. Overall, the reported

sensitivity ranges from ٢٠٪ to ٩٧,٣٪; specificity ranges from ٧٤٪ to ٩٩,٥٪ ***(Brimo et al., ٢٠٠٩)***.

B. Cystoscopy and biopsy: Conventional or white-light cystoscopy is the "gold standard" for the detection of bladder cancer. A disadvantage of conventional cystoscopy is the difficulty in detecting flat lesions such as carcinoma in situ (CIS) ***(Zhu et al., ٢٠١٩)***. This has led to the development of newer technologies such as photodynamic diagnosis (PDD) and narrow-band imaging (NBI) cystoscopy (Cheung et al., ٢٠١٣b).

- **Photodynamic diagnosis (PDD):** Photodynamic diagnosis or fluorescence cystoscopy aims to improve the visualization of bladder cancer based on cystoscopic detection of fluorescent signals from neoplastic tissue. This fluorescence is accomplished by the intravesical administration of photosensitizing agents ٥-aminolevulenic acid or its derivative hexaminolevulinate which cause selective accumulation of photoactive porphyrins in rapidly proliferating cells (e.g., tumor cells) (Figure ٣) ***(Cauberg et al., ٢٠١١)***.

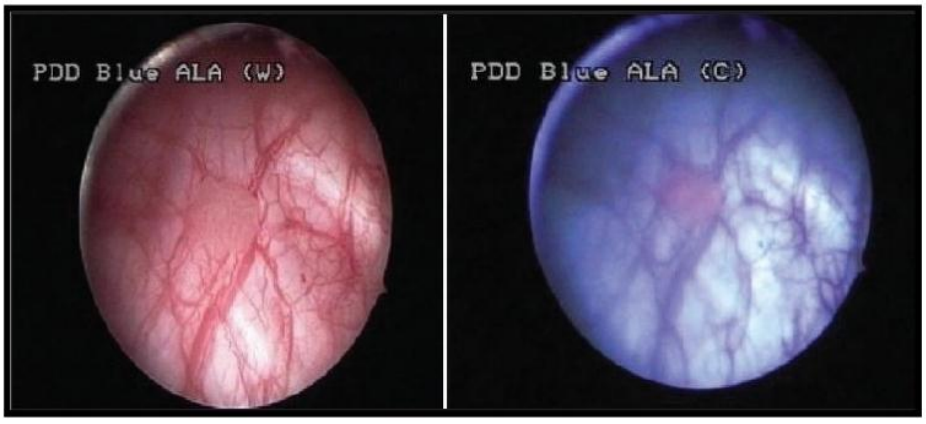


Figure (٣): Field of pTa G٢ bladder tumor with photodynamic diagnosis (right) versus white light cystoscopy (left) ***(Cauberg et al., ٢٠١١)***.

- **Narrow-band imaging (NBI):** Narrow-band imaging is a straight forward optical technique designed for endoscopy to enhance the visualization of (sub) mucosal vessels. The working mechanism is based on the filtering of white light into two narrow bandwidths of light that are centered around ٤١٥ nm (blue light) and ٥٤٠ nm (green light), which penetrate tissue only superficially and are specifically absorbed by hemoglobin ***(Song et al., ٢٠٠٨)***. Because bladder tumors tend to be well vascularised, narrow-band imaging will increase the contrast between these lesions and normal bladder mucosa (Figure ٣) ***(Cauberg et al., ٢٠١١).***

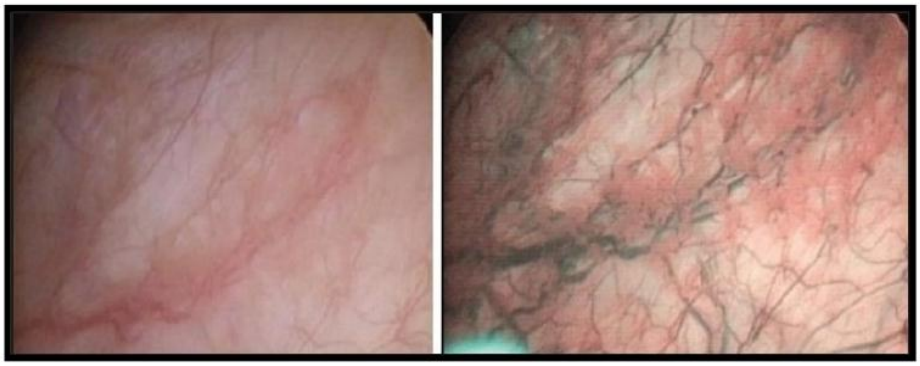


Figure (٤): *Field of pTa G ٢ bladder tumor with narrow-band imaging (right) versus white light cystoscopy (left)* ***(Cauberg et al., ٢٠١١).***

C. Imaging: Ultrasonography is being used more frequently and is advantageous because it does not require contrast ***agents (Jacobs et al., ٢٠١٠)***. Imaging with computerized tomography (CT) scan has essentially

replaced intravenous pyelograms (IVP) in many centers. Magnetic resonance imaging (MRI) had been a reliable modality for patients with renal failure. CT scan and MRI may be used to determine the stage of bladder cancer; however, they are unable to accurately detect early

metastatic disease particularly in lymph nodes less than ١٠ mm ***(Jacobs et al., ٢٠١٠)***.

D. Molecular markers: Numerous urinary markers have been investigated, with the aim of reducing frequency of cystoscopy ***(Yutkin et al., ٢٠١٠)***. Several are commercially available, but none has been adopted into routine standard of care, owing to poor sensitivity and/or expense. These markers may serve as an adjunctive diagnostic test in cases where urine cytology is equivocal ***(Cheung et al., ٢٠١٣)***.

- **Fluorescence in situ hybridization (FISH):** FISH can be used to detect urinary cells that have chromosomal abnormalities consistent with a diagnosis of bladder cancer. For example, The UroVysion Bladder Cancer Kit (UroVysion Kit) uses fluorescently labeled DNA probes to detect aneuploidy in chromosomes ٣, ٧, and ١٧ and loss of the ٩ p٢١ locus of the P١٦ tumor suppressor gene ***(Cheung et al., ٢٠١٣)***.
- **Microsatellite analysis (MSA):** Microsatellites are highly polymorphic, short, tandem DNA repeats found in the human genome. Two types of microsatellite alterations can be found in many cancers: loss of heterozygosity (LOH), an allelelic deletion, and somatic alteration of microsatellite repeat length ***(Vrooman & Witjes, ٢٠٠٨)***. In bladder cancer, most mutations are in the form of LOH. Microsatellite alterations in exfoliated urine are detected by a polymerase chain reaction (PCR) using DNA primers for a panel of known microsatellite markers ***(Vrooman & Witjes, ٢٠٠٨)***.
- **Immunocyte markers:** Immunocytology is based on the visualization of tumor associated antigens in urothelial carcinoma cells using monoclonal antibodies. Three fluorescently marked antibodies label

two mucin like proteins and a high molecular weight form of carcinoembryonic antigen. After this process the cells are examined under a fluorescent microscope ***(Vrooman & Witjes, ٢٠٠٨)***.

- **Telomerase:** Telomeres are repetitious sequences at the end of chromosomes that protect genetic stability during DNA replication. There is loss of telomeres during each cell division, which causes chromosomal instability and cellular senescence. Bladder cancer cells express telomerase, an enzyme that regenerates telomeres at the end of each DNA replication and therefore sets the cellular clock to immortality. Determination of telomerase activity is a PCR-based technology and must be performed in specialized laboratories ***(Vrooman & Witjes, ٢٠٠٨)***.
- **Bladder tumor antigen (BTA) BTA-TRAKTM and BTA-statTM:** BTA-TRAK and BTA-stat (Alidex Inc, Redmond, WA, USA) are both versions of the bladder tumor antigen assay that measures complement factor H–related protein in urine ***(Vrooman & Witjes, ٢٠٠٨)***.
- **Hyaluronic acid and hyaluronidase:** Hyaluronic acid (HA) is a glycosaminoglycan and a normal component of tissue matrices and body fluids. In tumor tissues, elevated HA is mostly localized to tumor stroma. In bladder carcinoma HA is found in tumor cells, and elevated HA levels have been shown in urinary samples of bladder cancer patients ***(Lokeshwar et al., ٢٠٠٥)***. The concentration of HA is also associated with tumor metastases ***(Lokeshwar et al., ٢٠٠٥)***. Hyaluronidase (HA-ase) is an enzyme that cleaves HA into fragments. HA-ase levels are elevated in bladder tumor tissue, and an increase is correlated with tumor grade ***(Vrooman & Witjes, ٢٠٠٨)***.

- **Nuclear matrix protein ٢٢ (NMP٢٢):** NMP٢٢ is a nuclear matrix protein and is an important regulator of mitosis. In tumor cells the nuclear mitotic apparatus is elevated and NMP٢٢ is released from cells in detectable levels ***(Vrooman & Witjes, ٢٠٠٨)***.
- **Cytokeratins:** Cytokeratins are intermediate filaments; their main function is to enable cells to withstand mechanical stress. In humans ٢٠ different cytokeratin isotypes have been identified. Cytokeratins ٨, ١٨, ١٩, and ٢٠ have been associated with bladder cancer. The Urinary Bladder Cancer test detects cytokeratin ٨ and ١٨ fragments in urine ***(Vrooman and Witjes, ٢٠٠٨)***. Cytokeratin ١٩ fragment (CYFRA ٢١-١) is a soluble fragment of cytokeratin ١٩, is analyzed with Enzyme-linked immunosorbent assay (ELISA), and is measurable in serum and urine (Y. L. Huang et al., ٢٠١٥).
- **Survivin:** Survivin is a member of the family of proteins that regulate cell death, the so-called inhibitor of apoptosis family. Its overexpression inhibits extrinsic and intrinsic pathways of apoptosis ***(Moussa et al., ٢٠٠٨)***. Survivin is expressed during fetal development but not in terminally differentiated adult tissues. However, it is one of the most commonly overexpressed genes in cancer. In bladder cancer, survivin is expressed in urine, and its expression is associated with disease recurrence, stage, progression and mortality ***(Shariat et al., ٢٠٠٧)***. Reverse transcription polymerase chain reaction (RT-PCR) provides a diagnostic tool to detect surviving messenger RNA (mRNA) in urine ***(Vrooman and Witjes, ٢٠٠٨)***.

E. microRNA: microRNAs are receiving growing attention because of numerous reports on their dysregulation in human diseases and their potential as diagnostic and therapeutic targets. Because of their stability

and presence in almost all body fluids, miRNAs constitute a novel class of noninvasive biomarkers ***(Brase et al., ٢٠١١)***.

Some miRNAs have been reported to be up-regulated in bladder cancer tissues. For example, miR-١٢٩ was the most commonly upregulated and its up-regulation was associated with poor outcome ***(Dyrskjøt et al., ٢٠٠٩)***; the expression of miR-٩٦ and miR-١٨٣ in urine were significantly correlated with tumor stage and grade, and their expressions were significantly decreased after radical surgery ***(Yamada et al., ٢٠١١)***; miR-١٣٣b and miR-٥١٨c were also strongly up-regulated in bladder cancer tissues ***(Dyrskjøt et al., ٢٠٠٩)***.

Meanwhile, some miRNAs were reported to be downregulated in cancer tissues and might function as tumor suppressors. miR-٢٠٠ family members were lower in urine sediment of bladder cancer patients and increased significantly following surgery which suggested this microRNA family could be used as diagnostic and prognostic markers of bladder cancer ***(Wang et al., ٢٠١٢)***. These aberrant expression miRNAs in bladder cancer are attractive as potential biomarkers and new targets for bladder cancer therapy ***(Feng et al., ٢٠١٤)***.

F. Metastatic work-up: For invasive bladder tumors, metastatic evaluation should include chest radiography, liver function tests, and alkaline phosphatase ***(Kirkali et al., ٢٠٠٥)***. Abdominal and pelvic imaging (MRI or CT) is not accurate for staging of the primary bladder tumor but may be useful when metastatic disease is suspected. A bone scan is unnecessary in all cases, but it should be performed in the presence of bone pain or elevated alkaline phosphatase ***(Kirkali et al., ٢٠٠٥)***.

Briefly, the diagnostic work-up includes, but is not limited to, physical examination, imaging and transurethral resection of the bladder tumor (TURBT) with subsequent histological evaluation. In patients with confirmed MIBC, Computer tomography (CT) of the chest, abdomen and pelvis is currently used for staging due to yet insufficient data supporting advantages of FDGPET/CT ***(Bray et al., ٢٠١٨)***.

Treatment of bladder cancer:

Because of the unpredictable disease course, high recurrence rate, and risk of progression, patients with bladder cancer require continuous, costly, follow-up monitoring which poses a burden both to the patient and the healthcare system. This makes bladder cancer one of the most expensive malignancies per patient ***(Yeung et al., ٢٠١٤)***.

The tumor stage is closely related to patient outcome and is the crucial factor for treatment selection. For patients with stage Ta, Tis, and T١ tumors the preferred treatment choice is local resection of the tumor, paired most commonly with intravesical immunotherapy in the form of Bacillus Calmette-Guerin (BCG) instillations or intravesical cytostatic chemotherapy using mitomycin C, a DNA crosslinking drug, or DNA intercalating agents such as epirubicin or pirarubicin ***(Svatek et al., ٢٠١٤)***. Intravesical therapy is particularly well suited for NMI bladder cancers because of their superficial confinement as well as the anatomical properties of the bladder, and aims to reduce the risk of recurrence and progression ***(Svatek et al., ٢٠١٤)***. Chemotherapy has been shown to significantly reduce the rate of recurrences, but shows little effect on the rate of progression ***(Sylvester et al., ٢٠١٦)***.

BCG was developed as a live attenuated vaccine against tuberculosis in the beginning of the ٢٠th century. In the ١٩٣٠s it was noted that tuberculosis patients had a lower cancer incidence ***(Alhunaidi & Zlotta, ٢٠١٩)***, and BCG was proposed as a potential cancer therapy. The first use of BCG for the treatment of bladder cancer was reported in ١٩٧٦ ***(Morales et al., ١٩٧٦)***.

Intravesical chemotherapy and BCG both significantly reduces the rate of tumor recurrences, but the reduction is greater with the use of BCG. BCG also has the advantage of reducing the rate of disease progression, and is considered the superior treatment of choice both for high and intermediate risk NMI bladder cancer ***(Spencer et al., ٢٠١٣)***. The use of BCG is primarily limited by the eligibility of the patients due to toxicity, underutilization by clinicians, as well as a recent supply shortage associated with its production ***(Mostafid et al., ٢٠١٥)***.

The mode of action of BCG immunotherapy is complex and not fully understood, depending on molecular interactions between the patient, the immune system, and the tumor ***(Redelman-Sidi et al., ٢٠١٤)***. While BCG is effective, the treatment fails in up to ٣٠-٤٠٪ of patients ***(Nepple et al., ٢٠١٠)***. It has been reported that the survival rate for patients progressing from NMI to muscle invasive disease may be worse than for patients with MIBC without a history of NMIBC ***(Van Den Bosch & Witjes, ٢٠١١)***.

The most appropriate treatment method for high risk T١ and operable primary or progressed muscle invasive tumors (stage ≥T٢) is radical cystectomy (RC). This is a surgery with curative intent involving removal of the urinary bladder and pelvic lymphadenectomy, and is

always accompanied by prostatectomy in males or usually hysterectomy in females. If the patient is eligible, the cystectomy may be paired by systemic neoadjuvant chemotherapy (NAC) to target potential micrometastatic disease prior to surgery, which has been shown to provide a ٥-٨٪ increase in overall survival (OS) and a ٩٪ increase in cancer specific survival (CSS) ***(Yin et al., ٢٠١٦)***.

The chemotherapy mainly consists of cisplatin-based combination e.g. gemcitabine and cisplatin (GC), or methotrexate, vinblastine, adriamycin, and cisplatin (MVAC). These regimes are sometimes also used in the adjuvant setting ***(Zargar et al., ٢٠١٨)***.

Open radical cystectomy (ORC) is the current gold-standard treatment for MIBC and for high-risk recurrent NMIBC. Ideally, all patients with MIBC should receive platinum-based neo-adjuvant chemotherapy ***(Grossman et al., ٢٠٠٣)***. The ٥-year survival after cystectomy is about ٥٠٪. Advanced bladder cancer metastasizes to lymph nodes, bones, lungs or liver and these patients can be treated with cisplatin systemically ***(Shah et al., ٢٠١١)***.

ORC has a peri-operative complication rate of ٢٥ to ٦٢٪. Therefore, minimally invasive techniques such as laparoscopic radical cystectomy (LRC) have been explored ***(Cheung et al., ٢٠١٣)***. The advantages of LRC include decreased blood loss, reduced postoperative pain, early return of bowel function and shorter hospital stay ***(Haber & Gill, ٢٠٠٧)***. Furthermore, LRC has good early oncologic outcomes with low morbidity in large cohorts with up to ٥ years follow up ***(Shah et al., ٢٠١١)***.

Chapter (II):

MicroRNA ٩٦ and ١٢٦

Definition of microRNA(miRNA)

miRNAs are endogenous, non-coding short RNA molecules ~٢٢ nucleotides (nt) long that are abundantly expressed across organs and species. They are considered to regulate the cleavage of target mRNAs or just repress their translation at posttranscriptional level.

According to miRNA Data base ***(miRBase, www.mirbase.org)***, December ٢٠١٦, there are ٢٥٨٨ mature human miRNAs annotated as well as ١٨٨١ human precursor miRNAs. It is hypothesized that mature miRNAs regulate transcription of more than ٦٠٪ of all protein coding genes ***(Martinez et al.,*** ٢٠١٩), and thereby regulate many developmental and cellular processes in the eukaryotic organism. It has to be taken into consideration that one miRNA can regulate and influence hundreds of mRNAs ***(O'Brien et al., ٢٠١٨)***.

Discovery of miRNAs

Lee et al. (١٩٩٣) discovered the first miRNA lin-٤ during a study of developmental timing in Caenorhabditis elegans (C. elegans). About few years later, a second miRNA lethal-٧ (let-٧) was characterized ***(Reinhart et al., ٢٠٠٠)***.

These two discoveries of miRNA encouraged further studies on discovery of new miRNAs, and consequently a large class of small non-coding RNAs emerged with a diverse range of biological functions, such as temporal regulation of development, cell death and proliferation, hematopoiesis and tumorigenesis ***(Kim & Bae, ٢٠٠٨)***.

Over ٢٥٠٠ miRNAs have been identified in the human genome, which are thought to regulate more than ٣٠٪ of the protein-coding genes ***(MacFarlane & R. Murphy, ٢٠١٠).***

Nomenclature

The name for a miRNA consists of the prefix “mir” followed by a dash and a number. The number indicates order of naming. For example, miR-١٢٣ was named and discovered prior to miR-٤٥٦. The uncapitalized (r) in "mir-" refers to immature miRNA, while a capitalized (R) in "miR" refers to the mature form. miRNAs with nearly identical sequences except for one or two nucleotides are annotated with an additional lower case letter. For example, miR-١٢٣a is closely related to miR-١٢٣b. Precursor-miRNAs (pre-miRNA) that lead to ١٠٠٪ identical mature miRNAs but that are located at different places in the genome are indicated with an additional dash-number suffix ***(Gonzalez et al., ٢٠١٤)***. For example, the premiRNAs mir-١٩٤-١ and mir-١٩٤-٢ lead to an identical mature miRNA (miR-١٩٤) but are located in different regions of the genome (Figure ٤) ***(Gonzalez et al., ٢٠١٤)***.

Species of origin is designated with a three-letter prefix, e.g., *Homo sapien*-miR-١٢٣ is a human miRNA (hsa-miR-١٢٣) and *Ovis aries*-miR-١٢٣ is a sheep miRNA (oar-miR-١٢٣). Other common prefixes include 'v' for viral miRNA encoded by a viral genome and 'd' for *Drosophila* miRNA (for example d-mir-٢٨١in *Drosophila*).When two mature microRNAs originate from opposite arms of the same premiRNA, they are denoted with a -٣p (٣′ arm) or -٥p (٥′ arm) suffix. In the past, this distinction was also made with 's' (sense) and 'as' (antisense) ***(Griffiths-Jones et al., ٢٠٠٦)***.

When relative expression levels are known, an asterisk following the name indicates a miRNA expressed at low levels relative to the miRNA in the opposite arm of a hairpin, for example, miR-١٢٣ and miR-١٢٣* would share a pre-miRNA hairpin, but more miR-١٢٣ would be found in the cell ***(Griffiths-Jones et al., ٢٠٠٦).***

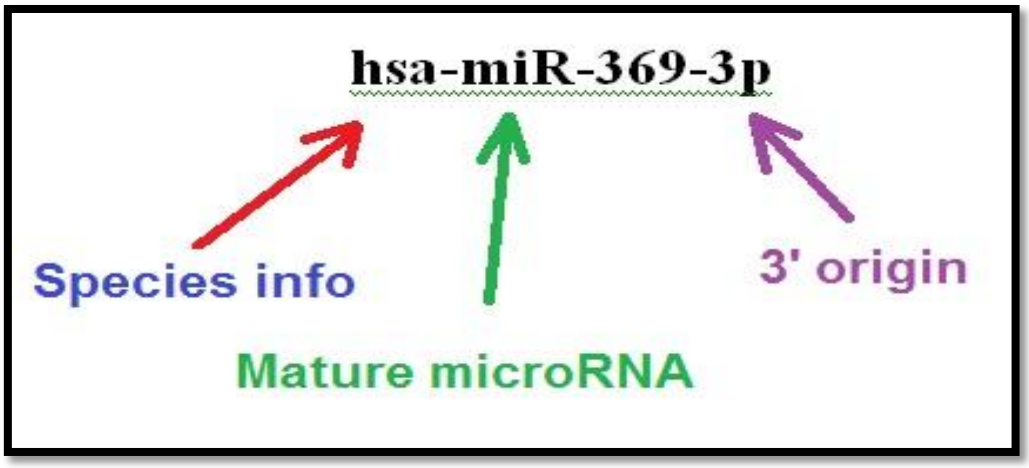


Figure (٥): *Nomenclature of miRNA*

http://blog-biosyn.com/٢٠١٣/٠٤/١١/how-can-microrna s-be-descriped/

miRNA classification:

According to genomic organization, miRNA genes can be classified as intergenic and intragenic miRNA gene. Intergenic miRNAs have independent transcription units (TUs), including promoter, transcript sequence, and terminator units, and they do not overlap with other genes ***(Yang et al., ٢٠١٢)***. However, intragenic miRNAs are located within other TUs (host genes) within either intronic or exonic regions ***(Olena & Patton, ٢٠١٠)***.

In addition, some miRNAs were located in both intergenic and intragenic regions and were denoted as mixed miRNAs ***(Jiu et al., ٢٠١٥).***

Biogenesis of miRNAs

MiRNAs can either be transcribed from specific genes or they can be expressed from introns of protein-coding genes. In Eukaryotes, miRNAs biogenesis begins when RNA polymerase II transcribes a primary product that is several thousand nucleotides long which contains stem–loop structures (pri-miRNA). Following transcription, cleavage of this pri-miRNA occurs in nucleus and pre-miRNA with a small hairpin structure is liberated. This step is performed by the nuclear RNAse III-endonuclease Drosha ***(Tétreault & De Guire, ٢٠١٣)***.

After its cleavage in nucleus, pre-miRNA is transported to the cytoplasm via a nucleo/cytoplasmic cargo transporter, Exportin-٥, as demonstrated in Figure ١,١٣. Another RNAse III-endonuclease located in cytosol, Dicer, cleaves the pre-miRNA hairpin structure into a small double-stranded RNA duplex that is made of a mature RNA stand and its complementary strand. These two strands are also termed as miR-٥p and miR-٣p ***(Tétreault and De Guire, ٢٠١٣)***.

Upon cleavage, the double stranded duplex generated by Dicer is loaded into an AGO protein and RNA-induced silencing complex (RISC) is formed. AGO proteins are part of the Argounate family proteins. As the integral parts of the RISC complex, they bind to small non-coding RNAs and play role in RNA silencing as effector molecules. When the AGO protein associates with RNA duplex, the pre-RISC complex is formed. In pre-RISC, the complementary strand of the duplex is degraded and mature RISC is generated ***(Ha & Kim, ٢٠١٤)***.

The RISC complex chooses the RNA strand with the lowest thermodynamic stability at its ٥' end as the guide strand, while the complementary strand is often degraded. The guide strand leads RISC to its target ***(Yamamoto et al., ٢٠١٧)***.

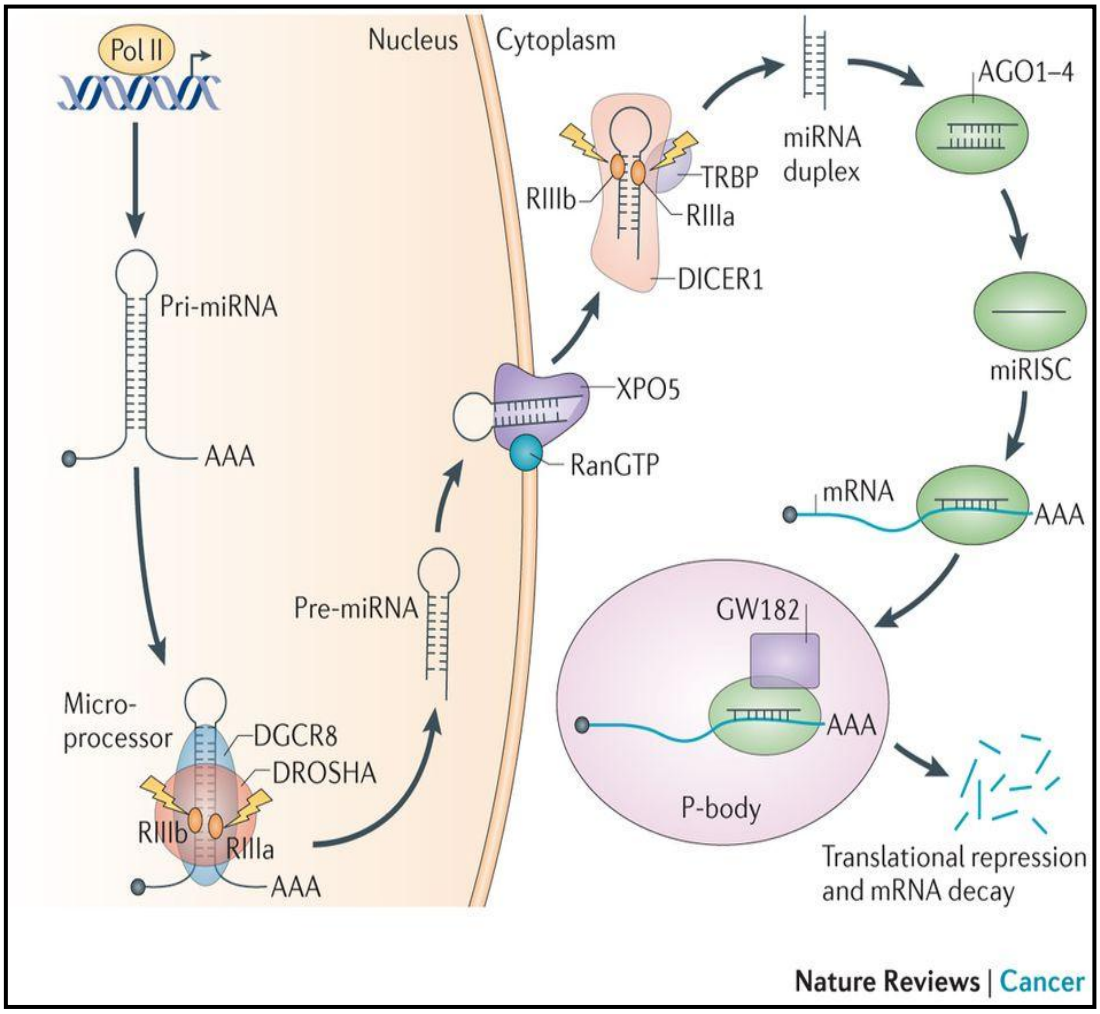


***Figure (٥)**: Biogenesis of miRNAs adopted from **(Ha and Kim, ٢٠١٤)**.*

Mechanisms of miRNAs regulation:

Regulation of microRNA expression can be exerted through several mechanisms, which result to be altered in human diseases, including cancer: chromosomal abnormalities, as suggested by the

evidence that microRNAs are frequently located in regions of the genome involved in alterations in cancer ***(Iorio & Croce, ٢٠١٢)***.

The deregulated microRNA expression in cancer can also be due to epigenetic changes, as altered DNA methylation. An extensive analysis of genomic sequences of miRNA genes have shown that approximately half of them are associated with CpG islands, suggesting that they could be subjected to this mechanism of regulation ***(Weber et al., ٢٠٠٧)***.

Several reports have indeed shown that aberrant methylation can result in aberrant miRNA expression in cancer. ***Saito and colleagues (٢٠٠٩)*** reported a strong upregulation of miR-١٢٧, a miRNA characterized by a CpG island promoter able to target the proto oncogene BCL-٦, found silenced in several cancer cells. Hypermethylation and down-modulation of miR-٩-١ has been described in breast cancer ***(Lehmann et al., ٢٠٠٨)***.

Conversely, upregulation of putative oncogenic miRNAs can result from DNA hypomethylation, as shown in lung adenocarcinoma or epithelial ovarian cancer for miR-٢١ ***(Brueckner et al., ٢٠٠٧)***. Methylation is not the only epigenetic modification that can affect miRNAs expression. ***Scott et al., (٢٠٠٦)*** showed that in breast carcinoma cells, an extensive and rapid alteration of miRNA levels followed histone deacetylase inhibition.

Finally, miRNA dysregulation can result from increased or decreased transcription activity of a transcription factor at the promoter. The miR-٣٤a, miR-٣٤b and miR-٣٤c family of miRNAs, for instance, was shown to be directly induced by the tumor suppressor p٥٣ and to be

partially responsible of the phenotype induced by this oncosuppressor ***(Chang, ٢٠٠٨).***

Nevertheless, despite the advances in our understanding of the mechanisms causing miRNA deregulation, the daunting task still remains the elucidation of the biological role of miRNAs in the initiation and in the development of cancer ***(Iorio and Croce, ٢٠١٢).***

Biological functions of miRNA

MiRNAs were found to regulate gene expression by targeting mRNAs through sequence specific targeting, resulting in mRNA degradation or translational repression. The recognition of the target mRNA is based on the complementarity of seven to eight nucleotides at the ٥'-end of the miRNA (seed sequence) to the specific motif along the ٣'-untranslated sequence of the target mRNA ***(O'Brien et al., ٢٠١٨)***. Perfect, or nearly perfect, complementarity can induce degradation of the mRNA, while imperfect base pairing can result in translational inhibition ***(O'Brien et al., ٢٠١٨)***.

Translational repression occurs more frequently and is mediated by blocking the initiation step therefore affecting mRNA stability ***(Guo et al., ٢٠١٠)***. MiRNAs have also been described as having decoy abilities, which is a less studied mechanism. In a decoy state, the miRNA would interfere with the function of proteins by preventing interaction between coordinating mRNAs ***(Eiring et al., ٢٠١٠)***.

miRNA and cancer:

The dysregulation of miRNAs in cancer was first reported in ٢٠٠٢, with the discovery of miR-١٥ and miR-١٦ being frequently

deleted in chronic lymphocytic leukemia (CLL) ***(Almeida et al., ٢٠١١)***. Since this discovery was made there have been several studies showing differential miRNA expression profiles in tumor versus normal tissues ***(Almeida et al., ٢٠١١)***.

MiRNAs have even been implicated in the initiation and progression of many cancers, suggesting that they play a key role in cancer biology ***(Croce, ٢٠٠٩)***.

Several studies have also shown that the overexpression of a single miRNA is sufficient to initiate tumor development ***(Medina et al., ٢٠١٠)***. Deregulated miRNAs can also influence tumorigenesis by decreasing or increasing the inhibition of their mRNA targets, which has led to the identification of oncogenic miRNAs and tumor suppressive miRNAs ***(Reinhart et al., ٢٠٠٠)***.

Oncogenic miRNAs are often upregulated in tumor tissues and target tumor suppressor genes, while tumor suppressive miRNAs are frequently downregulated in cancer and target oncogenes. However, subsequent studies have shown that miRNAs are more complex and may have dual functions depending on tumor type or stage in progression. While miRNAs are thought to act on downstream signaling, they are often involved in feedback loops, causing the expression of miRNA families to be regulated by transcription factors ***(Syeda et al., ٢٠٢٠)***.

MiRNA expression can also be regulated by epigenetic modifications, as demonstrated by miRNA expression changes after exposing cells to DNA methylation inhibitors and histone deacetylase inhibitors ***(Chuang & Jones, ٢٠٠٧)***.

While epigenetic modifications can control miRNAs, miRNAs have also been implicated in controlling epigenetic changes. In cancer, miRNAs have been shown to play an important role in controlling

metastasis, which is one of the primary causes of cancer-related deaths. There are multiple steps involved in the metastatic process, in which miRNAs can either promote or inhibit metastasis ***(Nicoloso et al., ٢٠٠٩)***.

For example, miRNA-١٠b has been shown to positively regulate migration and invasion, and is capable of initiating metastasis in mice by targeting repressors of metastasis ***(Ma et al., ٢٠٠٧)***.

However, more miRNAs have been implicated in metastasis suppression, including miR-٣٣٥, miR-١٢٦, miR-٢٠٦, and the miR-٢٠٠ family, by targeting transcription factors involved in metastasis initiation ***(Gregory et al., ٢٠٠٨)***.

➢ MicroRNA-٩٦

MiR-٩٦ belongs to the miR- ١٨٣ family ***(Yang & Liu, ٢٠٢٠)***. The coding sequence of miR-٩٦ is located in chromatin ٧q٣٢,٢, between two protein-coding regions. miRNA-٩٦ is one member of the miR-١٨٣-٩٦-١٨٢ cluster ***(Zhu et al., ٢٠١٩)***. miR-٩٦ has been found to be upregulated in various human cancers such as breast, lung, liver, colon, prostate, ovary, testis cancer and lymphoma, suggesting the expression of miR-٩٦ was associated with the progression of tumor ***(Yang et al., ٢٠١٩)***. It promotes the proliferation of esophageal cancer ***(Xia et al., ٢٠١٥)***, functions as a tumor suppressor gene in pancreatic cancer ***(Huang et al., ٢٠١٤)***, and promotes cell proliferation in prostate cancer cells. These results implied that miR-٩٦ is an onco-miRNA and might be a potential target of gene therapy of some human cancers ***(Abd-Aziz et al., ٢٠٢٠).***

The target genes of miR-٩٦ include the tumor suppressor genes *FOXO١* and *FOXO٣a* in breast cancer ***(Li et al., ٢٠١٧)*** and other validated targets of miR-٩٦ include *RECK* in esophageal cancer ***(Xia et***

al., ٢٠١٥), *EphrinA٥* in Hepatocellular carcinoma ***(Wang et al., ٢٠١٦)***, *SAMD٩* in non-small cell lung cancer (NSCLC) ***(Wu et al., ٢٠١٦)***.

Transforming growth factor β (TGF β) receptor signaling pathway plays a significant role during various biological processes, including cell proliferation, apoptosis, phenotype modification, and formation of neoplasms ***(Seoane & Gomis, ٢٠١٧)***. Previous study identified that miR ٩٦ is a key factor in metastatic prostate cancer in response to TGF β signaling ***(Siu et al., ٢٠١٥)***.

Forkhead box O٣ (FOXO٣, also known as FOXO٣a) functions as a trigger for apoptosis through the upregulation of genes necessary for cell death ***(Anderson et al., ١٩٩٨).*** miR- ٩٦ has recently been found to directly bind to the ٣′- untranslated region (UTR) of FOXO٣ messenger RNA, which subsequently inhibits its function ***(Wu et al., ٢٠١٦)***.

The FOXO subfamily of forkhead transcription factors, including FOXO١, FOXO٣a, FOXO٤, and FOXO٦ contains evolutionarily conserved transcriptional activators that are characterized by a highly conserved forkhead domain with a DNA-binding motif ***(Y. Wang et al., ٢٠١٤).***

FOXO proteins play a pivotal role in biological processes, such as apoptosis, cell cycle control, differentiation, stress response, DNA damage repair, and glucose metabolism ***(Farhan et al., ٢٠١٧).*** Inhibition of the transcriptional activity of FOXO٣a protein in human breast cancer cells can promote cell transformation and tumor progression (Y. Jiang et al., ٢٠١٣).

Furthermore, miR-٩٦ represses neural induction from human embryonic stem cells, suggesting that miR-٩٦ may play a role in the maintenance of pluripotency or the self-renewal of embryonic stem cells ***(Du et al., ٢٠١٣)***.

➢ MicroRNA-١٢٦

MicroRNA-١٢٦ originates from the precursor molecule pre-miR-١٢٦. Unlike many miRNAs, passenger strand of miR ١٢٦-٣p, miR ١٢٦-٥p (also shown as miR-١٢٦*) is not degraded ***(Meister & Schmidt, ٢٠١٠)***.

The host gene of miR ١٢٦, epidermal growth factor (EGF)-like domain ٧ (*EGFL٧*) is located on chromosome ٩q٣٤,٣ in humans. Along with the two miRNAs mentioned before, it encodes EGFL٧ that is highly conserved in vertebrates. The ٤١ kDA angiogenic factor EGFL٧ is specific to endothelial cells. It acts solely on endothelial cells, binds to components of the extracellular matrix and regulates blood vessel development via Notch signaling ***(Chistiakov et al., ٢٠١٦)***.

Although miR-١٢٦ upregulation was reported to be associated with oxidative stress, the mechanism miR-١٢٦ upregulation is not clear ***(Matsuzaki & Ochiya, ٢٠١٨)***.

In this context, aberrant promoter methylation is one of the mechanisms that regulate gene expression ***(Cui et al., ٢٠١٦).*** DNA methyl transferases (DNMT١, DNMT٣A and DNMT٣B) in combination with the ten-eleven translocation (TET) family of proteins that catalyze demethylation, have been reported to regulate stress-induced miR-١٢٦ expression ***(Gaetani et al., ٢٠٢٠).***

MiR-١٢٦ is up-regulated in embryonic stem cell- derived endothelial progenitor cells during embryonic developmental stages. In the embryogenic stage, for generation of initial vascular plexus, VEGF is

needed, and VEGF-driven angiogenesis is modulated by miR-١٢٦. The VEGF signaling suppressor molecules, Sprouty-related protein SPRED١ and phosphoinositol-٣ kinase regulatory subunit ٢ (PIK٣R٢) are targeted by miR-١٢٦ ***(Fish et al., ٢٠٠٨)***.

The inhibition of *Pik٣r٢* results in an up-regulation in angiopoietin-١, which functions as a proangiogenic signaling factor with a role in stabilizing and maturation processes of the vessels, which is normally down-regulated by PI٣K/Akt signaling ***(Jiang et al., ٢٠٢٠)***.

MiR ١٢٦-٣p is highly expressed in vascularized tissues such as heart, liver and lungs. Expression studies demonstrated its specific expression in the endothelial cell lineage, hematopoietic progenitor cells and endothelial cell lines ***(Meister and Schmidt, ٢٠١٠)***.

Deletion of MiR-١٢٦ in mice (without altering Egfl٧ expression) results in major defects in blood vessel development, such as delays in angiogenesis, hemorrhage and early embryonic lethality ***(Kuhnert et al., ٢٠٠٨)***.

The observation of similar defects when studies were performed with loss of VEGF signaling in mice and zebrafish suggests that miR-١٢٦ modulates VEGF signaling. In endothelial cells, decreased phosphorylation of AKT and ERK١/٢ in response to VEGF treatment was observed in knockdown of miR-١٢٦. MiR-١٢٦ targets PIK٣R٢ and SPRED١, the negative regulators of VEGF pathway ***(Wu et al., ٢٠١٩).***

In adults, the roles of miR-١٢٦ are quite different ***(Chistiakov et al., ٢٠١٦)***. MiR-١٢٦ induces angiogenesis, as well as vascular tissue remodeling in response to vascular injury. It has been demonstrated by ***Voellenkle et al. (٢٠١٢)***, under hypoxic conditions, the levels of miR-١٢٦ increase.

The protein sequence of EGFL٧ is composed of a putative amino-terminal signal peptide domain, an Emilin-like domain (EMI) and ٢ centrally located EGF-like domains; the centrally located one for Notch receptor-ligand interactions and the distal one for Ca٢+-binding as can be seen in Figure ١,١٦. These protein motifs are often found in secreted and extracellular matrix–bound proteins. EGFL٧ expression is highest during embryogenic development while after birth expression is down regulated in the vascular system. In adults, significant protein levels are maintained only in vascularized tissues such as the lung, heart, kidney, spleen and uterus. In cases of physiological and pathological angiogenesis, increase in expression levels is observed ***(Nichol & Stuhlmann, ٢٠١٢)***.

The role of *EGFL٧* in sprouting angiogenesis has been demonstrated by studies conducted on *EGFL٧* knock-out mice, in which endothelial cells without EGFL٧ had impaired migration and created clumps and over-sized sprouts instead of proper vessel sprouts. These findings suggested that EGFL٧ created an environment where cells can be properly positioned to construct new vessels. A study conducted by ***Bill et al. (٢٠١٩)*** has provided a link between Notch signaling and EGFL٧ by showing the binding of EGFL٧ to NOTCH receptors. EGFL٧ is suggested to compete with NOTCH ligands and inhibit NOTCH receptor activation, therefore affect angiogenesis ***(Nikolic et al., ٢٠١٠)***.

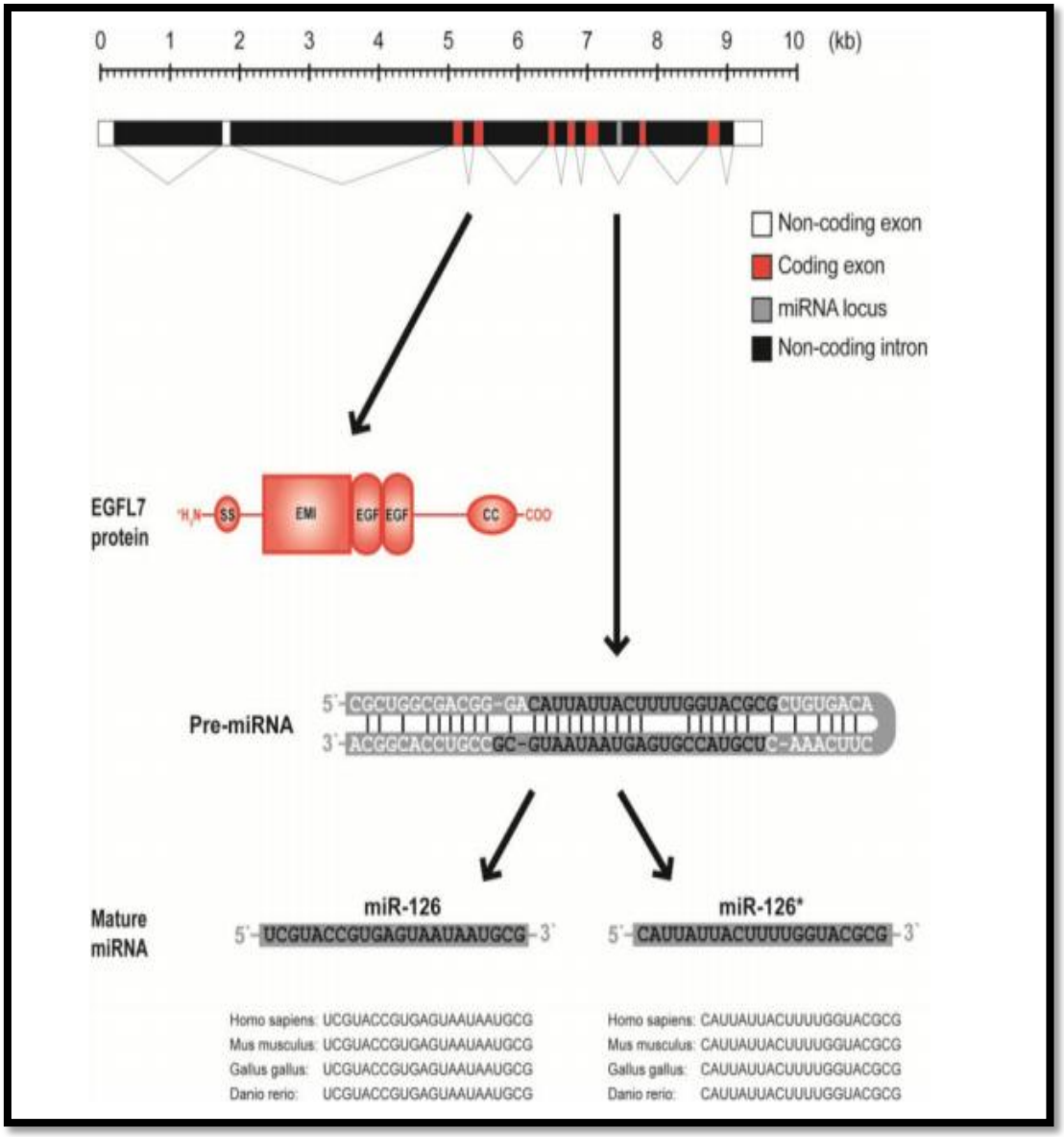


Figure (٦): MiR-١٢٦, miR ١٢٦* and putative protein sequence of EGFL٧

(Meister and Schmidt, ٢٠١٠)

Chapter (III):

Relationship between miR-٩٦, miR-١٢٦ and bladder cancer

miRNAs and bladder cancer:

The first indication that miRNAs were deregulated in bladder cancer occurred in ٢٠٠٧, with the identification of ١٠ distinct miRNAs that were up-regulated in bladder cancer when compared to normal tissues ***(Gottardo et al., ٢٠٠٧)***. This study identified miR-٢٢٣, miR-٢٦b, miR-٢٢١, miR-١٠٣-١, miR-١٨٥, miR-٢٣b, miR-٢٠٣, miR-١٧-٥p, miR-٢٣a, and miR-٢٠٥ to be significantly overexpressed in bladder tumors ***(Gottardo et al., ٢٠٠٧)***.

Since this initial discovery, several large-scale experiments have identified differences in miRNA expression patterns across the different stages and grades of bladder cancer. Low grade bladder cancer have been shown to have downregulation of several miRNAs, while high-grade bladder cancer are often associated with upregulation of miRNAs ***(Homami & Ghazi, ٢٠١٦)***.

Very few differences in miRNA expression were previously identified between high grade NMIBC and MIBC. While studies have identified differences in miRNA expression between NMIBC and MIBC, the reproducibility of these miRNA expression signatures has not been possible due to the heterogeneity of tumor specimens and research methods. The ١٩ majority of miRNA studies to date in bladder cancer consist of profiling experiments to compare miRNA expression profiles in normal bladder versus NMIBC or MIBC ***(Guancial et al., ٢٠١٤)***.

miR-٩٦ and bladder cancer

miR-٩٦ is a conserved miRNA that may be involved in the occurrence and development of bladder cancer ***(Wu et al., ٢٠١٥)***. Further research also demonstrated that miR-٩٦ was highly expressed in bladder cancer tissue and the blood plasma obtained from patients with bladder cancer ***(Kriebel et al., ٢٠١٥)***.

Yamada *et al,* ٢٠١١ confirmed that miR-٩٦ expression in urine samples from bladder urothelial carcinoma was significantly increased compared with that from healthy subjects. Therefore, miR-٩٦ is considered to be an important tumor diagnostic marker for bladder cancer. However, to the best of our knowledge, the influence of miR-٩٦ on tumor cell biology and its mechanisms have not been reported previously.

It has been demonstrated that miR-٩٦ was highly expressed in bladder cancer cell lines. miR-٩٦ inhibition resulted in a number of anticancer effects on bladder cancer cells, including inhibition of proliferation and invasion, and promotion of apoptosis ***(Xu et al., ٢٠١٨)***.

miR-١٢٦ and bladder cancer

The oncogenic roles of miR-١٢٦ have been well validated in bladder cancer, which could promote tumor growth and progression both *in vitro* ***(Xiao et al., ٢٠١٦)*** and *in vivo* experiments ***(Enokida et al., ٢٠١٦)***.

Previous studies demonstrated that aberrant miRNAs levels may be introduced as new biomarkers for early diagnosis and prognosis in bladder cancer ***(Yun et al., ٢٠١٢)***. MicroRNAs were frequently altered in most urologic cancers, playing significant roles as oncogenes or tumor suppressors, and miRs also target common pathways involved in the regulation of cell growth, proliferation, invasion, and apoptosis in bladder carcinogenesis ***(Mlcochova et al., ٢٠١٤).***

The invasive potential of bladder cancer cells can be attenuated with increased miR-١٢٦ levels by mechanistically targeting disintegrin and metalloproteinase domain-containing protein ***(Jia et al., ٢٠١٤)***.

By synergistically targeting oncogenes (*PI٣KR٢* and *adaptor protein Crk*) and tumor suppressors (*polo-like kinase* ٢), miR-١٢٦ was shown to have an inhibitory effect on the growth of gastric cancer cell lines ***(Liu et al., ٢٠١٧)***. Mechanistically, silencing miR-١٢٦ may alter the activation of the PI٣K/Akt pathway, suggesting an important regulatory role for miR-١٢٦ in PI٣K/Akt pathway transduction, which is considered to play a major role in bladder carcinogenesis ***(Calderaro et al., ٢٠١٤)***.

However, most researchers detected the miRNAs level in tissue and blood samples ***(Mitash et al., ٢٠١٧)***. Considering that bladder cancer as a genitourinary tumor and the convenience of urine collections, we further examined whether urinary miRNAs detection can serve as a

diagnostic marker for bladder cancer, especially in patients with hematuria. findings on the detection of urine miR-١٢٦ level for predicting bladder cancer may provide new insights into the developing a new effective diagnostic and prognostic marker for bladder cancer, which can enlighten future researches and optimize the current management ***(Mitash et al., ٢٠١٧)***.

Patients and methods

Patients:

This study was carried out between July ٢٠١٨ and July ٢٠٢٠ after approval of the study scheme by the research ethical committee of Benha Faculty of Medicine and obtaining informed consent from the included subjects.

Sample size justification:

Using data of previous studies ***(Wu et al, ٢٠١٥)***, setting the type-١ error (α) at ٠,٠٥, the power (١-β) at ٠,٨ and assuming a ٥٪ dropout rate, the number of participants needed to produce a statistically acceptable figure is ٧٠ participants.

Inclusion criteria:

The study included ٧٠ subjects of both sex selected from Urology Department, Faculty of Medicine, Benha University Hospital.

The subjects were categorized into ٢ groups:

A. **Bladder cancer group**: Included ٥٠ patients, diagnosed as bladder cancer patients by clinical, radiological, cystoscopic and histopathological examinations.

B. **Control group**: Included ٢٠ persons, age and sex matched, with histopathologically normal urothelium.

Exclusion criteria:

Patients who had any cancer other than bladder cancer will be excluded from the study.

All patients were subjected to:

١. Full history taking with attention to:

a) Special habits including, tobacco smoking.

b) Urological symptoms including, dysuria, frequency and hematuria

٢. General and local urological examinations specially P/R.

٣. Investigations include:

- Routine Laboratory investigations.
- Radiological investigations:
  - Abdominopelvic plain X-ray.
  - Abdominopelvic ultrasonography to detect stone or mass in the urinary bladder.
  - Abdominopelvic CT for detection and staging of urinary bladder cancer.

٤. Diagnostic cystoscopy and biopsy for histopathology.

٥. Molecular biology investigations: Quantitative reverse transcription polymerase chain reaction (qRT-PCR) for detection of miRNAs expression levels ***(Longo et al., ١٩٩٠)***.

Methods:

- ***Sampling*:**

Random sample of morning voided urine (٣٠–٦٠ mL) samples were obtained from all individuals. Urine samples were collected using sterile urine collection cups, sealed immediately and placed on ice stored at -٨٠^{o}C and centrifuged at ٢،٥٠٠–٤،٠٠٠ xg for ١٥–٢٠ min.

- ***Estimation miRNAs expression levels according to the following steps:***

I. **Extraction of total RNA including miRNA from urine pellet samples:** Using the PureLink® RNA Mini Kit (Ambion, USA) according to manufacturer's instructions.

II. **cDNA synthesis:** Using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) according to manufacturer's instructions.

III. **qRT-PCR for detection of miR- ٩٦ and miR-١٢٦:** Using Maxima SYBR Green qPCR Master Mix (٢X) (Thermo Scientific, USA) according to manufacturer's instructions.

I. Extraction of miRNA from urine pellet samples: using the PureLink® RNA Mini Kit (Ambion, USA) according to manufacturer's instructions *(Naccache et al., ٢٠١٣).*

Procedure:

١. Freshly prepared RNA Lysis Solution (٣٠٠ µl) containing ß-mercaptoethanol was added to urine pellet and was homogenized thoroughly with a syringe until no visible pellet can be seen.

٢. ١٠٠٪ ethanol (٣٠٠µl) was added and the precipitate was dispersed completely by pipetting up and down >٥ times.

٣. The sample was transferred to the RNA Spin Cartridge.

٤. Centrifugation was done at ١٢،٠٠٠ xg for ٣٠s at ٢٥^{o}C.
The flow-through was discarded.

٥. Wash Buffer I (٦٥٠ µl) was added to the cartridge. Centrifugation was done at ١٢،٠٠٠ x g for ١٥s at ٢٥^{o}C.
The flow-through was discarded.

٦. Repeat step ٥. Additional washes were added if necessary, especially for bloody urine. The flow-through must be completely clear and colorless. More washes yield better RNA.
After the final wash, the flow-through and the tube were discarded. The spin cartridge was placed into a clean ٢ ml RNA wash tube.

٧. Wash Buffer II (٥٠٠ µl) was added to the cartridge. Centrifugation was done at ١٢،٠٠٠ x g for ١٥s at ٢٥^{o}C.
The flow-through was discarded.

٨. Step ٧ was repeated three more times, for a total of four washes with Wash Buffer II.

٩. Centrifugation of the cartridge was done for ١ min to dry the membrane.

١٠. The cartridge was removed from the wash tube and was placed into an RNA Recovery Tube. ٣٠µl of RNase-free Water was added to the cartridge membrane. Centrifugation was done at ١٢،٠٠٠ x g for ٢ min at ٢٥^{o} C. The cartridge was discarded.

II. cDNA synthesis:

Using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) ***(Wiame et al., ٢٠٠٠)*** according to manufacturer's instructions.

Procedure:

١. Template RNA was left to thaw on ice and mixed by pipetting.

٢. RNA quantity(µg/١µl) was measured by NanoDrop ND-١٠٠٠ spectrophotometer (Thermo Scientific, USA).

٣. In different tubes the following were added (table ٣):

Table (٣): Volumes of miRNA and primers used for cDNA synthesis.

MiR-٩٦	**MiR-١٢٦**	**GAPDH**
Total miRNA equivalent to ١µg	Total miRNA equivalent to ١µg	Total miRNA equivalent to ١µg
miR-٩٦ RT primer* ١µl	miR-١٤٥ RT primer* ١µl	GAPDH RT primer* ١µl
Different volumes of nuclease free water were added to complete total volume ١٢µl		

**Sequences of these primers are mentioned in (table ٢).*

٤. Gentle mixing and brief centrifugation were done.

٥. The tubes were incubated at ٦٥ ºC for ٥ min. then returned on ice.

٦. Brief centrifugation was done (done at ١٢،٠٠٠ xg for ٣٠s at ٢٥^{o}C), then the tubes were returned on ice again.

٧. The following components were added to the previous tubes in the indicated order as in (table ٤) to complete final volume ٢٠µl.

Table (٤): Reaction setup for reverse transcription master mix preparation.

Component	Volume/reaction
٥X Reaction Buffer	٤µl
RiboLock RNase Inhibitor	١µl
١٠ mM dNTP Mix	٢µl
RevertAid M-MuLV Reverse Transcriptase	١µl
Total volume	٨µl

٨. Centrifugation was done briefly.

٩. The tubes were placed in G-Storm Thermal Cycler (Gene Technologies, UK) for cDNA synthesis according to the following program: Incubation at ٤٢ ºC for ٦٠min followed by incubation at ٧٠ ºC for ٥ min.

١٠. The cDNA was immediately used for qRT-PCR.

iii. qRT-PCR for detection of mRNAs and mature miRNAs:

Using Maxima SYBR Green qPCR Master Mix (٢X) ***(Longo et al., ١٩٩٠)*** (Thermo Scientific, USA) according to manufacturer's instructions.

Procedure:

١. The PCR reaction mix was prepared in a total volume of ٢٥µl / well (used in ٩٦-well plate) as in (table ٥).

Table (٥): Reaction setup for real-time PCR.

Component	**Volume/reaction**
Maxima SYBR Green qPCR Master Mix (٢X)	١٢,٥µl
Forward Primer	٠,٤*µl
Reverse Primer	٠,٤*µl
ROX Solution	٠,١µl
Template DNA	٢,٥µl
Water, nuclease-free	٩,١µl
Total volume	٢٥µl

٢. The plate was placed in the ٧٩٠٠HT Fast Real-Time PCR System, (Applied Biosystem, Singapore) and the run was started according to the following program:

a) Initial activation step for ١٠ min. at ٩٥ºC.

b) Cycling step (٤٠ cycles): Denaturation at ٩٥ ºC for ١٥ seconds followed by annealing at ٥٤ ºC for ٣٠ seconds then extension at ٧٢ ºC for ٤٥ seconds.

c) Final extensions step at ٧٢ ºC for ٢ min.

Table (٦): Primer sequence of target and endogenous control genes.

Gene	Forward Primer	Reverse Primer	Reference
Mir ٩٦	٥'-TTTGGCACTAGCACAT-٣'	٥'-GAGCAGGGTGGAGAA-٣'	***(Zhang et al., ٢٠١٥)***
Mir ١٢٦	٥'-GCCGGCGCCCGAGCTCTGGCTC-٣'	٥'-CATTATTACTTTTGGTACGCG-٣'	***(Xiao et al., ٢٠١٦)***
GAPDH	٥'-CCACTCCTCCACCTTTGACG-٣'	٥'-CCACCACCCTGTTGCTGTAG-٣'	***(Zhang et al., ٢٠١٥)***

❖ ***Data analysis***

According to the RQ Manager Program ABI SDS Software (ABI ٧٩٠٠), the data are produced as sigmoid shaped amplification plots in which the number of cycle is plotted against fluorescence (when using linear scale).

The threshold cycle (CT) serves as a tool for calculation of the starting template amount in each sample. Because the samples of control group are used as calibrators, the expression levels are set to ١. But because the gene expression levels were plotted as log ١٠ values (log ١٠ of ١ is ٠), the expression level of the calibrator samples appear as ٠ in the graph ***(Alhusseini et al., ٢٠١٠)***.

Because the relative quantities of the miRNA ٩٦ and miRNA ١٢٦, they are normalized against the relative quantities of endogenous control (GAPDH). Fold expression changes are calculated using the equation $٢^{-\Delta\Delta}$CT and expressed as relative units (RU) ***(Livak & Schmittgen, ٢٠٠١)***.

❖ ***Statistical analysis*:**

The collected data were summarized in terms of mean ± Standard Deviation (SD) and range for quantitative data; frequency and percentage for qualitative data. Comparisons between case and control groups were carried out using the student T- test, to compare quantitative data between two groups and Chi- squared (χ^2) test, to compare proportions of two or more groups. Pearson correlation was used to estimate the correlation between miRNA-٩٦, miRNA-١٢٦ and age of the studied groups. The corresponding test statistics were calculated and the corresponding P-values were obtained. P-value ≤ ٠,٠٥ was considered statistically significant, while P-value > ٠,٠٥ was considered statistically non-significant. The cut-off scores for the analysis were obtained by analyzing the receiver-operating characteristic (ROC) curve using MedCalc software (Med-Calc Software, Mariakerke, Belgium). All analysis except ROC is performed using the Statistics Program for Social Sciences (SPSS) and Microsoft Office Excel is used for the data processing and data analysis ***(Knapp and Miller, ١٩٩٢).***

Results

Table (٧): Comparison between the studied groups regarding age.

Group / Age(years)	Control group	Bladder cancer group	T- Test	P-value
Mean± SD	٥٧,٤±٦,٤٥	٦١,٠± ٨,٩	١,٦٦	٠,١٠
Range	٣٢-٦٧	٣٤-٨٤		

SD: Standard deviation, P ≤ ٠,٠٥ significant, P > ٠,٠٥ non-significant

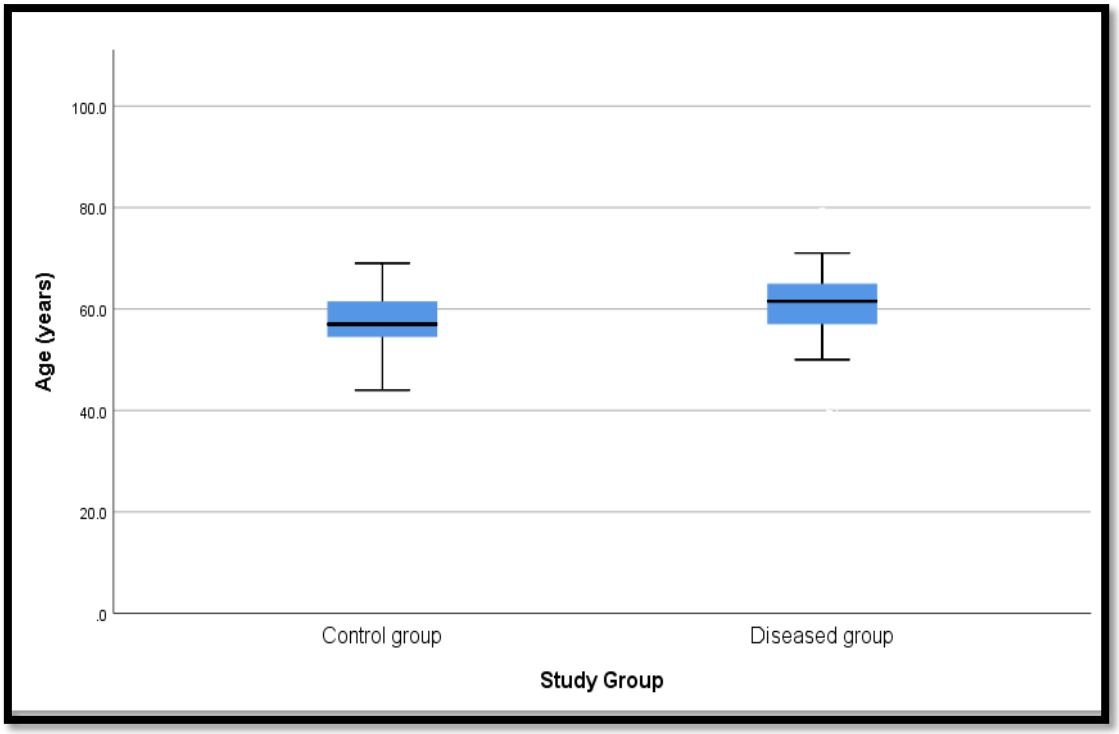


Figure (٧): *Box-plot showing difference between the studied groups regarding age.*

Table (٧) and figure (٧) show mean ± SD and range among the studied groups with no statistically significant difference between the study groups regarding age.

Table (٨): Gender distribution among the studied groups.

Gender \ Group	Bladder cancer group	Control group	P-value
Male:			٠,٧٣٥
No.	٤٤	١٧	
Percent (%)	٨٨٪	٨٥٪	
Female:			
No.	٦	٣	
Percent (%)	١٢٪	١٥٪	

*p≤٠,٠٥ is considered statistically significant, * Comparison between the study groups was done by Pearson Chi-Square Test.*

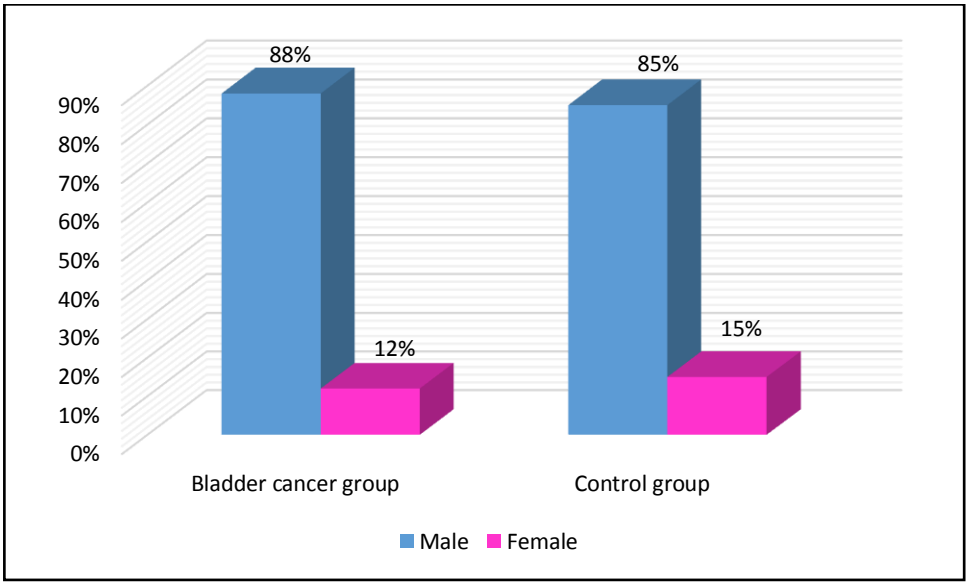


Figure (٨): *Gender distribution among the studied groups.*

Table (٨) and Figure (٨): Show gender distribution in control group as well as bladder cancer group. There was no significant statistical difference between the two groups.

Table (٩): Comparison between bladder cancer group and control group regarding smoking.

Study Group / Smoking habit		Bladder cancer group	Control group	Test	P-value
Smokers	No.	٣٨	١٥	$\chi^{٢}$ =٠,٠٠٨	٠,٩٣
	%	٧٦,٠٪	٧٥,٠٪		
Non-Smokers	No.	١٢	٥		
	%	٢٤,٠٪	٢٥,٠٪		

P ٠,٠٥ significant, P > ٠,٠٥ non-significant, $X^{٢}$: Chi-square test

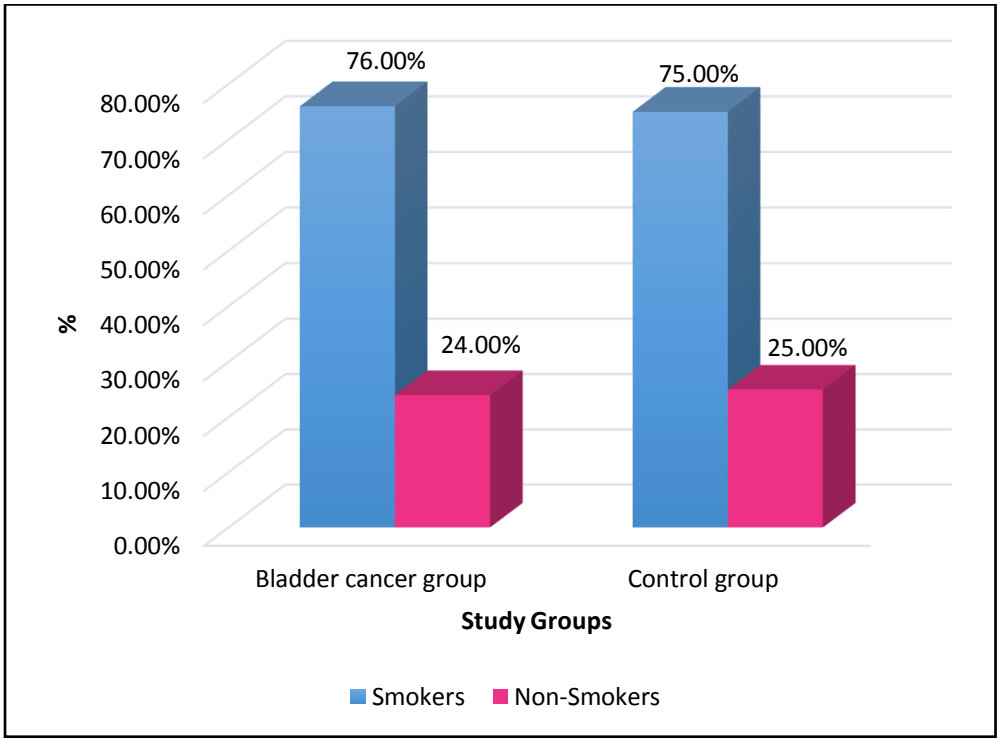


Figure (٩): *Comparison between the studied groups regarding smoking.*

Table (٩) and figure (٩): Show statistically non-significant difference regarding smoking habit distribution in bladder cancer group as compared to control group.

Table (١٠): Difference between bladder cancer group and control group regarding miR-٩٦ relative expression.

Study Group / MiR-٩٦ FRU (Log)	Control group	Bladder cancer group	T- Test	P-value
Mean ± SD	٤,١٥ ± ٠,٥٣	٥,٢٢ ± ٠,٨٧	٦,٢٥	< ٠,٠٠١

SD: Standard deviation, P ٠,٠٥ ≤ significant, P > ٠,٠٥ non-significant, analysis done by independent samples Student T test.

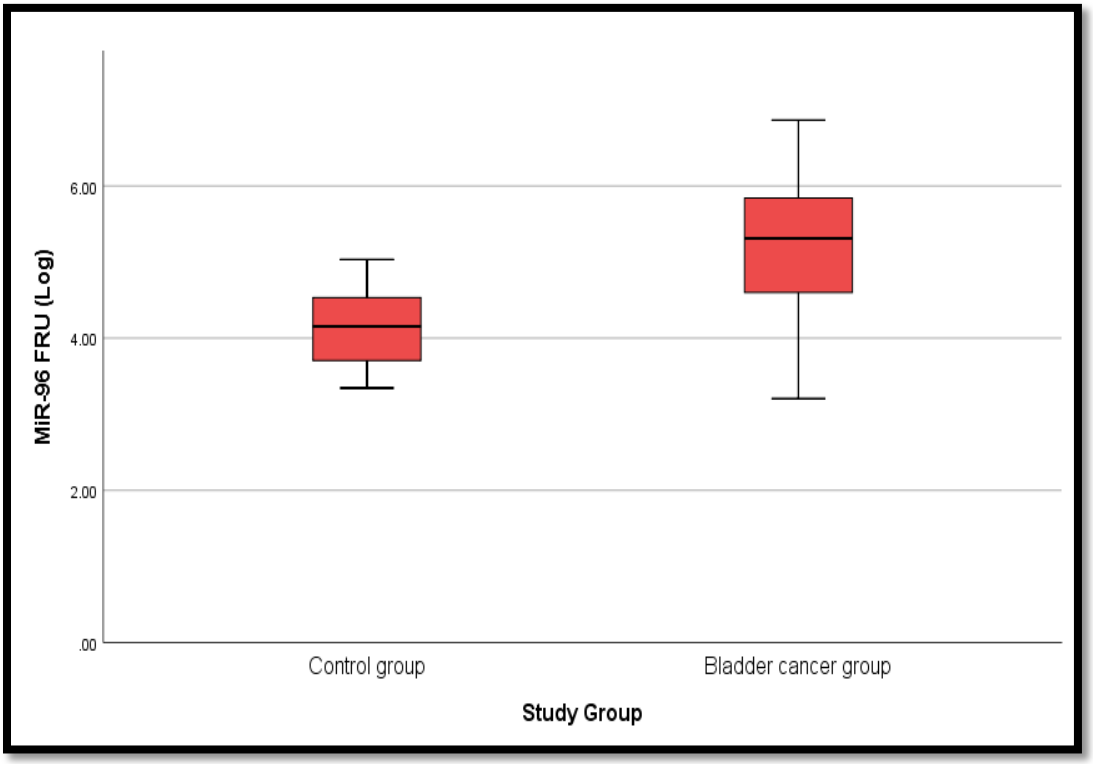


Figure (١٠): *Box-plot showing difference between the studied groups regarding miR-٩٦ relative expression*

Table (١٠) and Figure (١٠): Show statistically high significant increase of miRNA-٩٦ relative expression in bladder cancer group compared to that of control group.

Table (١١): Difference between bladder cancer group and control group regarding miR-١٢٦ relative expression.

Study Group / MiRNA-١٢٦ FRU (Log)	Control group	Bladder cancer group	T- Test	P-value
Mean ± SD	٤,٠٤ ±١,٠٥	٤,٨٢ ±١,٢٣	٢,٥١	.٠١٥

SD: Standard deviation, P ٠,٠٥≤ significant, P > ٠,٠٥ non-significant, analysis done by independent samples Student T test.

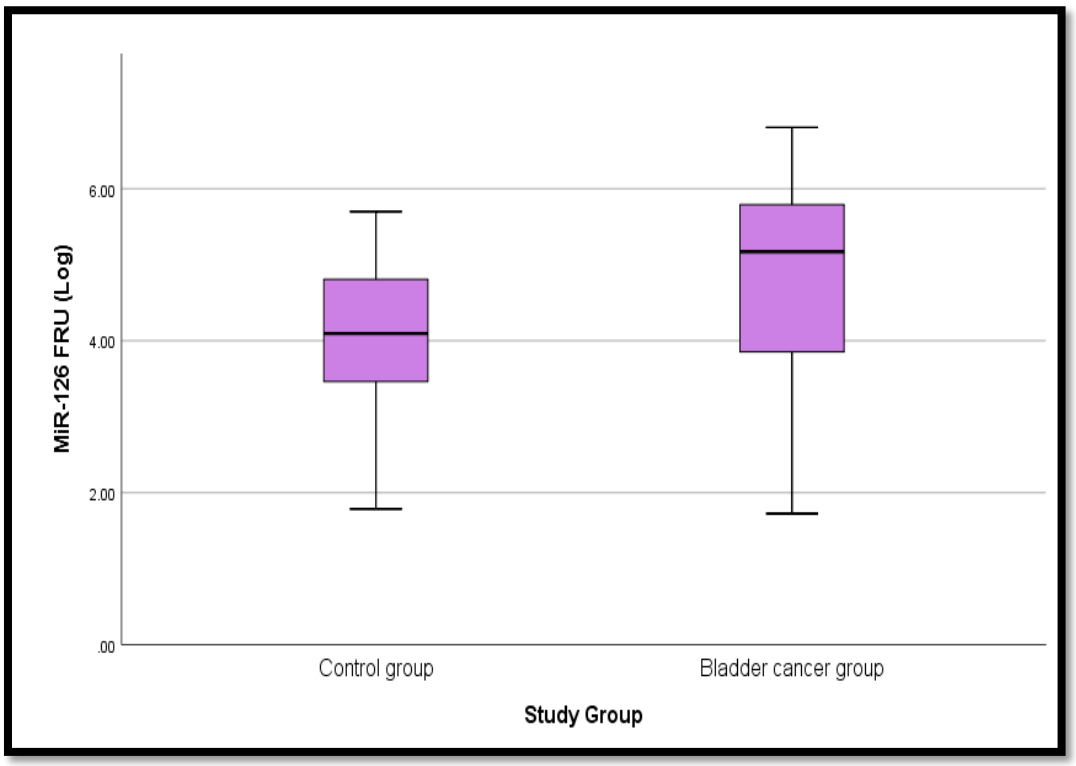


Figure (١١): *Box-plot showing difference between the studied groups regarding miRNA-١٢٦ relative expression.*

Table (١١) and figure (١١): Show statistically significant increase of miRNA-١٢٦ relative expression in bladder cancer group compared to that of control group.

Table (١٢): Correlation between miR-٩٦ relative expression and the age in the studied groups.

Variable	Age /years			
	Control group (n=٢٠)		Bladder cancer group (n=٥٠)	
	Pearson correlation	P-value	Pearson correlation	P-value
miR-٩٦ (FRU)	- ٠,١٥	٠,٥٢	.٠٥٧	٠,٦٩

P ٠,٠٥ ≤ significant, P > ٠,٠٥ non-significant

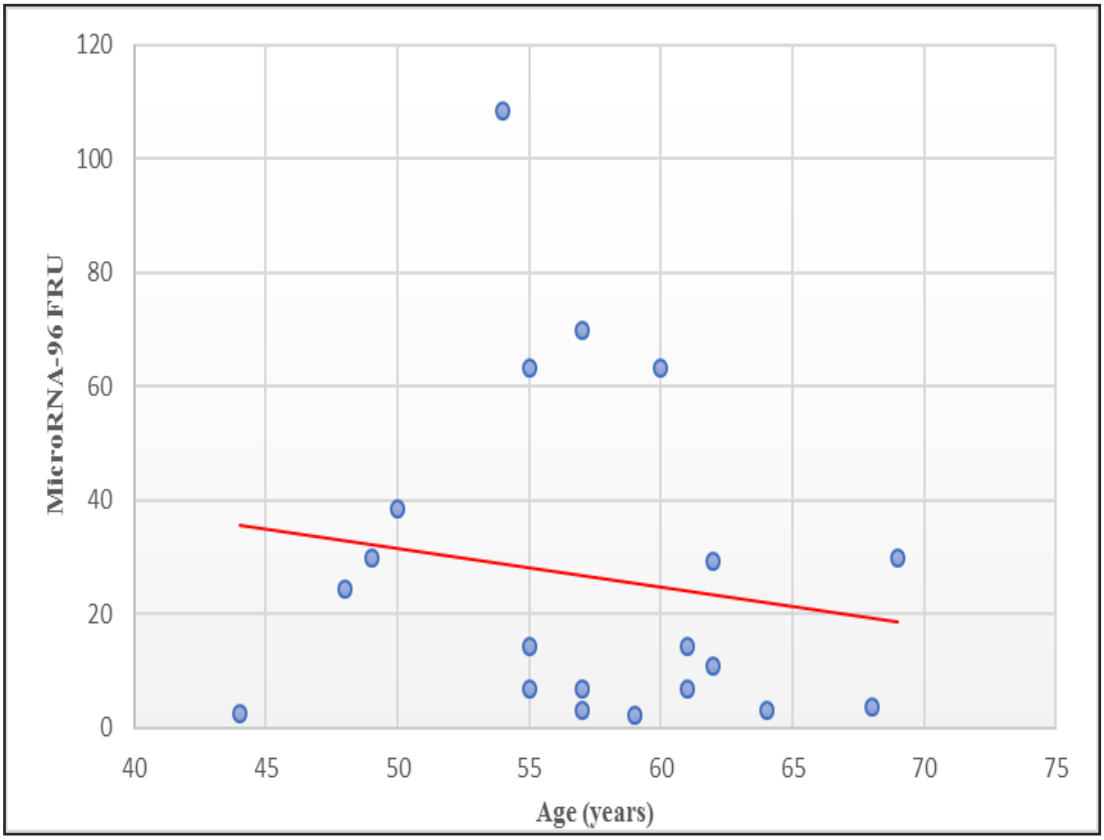


Figure (١٢): *Correlation between miR-٩٦ relative expression and the age of control group.*

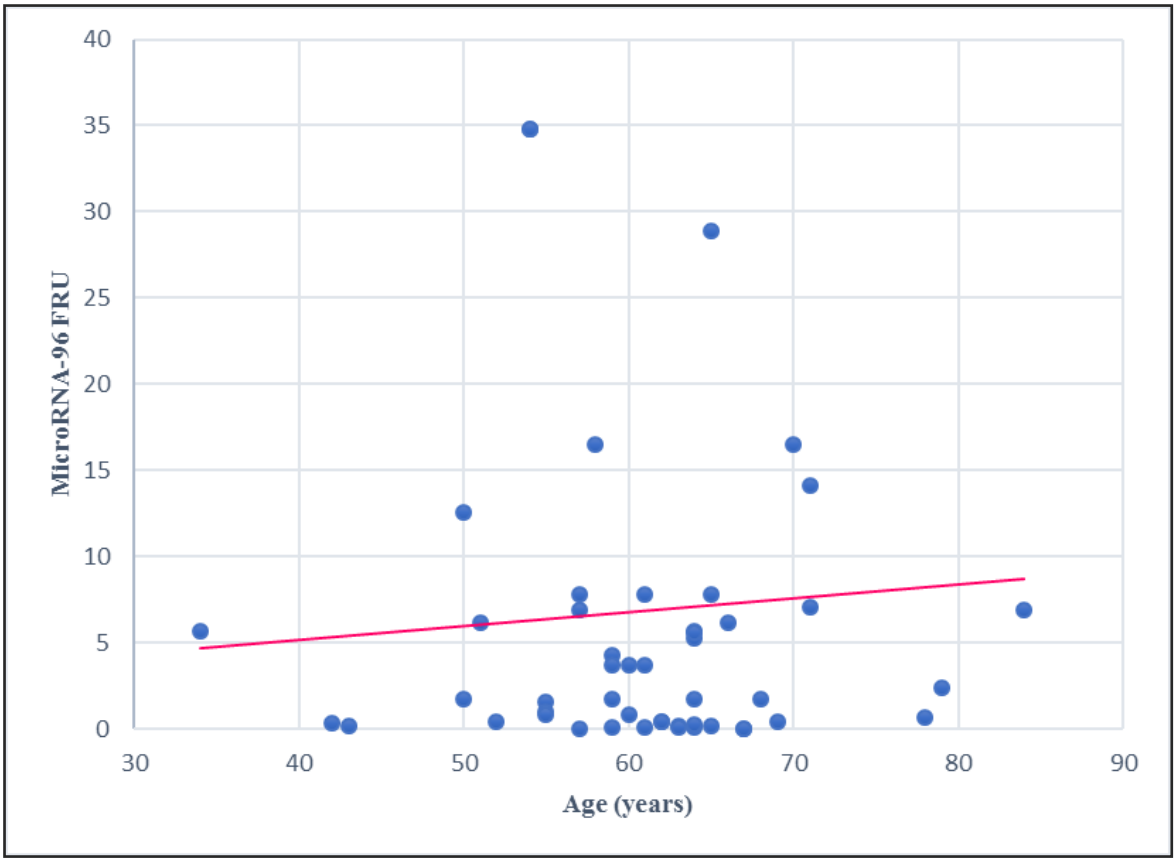


Figure (١٣): *Non-significant correlation between miR-٩٦ relative expression and the age of bladder cancer group.*

Table (١٢) and figure (١٢): Show statistically non-significant weak negative correlation between miR-٩٦ relative expression and the age of control group.

Table (١٢) and figure (١٣): Show statistically non-significant weak positive correlation between miR-٩٦ relative expression and the age of bladder cancer group.

Table (١٣): Correlation between miR-١٢٦ relative expression and the age in the studied groups.

Variable	Age /years			
	Control group (n=٢٠)		Bladder cancer group (n=٥٠)	
	Pearson correlation	P-value	Pearson correlation	P-value
miR-١٢٦ (FRU)	٠,١١٤	٠,٦٣	- ٠,١٨٨	٠,١٩

P ٠,٠٥ ≤ significant, P > ٠,٠٥ non-significant

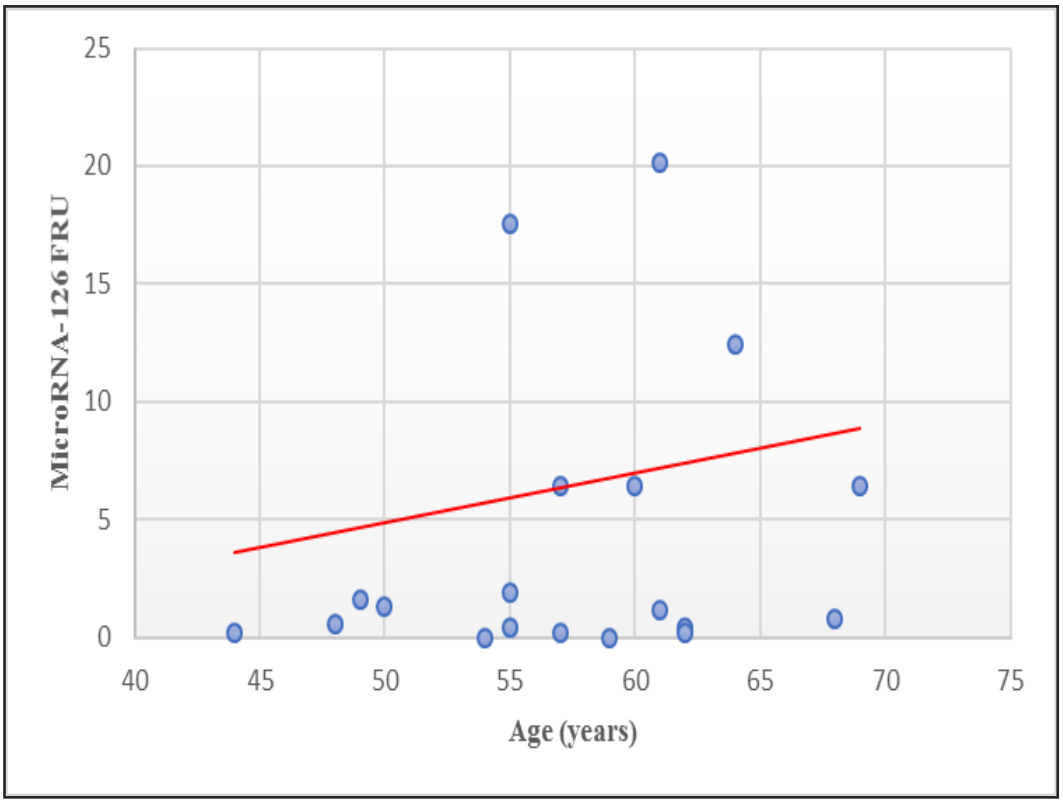


Figure (١٤): *Correlation between miR-١٢٦ relative expression and the age of control group.*

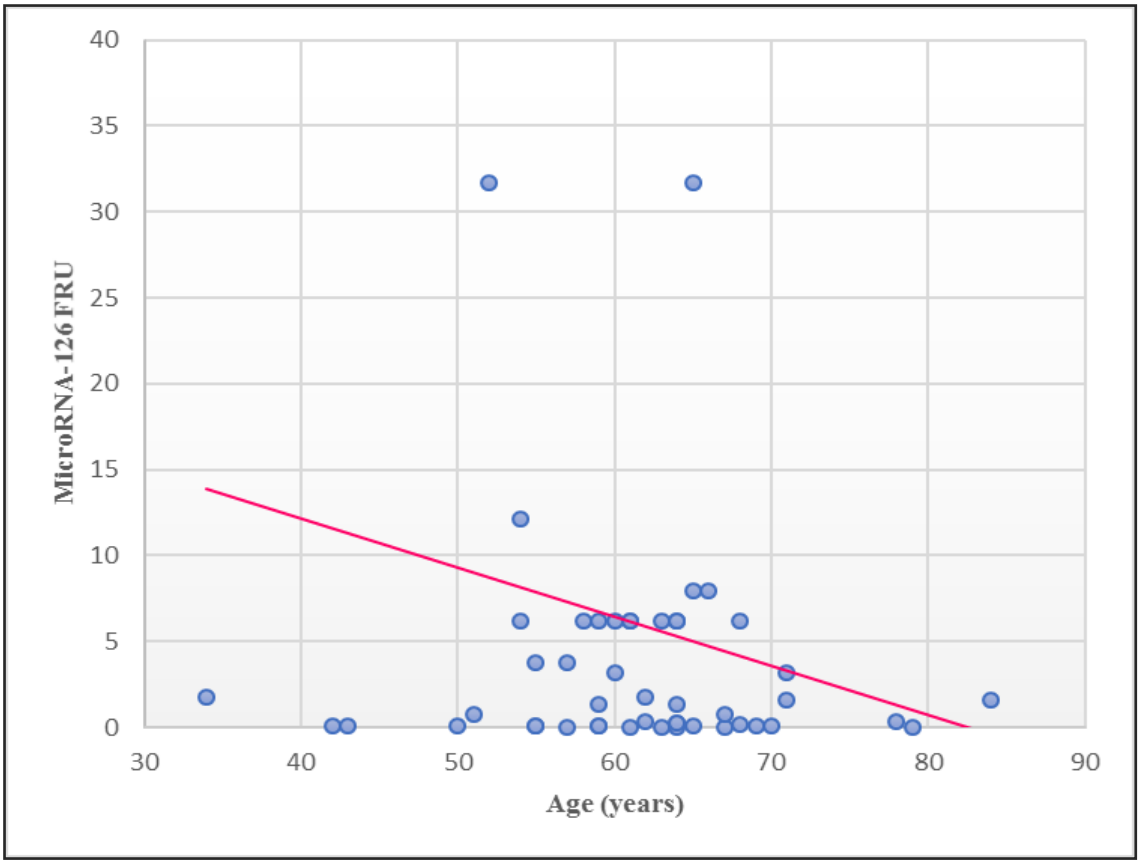


Figure (١٥): *Correlation between miR-١٢٦ relative expression and the age of bladder cancer group.*

Table (١٣) and Figure (١٤): Show statistically non-significant weak positive correlation between miR-١٢٦ relative expression and the age of control group.

Table (١٣) and Figure (١٥): Show statistically non-significant weak negative correlation between miR-١٢٦ relative expression and the age of bladder cancer group.

Table (١٤): Difference in mean values of miR-٩٦ relative expression in female as compared to male subjects in the control group.

Variable		Gender in control group			
		Female	Male	T-Test	P-value
MiR-٩٦ FRU (Log)	Mean ± SD	٣,٩٩ ± ٠,٧٤	٤,١٨ ± ٠,٥١	٠,٥٦	٠,٥٨

SD: Standard deviation, P ٠,٠٥ ≤ significant, P > ٠,٠٥ non-significant

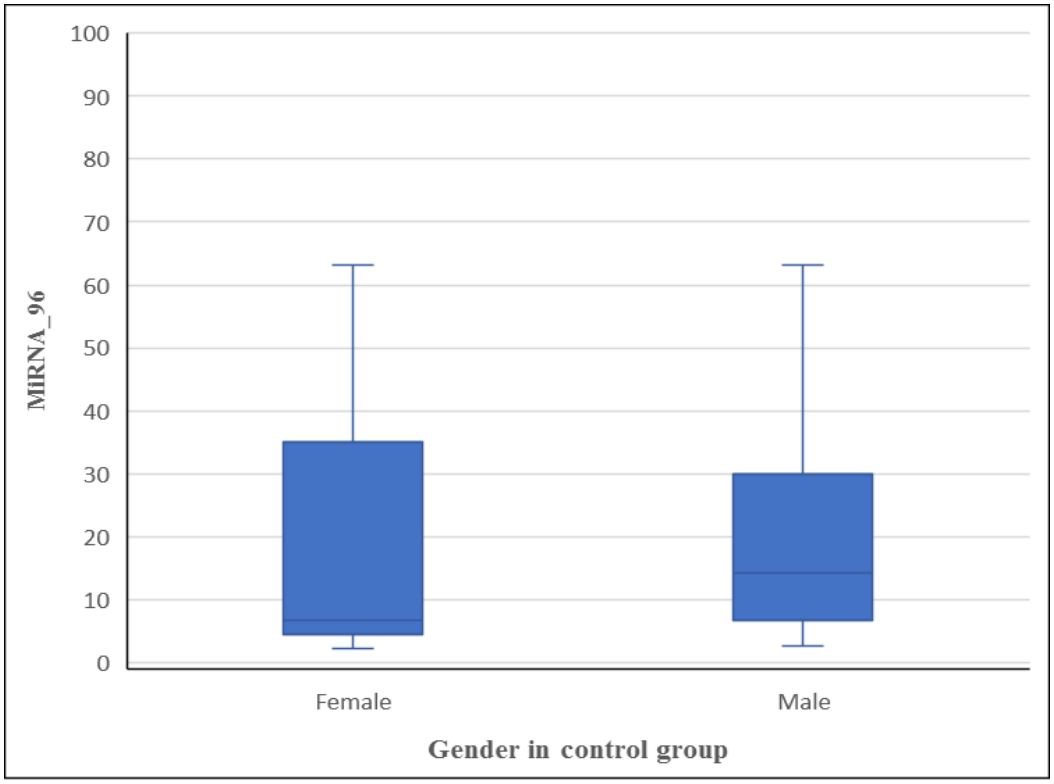


Figure (١٦): *Difference in mean value of miR-٩٦ expression regarding gender in control group.*

Table (١٤) and Figure (١٦): Show statistically non-significant statistical difference in mean value of miR-٩٦ relative expression in female as compared to male subjects in the control group.

Table (١٥): Difference in mean values of miR-٩٦ relative expression in female as compared to male subjects in the bladder cancer group.

Variable		Gender in bladder cancer group			
		Female	Male	T- Test	P-value
MiR-٩٦ FRU (Log)	Mean ± SD	٤,٧٢ ± ١,٠١	٥,٢٩ ± ٠,٨٤	-١,٥٢	٠,١٤

SD: Standard deviation, P ٠,٠٥≤ significant, P > ٠,٠٥ non-significant

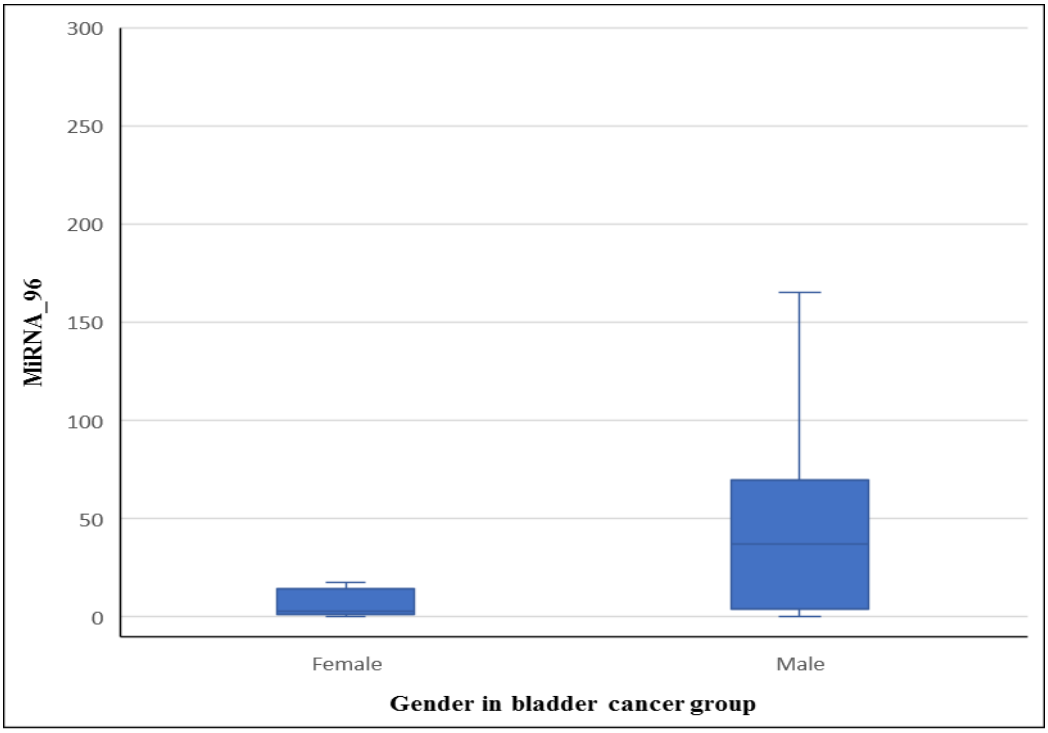


Figure (١٧): *Difference in mean value of miR-٩٦ expression regarding gender in bladder cancer group.*

Table (١٥) and figure (١٧): Show statistically non-significant difference in mean value of miR-٩٦ relative expression in female as compared to male subjects in the bladder cancer group.

Table (١٦): Difference in mean values of miR-١٢٦ relative expression in female as compared to male subjects in the control group.

Variable		Gender in control group			
		Female	Male	Test	P-value
MiR-١٢٦ FRU (Log)	Mean ± SD	٣,٣٩ ± ١,٥٢	٤,١٥ ± ٠,٩٧	١,١٥	٠,٢٧

SD: Standard deviation, P ٠,٠٥≤ significant, P > ٠,٠٥ non-significant

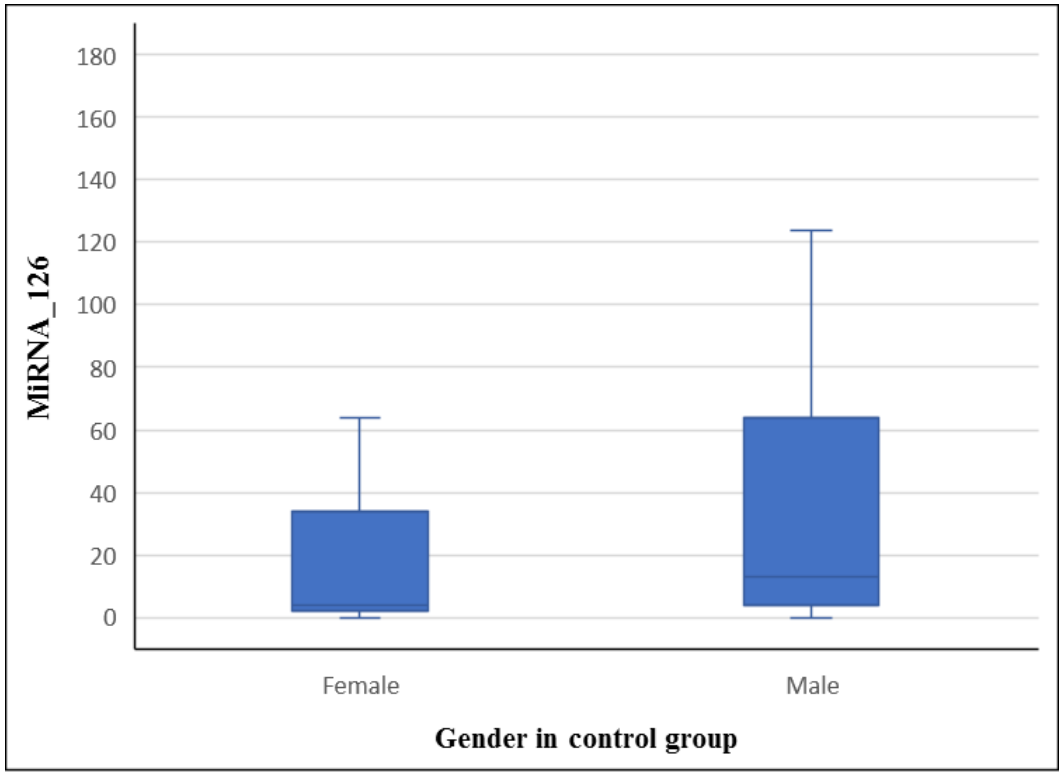


Figure (١٨): *Difference in mean value of miR-١٢٦ expression regarding gender in control group.*

Table (١٦) and figure (١٨): Show statistically non-significant difference in mean value of miR-١٢٦ relative expression in female as compared to male subjects in the control group.

Table (١٧): Difference in mean values of miR-١٢٦ relative expression in female as compared to male subjects in the bladder cancer group.

Variable		Gender in bladder cancer group			
		Female	Male	T- Test	P-value
MiR-١٢٦ FRU (Log)	Mean ± SD	٤,٧٤ ± ١,٩٦	٤,٨٣ ± ١,١٣	٠,١١	٠,٩٢

SD: Standard deviation, P ٠,٠٥≤ significant, P > ٠,٠٥ non-significant

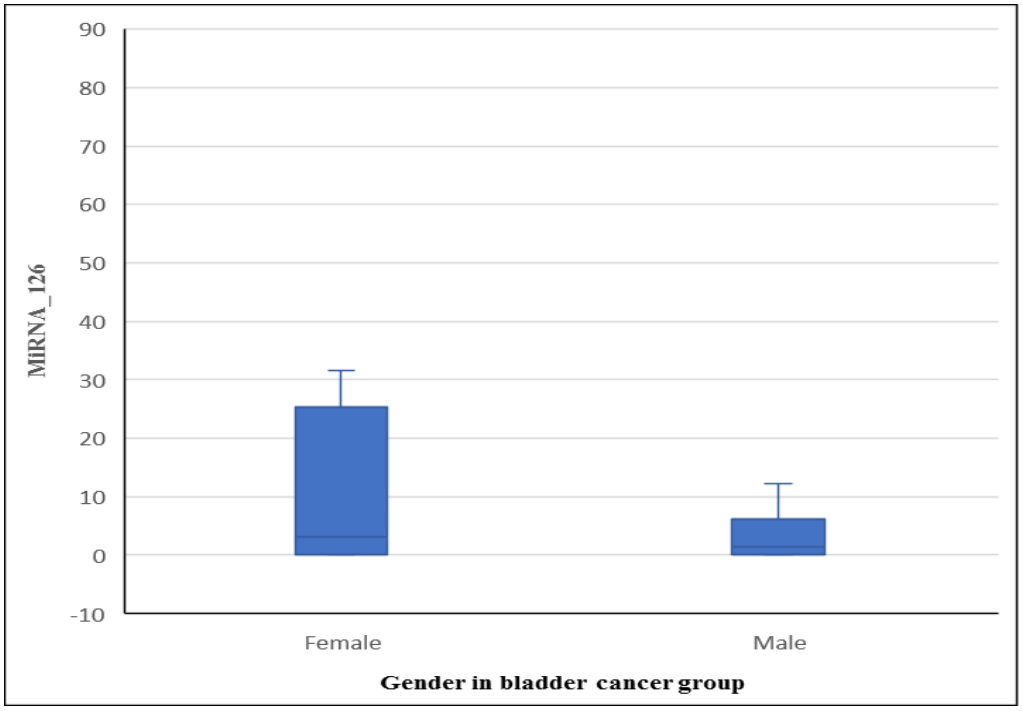


Figure (١٩): *Difference in mean value of miR-١٢٦ expression regarding gender in bladder cancer group.*

Table (١٧) and figure (١٩): Show statistically non-significant difference in mean value of miR-١٢٦ relative expression in female as compared to male subjects in the bladder cancer group.

Table (١٨): Difference in mean values of miR-٩٦ relative expression in smokers as compared to that of non-smokers subjects in control group.

Variable		Smoking in control group			
		Smokers	Non-smokers	T- Test	P-value
MiR-٩٦ FRU (Log)	Mean ± SD	٤,١٩ ± ٠,٥٣	٤,٠٤ ± ٠,٥٦	٠,٥٤	٠.٥٩

P ٠,٠٥ ≤ significant

P > ٠,٠٥ non-significant

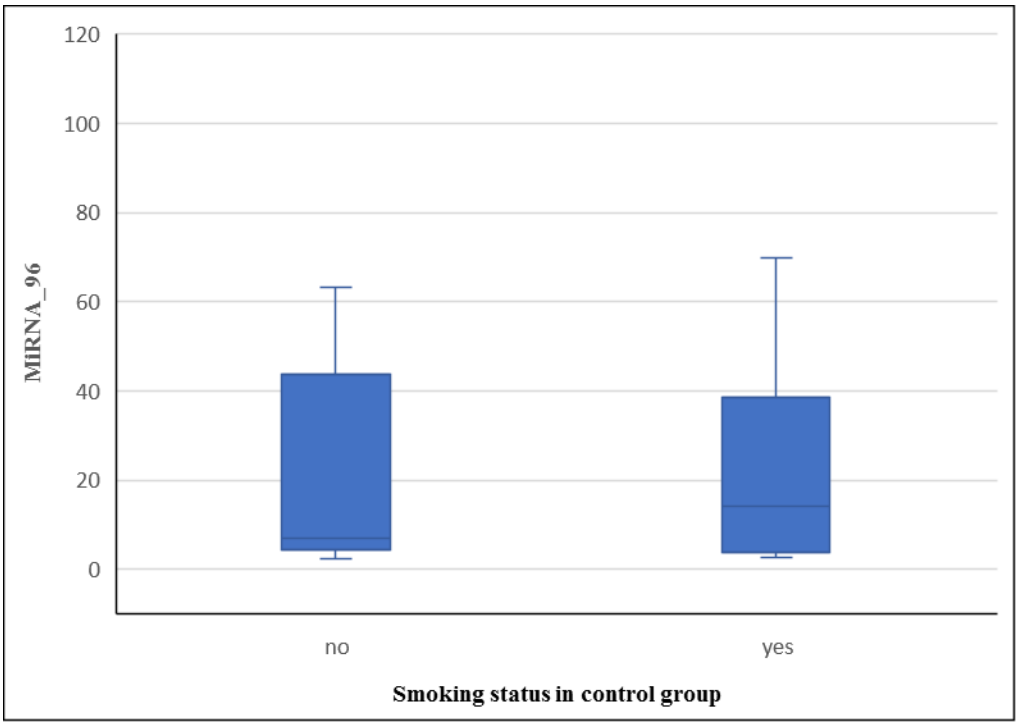


Figure (٢٠): *Difference in mean value of miR-٩٦ expression regarding smoking in control group.*

Table (١٨) and figure (٢٠): Show statistically non-significant difference in mean value of miR-٩٦ relative expression in smokers as compared to non-smokers in the control groups.

Table (١٩): Difference in mean values of miR-٩٦ relative expression in smokers as compared to that of non-smokers in the bladder cancer group.

Variable		Smoking in bladder cancer group			
		Smokers	Non-smokers	T- Test	P-value
MiR-٩٦ FRU (Log)	Mean ± SD	٥,١٦ ± ٠,٧٩	٥,٤٢ ± ١,١٠	٠,٩٢	٠.٣٦

P ٠,٠٥≤ significant

P > ٠,٠٥ non-significant

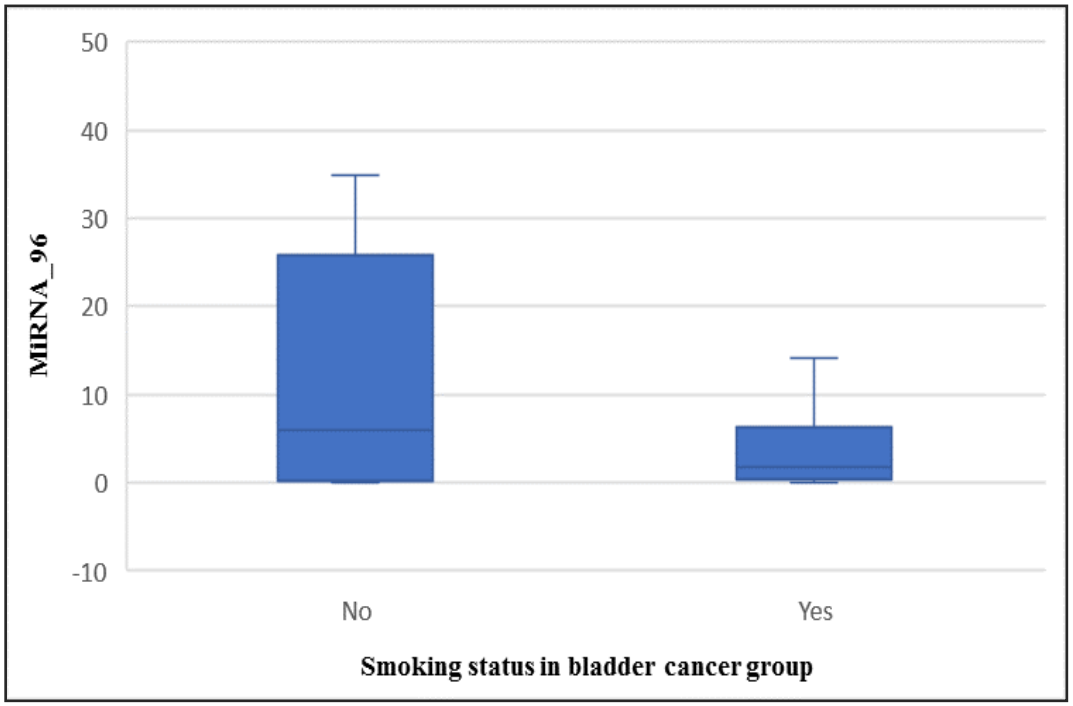


Figure (٢١): *Difference in mean value of miR-٩٦ expression regarding smoking in bladder cancer group.*

Table (١٩) and Figure (٢١): Show statistically non-significant difference in mean value of miR-٩٦ relative expression in smokers as compared to non-smokers in the bladder cancer group.

Table (٢٠): Difference in mean values of miR-١٢٦ relative expression in smokers as compared to non-smokers in control group.

Variable		Smoking in control group			
		Smoker	Non-smoker	T- Test	P-value
MiR-١٢٦ FRU (Log)	Mean ± SD	٤,٢٤ ± ٠,٩٩	٣,٤٤ ± ١,٠٩	١,٥٣	٠,١٥

P ٠,٠٥≤ significant

P > ٠,٠٥ non-significant

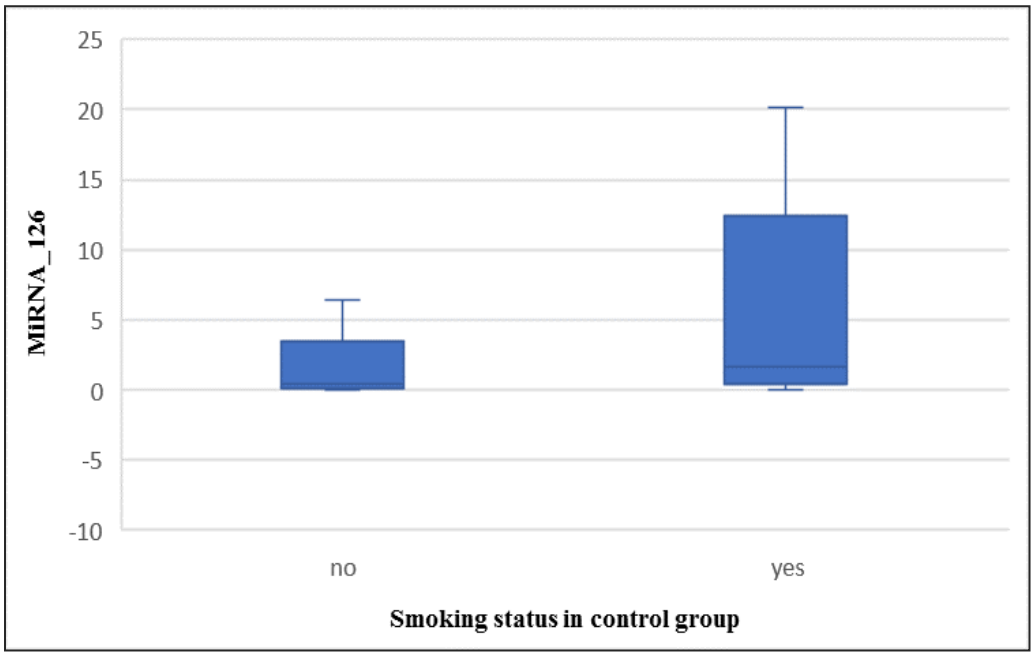


Figure (٢٢): *Difference in mean value of miR-١٢٦ expression regarding smoking in control group.*

Table (٢٠) and figure (٢٢): Show statistically non-significant difference in mean value of miR-١٢٦ relative expression in smokers as compared to non-smokers in the control groups.

Table (٢١): Difference in mean values of miR-١٢٦ relative expression in smokers as compared to non-smokers in the bladder cancer group.

Variable		Smoking in bladder cancer group			
		Smokers	Non-smokers	T- Test	P-value
MiR-١٢٦ FRU (Log)	Mean ± SD	٤,٨٦ ± ١,٢٩	٤,٦٨ ± ١,٠٤	٠,٤٤	٠,٦٦

P ٠,٠٥ ≤ significant

P > ٠,٠٥ non-significant

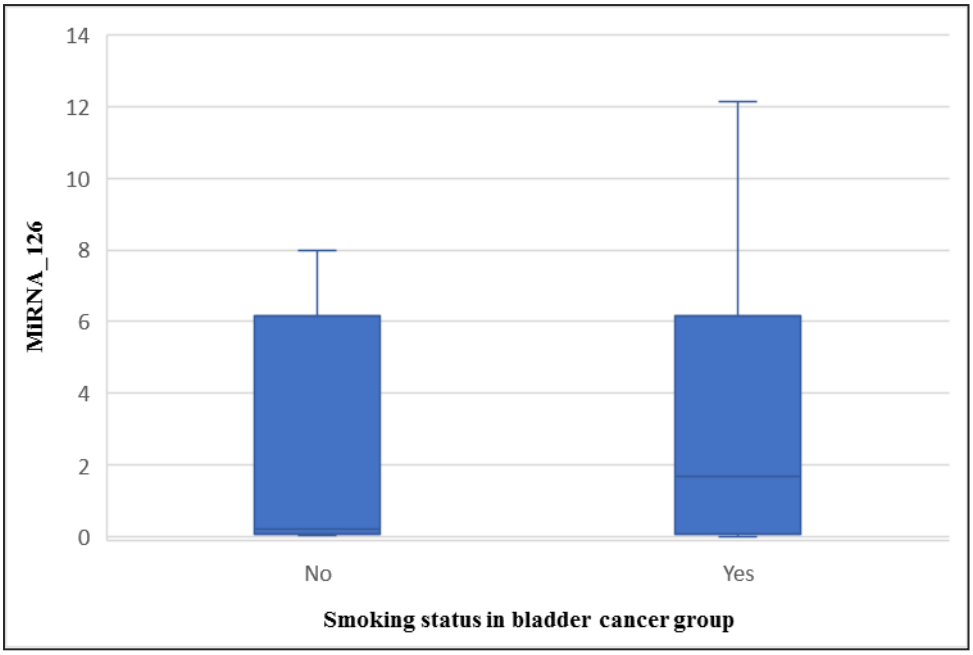


Figure (٢٣): *Difference in mean value of miR-١٢٦ expression regarding smoking in bladder cancer group.*

Table (٢١) and figure (٢٣): Show statistically non-significant difference in mean value of miR-١٢٦ relative expression in smokers as compared to non-smokers in the bladder cancer group.

Table (٢٢): Pathological data of bladder cancer group.

	Tumor Stage n=٥٠		Tumor grade n=٥٠			Tumor type n=٥٠	
	NMIBC* (Ta,CIS,T١)	MIBC* (T٢,T٣,T٤)	Grade (١)	Grade (٢)	Grade (٣)	Transitional cell carcinoma (TCC)	Small cell carcinoma (SCC)
Number (n)	١٤	٣٦	٧	١٩	٢٤	٤٦	٤
Percent %	٢٨,٠٪	٧٢,٠٪	١٤,٠٪	٣٨,٠٪	٤٨,٠٪	٩٢,٠٪	٨,٠٪

**NMIBC: Non-muscle invasive bladder cancer*

**MIBC: Muscle invasive bladder cancer*

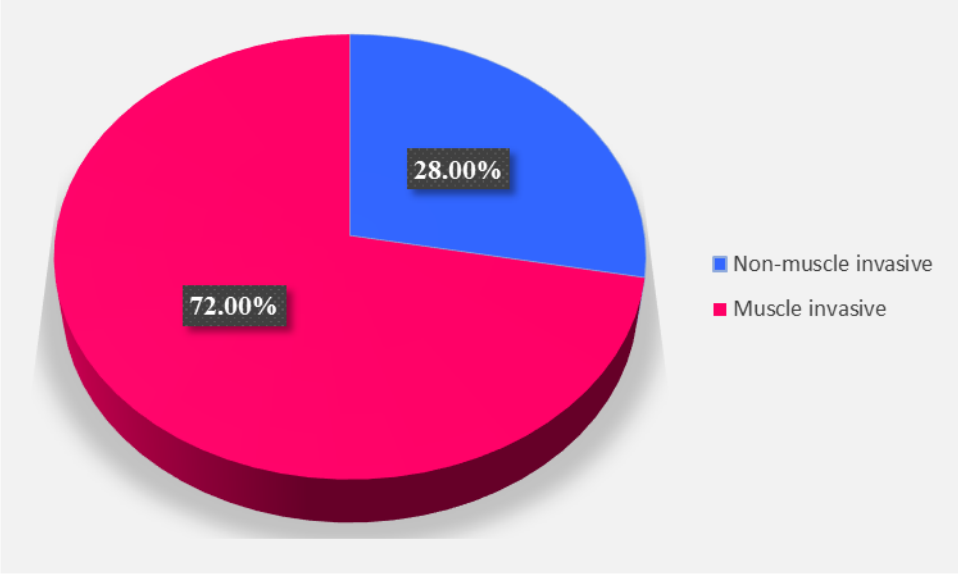


Figure (٢٤): *Distribution of bladder cancer patients regarding tumor stage.*

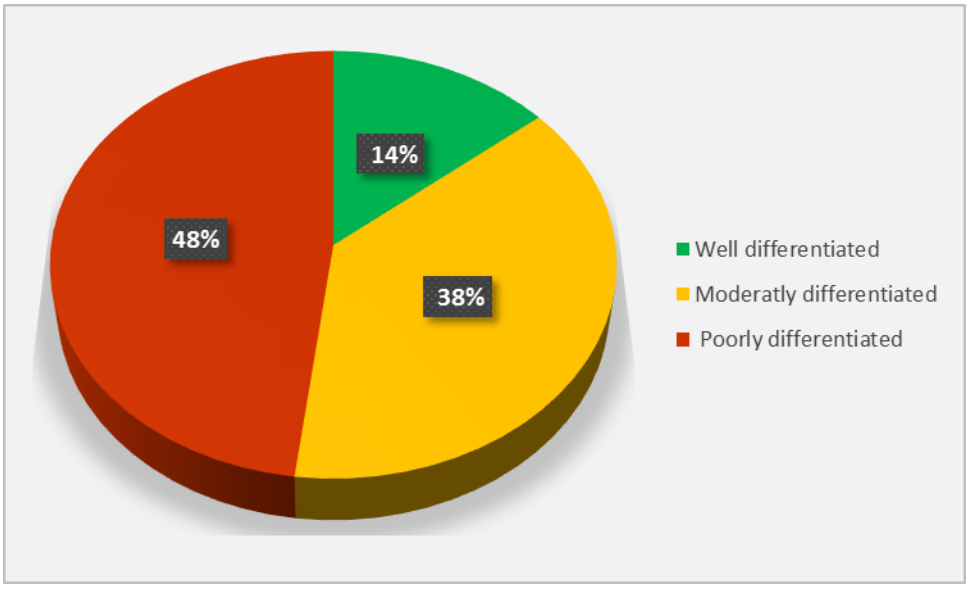


Figure (٢٥): *Distribution of bladder cancer patients regarding tumor grade.*

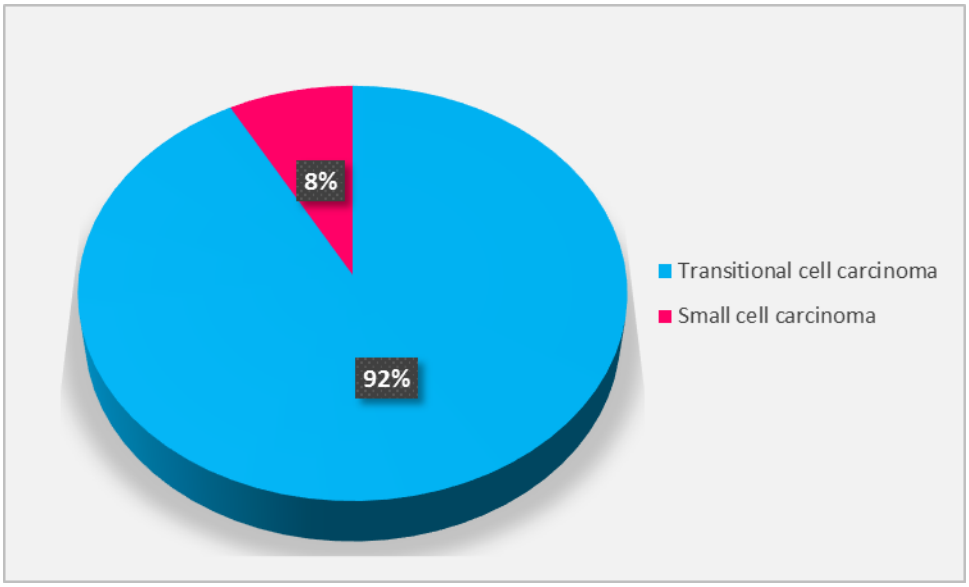


Figure (٢٦): *Distribution of bladder cancer patients regarding tumor type.*

Table (٢٣): Difference in mean values of miR-٩٦ relative expression in patients with small cell carcinoma as compared to those with transitional cell carcinoma.

Variable		Tumor type			
		Transitional cell carcinoma (TCC)	Small cell carcinoma (SCC)	T- Test	P-value
MiR-٩٦ FRU (Log)	Mean ± SD	٥,٢١ ± ٠,٨٩	٥,٣٩ ± ٠,٧٥	٠,٤٥	٠,٦٩

P ٠,٠٥ ≤ significant
P > ٠,٠٥ non-significant

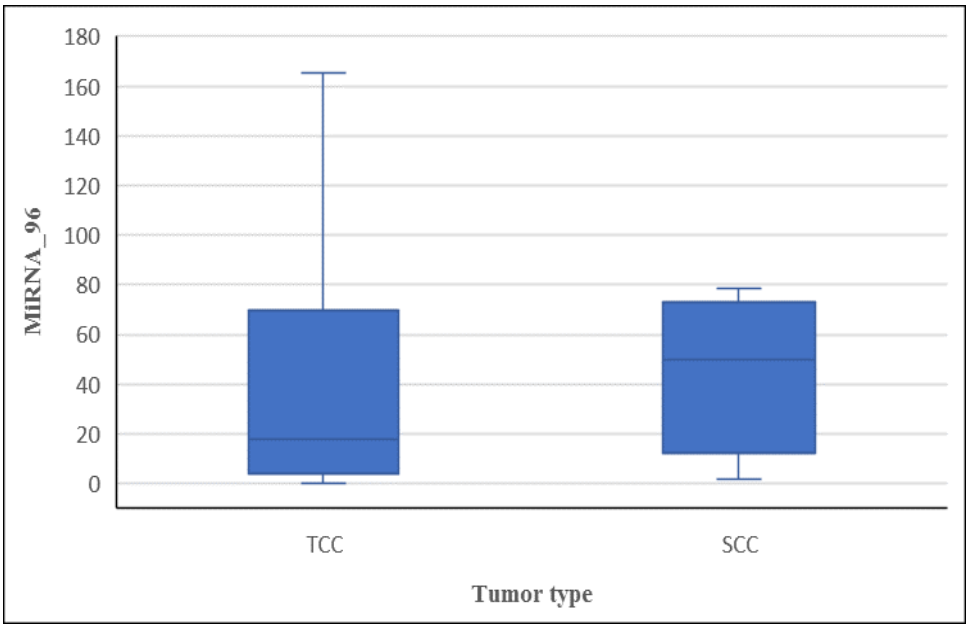


Figure (٢٧): *Difference in mean value of miR-٩٦ expression regarding tumor type.*

Table (٢٣) and figure (٢٧): Show statistically non-significant difference in mean value of miR-٩٦ relative expression in patients with small cell carcinoma as compared to that of patients with transitional cell carcinoma.

Table (٢٤): Difference in mean values of miR-١٢٦ relative expression in patients with small cell carcinoma as compared to those with transitional cell carcinoma.

Variable		Tumor type			
		Transitional cell carcinoma (TCC)	Small cell carcinoma (SCC)	T- Test	P-value
MiR-١٢٦ FRU (Log)	Mean ± SD	٤,٧٦ ± ١,٢٦	٥,٥٢ ± ٠,٣٩	٢,٨٣	٠,٠١٧

P ٠,٠٥ ≤ significant

P > ٠,٠٥ non-significant

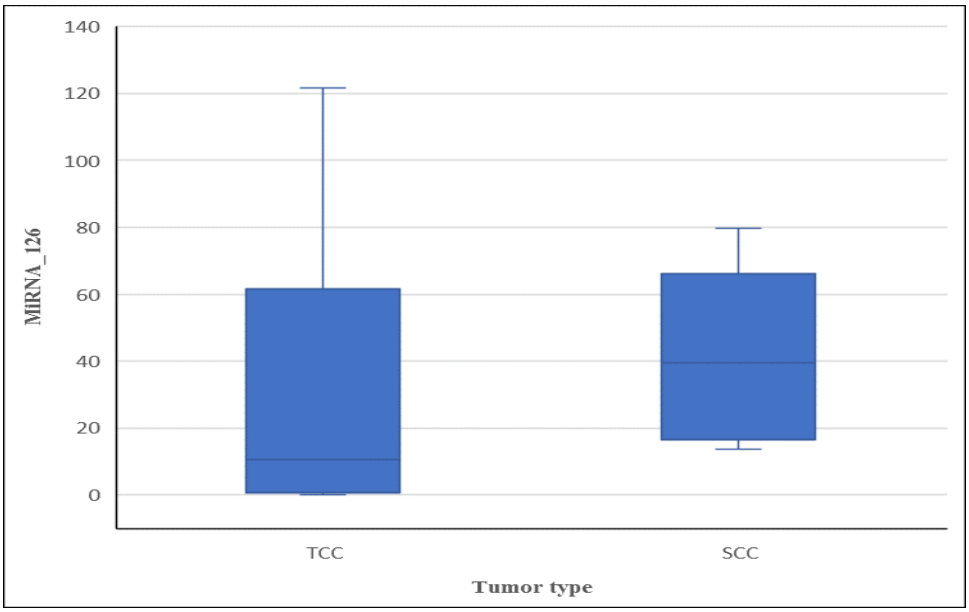


Figure (٢٨): *Difference in mean value of miR-١٢٦ expression regarding tumor type.*

Table (٢٤) and Figure (٢٨): Show statistically significant difference in mean value of miR-١٢٦ relative expression in patients with small cell carcinoma as compared to that of patients with transitional cell carcinoma.

Table (٢٥): Difference in mean values of miR-٩٦ relative expression in patients with muscle invasive tumors as compared to those with non-muscle invasive tumors.

Variable		Tumor Stage			
		Non muscle invasive (Ta,CIS,T١)	Muscle invasive (T٢,T٣,T٤)	T- Test	P-value
MiR-٩٦ FRU (Log)	Mean ± SD	٤,٤٨ ± ٠,٨١	٥,٥١ ± ٠,٧١	٤,٤٢	< ٠,٠٠١

P ٠,٠٥ ≤ significant

P > ٠,٠٥ non-significant

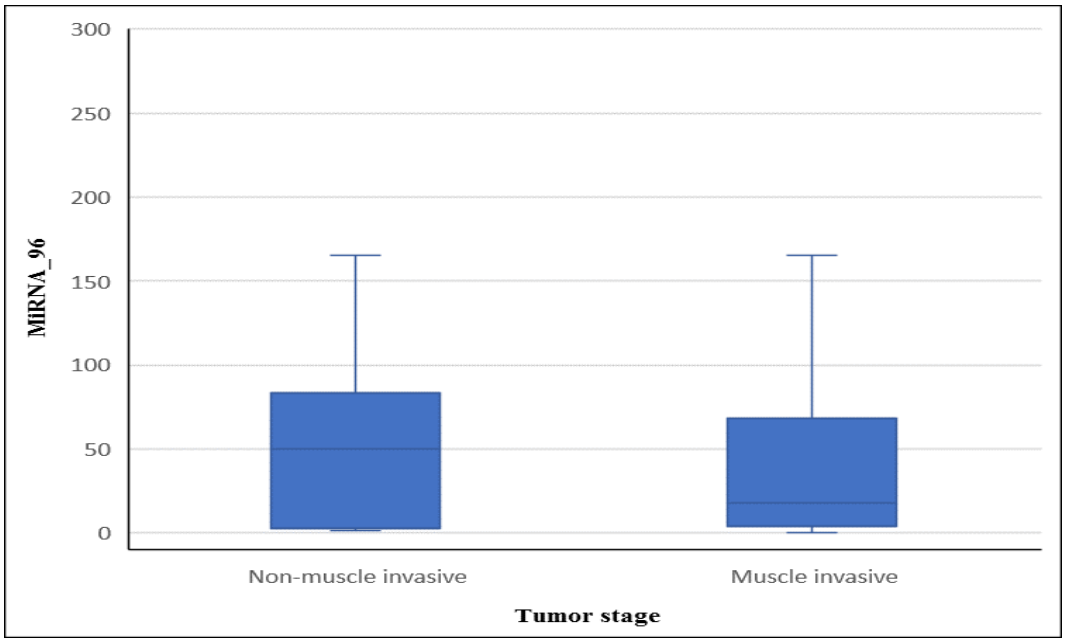


Figure (٢٩): *Difference in mean values of miR-٩٦ expression of patients with muscle invasive tumors as compared to that of patients with non-muscle invasive tumors.*

Table (٢٥) and figure (٢٩): statistically significant increase in mean value of miR-٩٦ relative expression in patients with muscle invasive tumors as compared to that of patients with non-muscle invasive tumors.

Table (٢٦): Difference in mean values of miR-١٢٦ relative expression in patients with muscle invasive tumors as compared to those with non-muscle invasive tumors.

Variable		Tumor stage			
		Non muscle invasive (Ta,CIS,T١)	Muscle invasive (T٢,T٣,T٤)	T- Test	P-value
MiR-١٢٦ FRU (Log)	Mean ± SD	٤,٩٠ ± ١,١١	٤,٧٩ ± ١,٢٩	٠,٢٨	٠,٧٨

P ٠,٠٥ ≤ significant

P > ٠,٠٥ non-significant

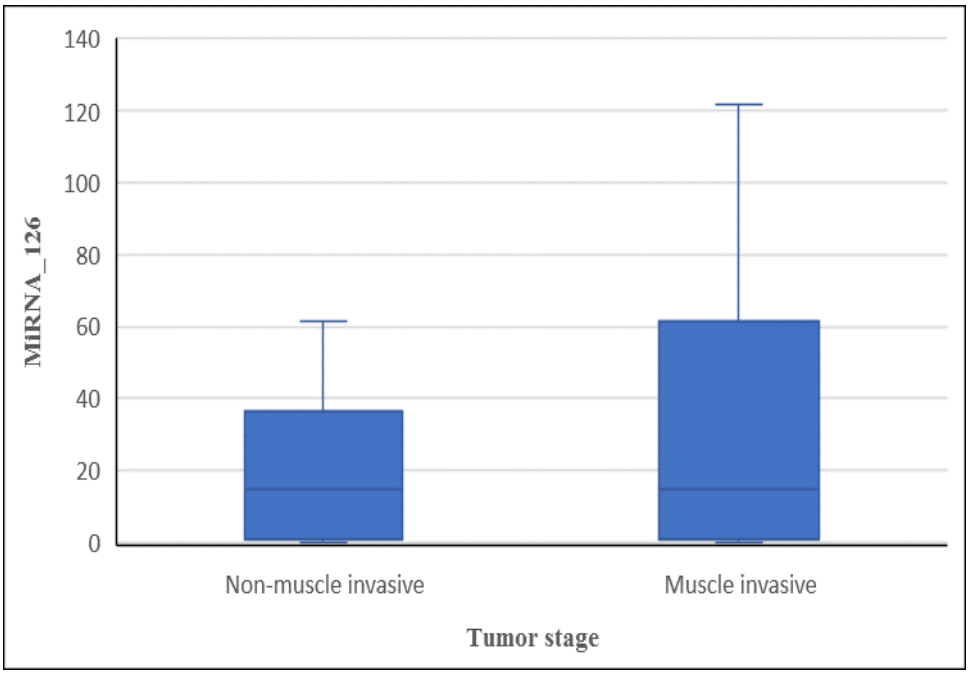


Figure (٣٠): *Difference in mean value of miR-١٢٦ expression regarding tumor stage.*

Table (٢٦) and figure (٣٠): Show statistically non-significant difference in mean value of miR-١٢٦ relative expression in patients with muscle invasive tumors as compared to that of patients with non-muscle invasive tumors.

Table (٢٧): Difference in mean values of miR-٩٦ relative expression in patients with moderately differentiated tumors as compared to those with well differentiated tumors.

Variable		Tumor grade			
		Well differentiated Grade (١)	Moderately Differentiated Grade (٢)	Test	P-value
MiR-٩٦ FRU (Log)	Mean ± SD	٣,٩٢ ± ٠,٤٣	٥,٠١ ± ٠,٦٩	٤,٨٣	٠,٠٠١

P ٠,٠٥ ≤ significant
P > ٠,٠٥ non-significant

Table (٢٧): Shows statistically significant increase in mean value of miR-٩٦ relative expression in patients with moderately differentiated tumors as compared to that of patients with well differentiated tumors.

Table (٢٨): The mean ± SD and test of significance of miR-٩٦ relative expression in patients with poorly differentiated tumors as compared to those with well differentiated tumors.

Variable		Tumor grade			
		Well differentiated Grade (١)	Poorly differentiated Grade (٣)	Test	P-value
MiR-٩٦ FRU (Log)	Mean ± SD	٣,٩٢ ± ٠,٤٣	٥,٥٣ ± ٠,٥٨	٧,٨٦	< ٠,٠٠١

P ٠,٠٥ ≤ significant
P > ٠,٠٥ non-significant

Table (٢٨): Shows statistically significant increase in mean value of miR-٩٦ relative expression in patients with poorly differentiated tumors as compared to those with well differentiated tumors.

Table (٢٩): The mean ± SD and test of significance of miR-٩٦ relative expression in patients with poorly differentiated tumors as compared to those with moderately differentiated tumors.

Variable		Tumor garde			
		Moderately differentiated Grade (٢)	Poorly differentiated Grade (٣)	Test	P-value
MiR-٩٦ FRU (Log)	Mean ± SD	٥,٠١ ± ٠,٦٩	٥,٥٣ ± ٠,٥٨	٣,٩٤	٠,٠٠١

P ٠,٠٥ ≤ significant

P > ٠,٠٥ non-significant

Table (٢٩): Shows statistically significant increase in mean value of miR-٩٦ relative expression in patients with poorly differentiated tumors as compared to those with moderately differentiated tumors.

Table (٣٠): Differences in mean values of miR-٩٦ relative expression in bladder cancer patients regarding tumor grades.

	Tumor Grade				
	Well differentiated Grade (١) (n=٧)	**Moderately differentiated Grade (٢) (n=١٩)**	**Poorly differentiated Grade (٣) (n=٢٤)**	**ANOVA Test**	**P-value***
	Mean ± Standard Deviation	**Mean ± Standard Deviation**	**Mean ± Standard Deviation**		
MiR-٩٦ FRU (Log)	٣,٩٢ ± ٠,٤٣	٥,٠١ ± ٠,٦٩	٥,٥٣ ± ٠,٥٨	F= ٢٧,١٤	<٠,٠٠١

** P-value between groups obtained using Bonferroni test, P-value is considered significant if < ٠,٠٥.*

Table (٣٠) and Figure (٣١): Show that miR-٩٦ relative expression showed significant increase in moderately and poorly differentiated tumors as compared to well differentiated ones and also between each other.

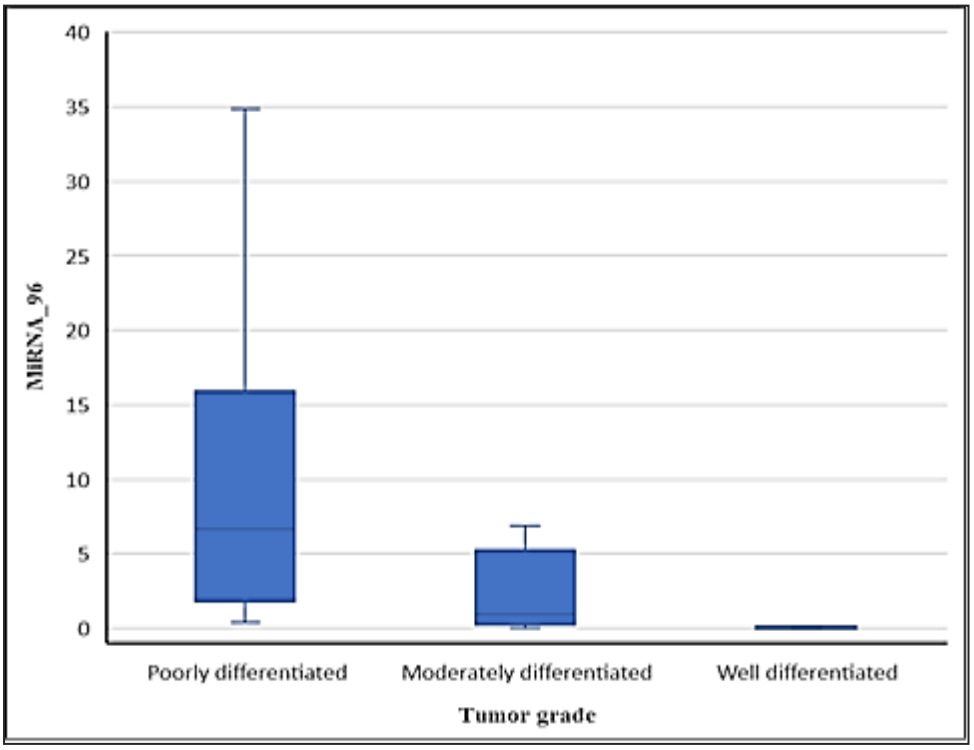


Figure (٣١): *Differences in mean values of miR-٩٦ relative expression of bladder cancer patients regarding tumor grade.*

Table (٣١): Difference in mean values of miR-١٢٦ relative expression in patients with moderately differentiated tumors as compared to those with well differentiated tumors.

Variable		Tumor grade			
		Well differentiated Grade (١)	Moderately Differentiated Grade (٢)	T-Test	P-value
MiR-١٢٦ FRU (Log)	Mean ± SD	٥,٠٠ ± ١,٣٩	٤,٥٠ ± ١,٤٢	٠,٨١	٠,٤٣

P ٠,٠٥ ≤ significant, P > ٠,٠٥ non-significant

Table (٣١): Shows statistically non-significant difference in mean value of miR-١٢٦ relative expression of patients with moderately differentiated tumors as compared to that of patients with well differentiated tumors.

Table (٣٢): Difference in mean values of miR-١٢٦ relative expression in patients with poorly differentiated tumors as compared to those with well differentiated tumors.

Variable		Tumor grade			
		Well differentiated Grade (١)	Poorly differentiated Grade (٣)	T-Test	P-value
MiR-١٢٦ FRU (Log)	Mean ± SD	٥,٠٠ ± ١,٣٩	٥,٠٢ ± ١,٠١	٠,٠٤	٠,٩٧

P ٠,٠٥ ≤ significant, P > ٠,٠٥ non-significant

Table (٣٢): Show statistically non-significant difference in mean value of miR-١٢٦ relative expression in patients with poorly differentiated tumors as compared to those with well differentiated tumors.

Table (٣٣): Difference in mean values of miR-١٢٦ relative expression in patients with poorly differentiated tumors as compared to those with moderately differentiated tumors.

Variable		Tumor grade			
		Moderately differentiated Grade (٢)	**Poorly differentiated Grade (٣)**	**T-Test**	**P-value**
MiR-١٢٦ FRU (Log)	**Mean ± SD**	٤,٥٠ ± ١,٤٢	٥,٠٢ ± ١,٠١	١,٤٢	٠,١٧

P ٠,٠٥≤ significant, P > ٠,٠٥ non-significant

Table (٣٣): Shows statistically non-significant difference in mean value of miR-١٢٦ relative expression in patients with poorly differentiated tumors as compared to those with moderately differentiated tumors.

Table (٣٤): Differences in mean values of miR-١٢٦ relative expression in bladder cancer patients regarding tumor grades.

	Tumor Grade			ANOVA-Test	P-value*
	Well differentiated Grade (١) (n=٧)	Moderately differentiated Grade (٢) (n=١٩)	Poorly differentiated Grade (٣) (n=٢٤)		
	Mean ± Standard Deviation	Mean ± Standard Deviation	Mean ± Standard Deviation		
MiR-١٢٦ FRU (Log)	٥,٠٠ ± ١,٣٩	٤,٥٠ ± ١,٤٢	٥,٠٢ ± ١,٠١	F= ١,٠٥	٠,٥٢

Table (٣٤) and Figure (٣٢): Show that miR-١٢٦ relative expression had statistically non-significant significant difference in moderately and poorly differentiated tumors as compared to well differentiated ones and also between each other.

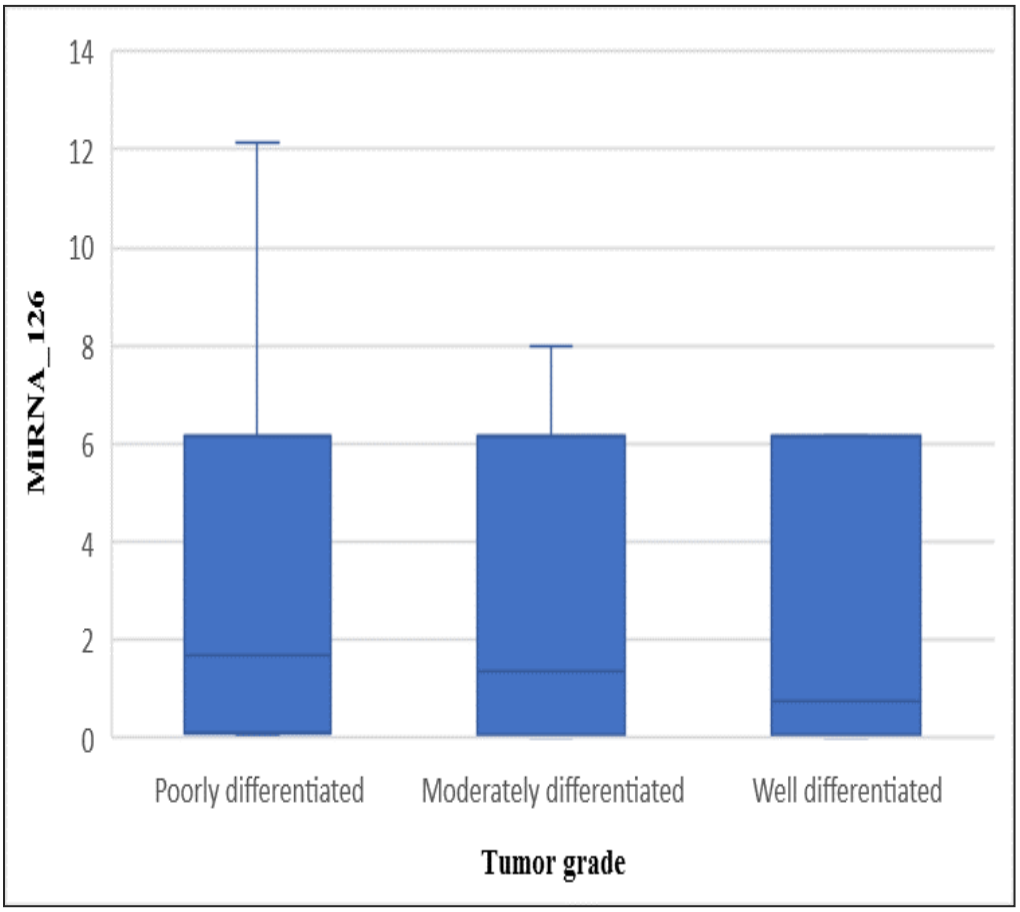


Figure (٣٢): *Differences in mean values of miR-١٢٦ relative expression of bladder cancer patients regarding tumor grade.*

Table (٣٥): Pearson correlation between miR-٩٦ and miR-١٢٦ relative expression in the studied samples (n=٧٠).

Variable	MiR-١٢٦ FRU (log.)	
	Samples (n=٧٠)	
	Pearson correlation	P-value
MiR-٩٦	٠,٢٩	٠١٥.

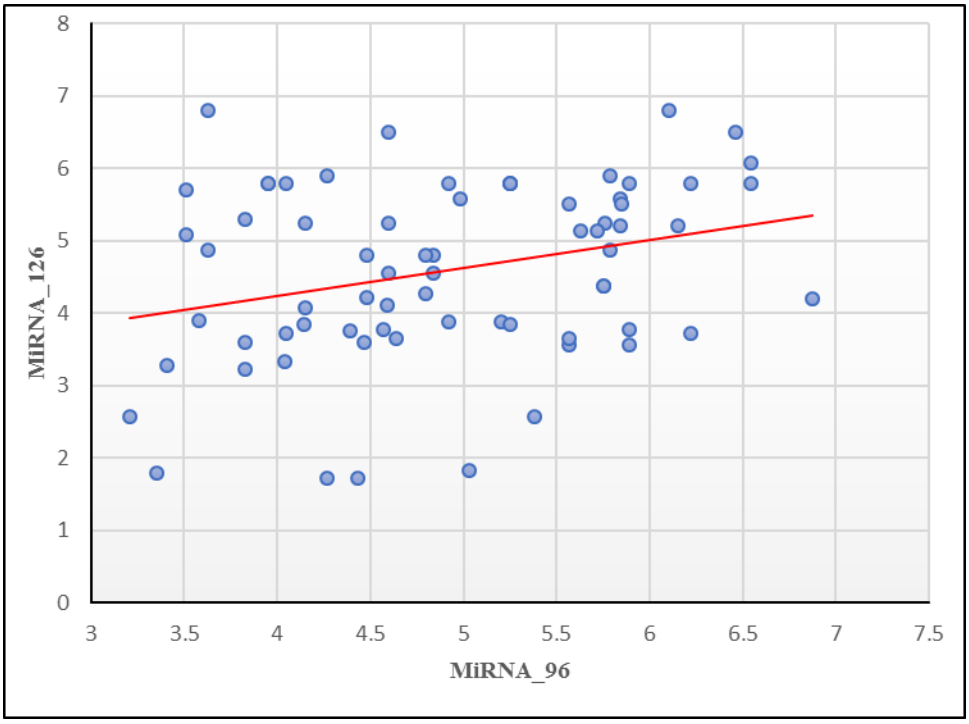


Figure (٣٣): Significant positive correlation between miR-٩٦ and miR-١٢٦ relative expression in the studied samples (n=٧٠).

Table (٣٥) and figure (٣٣): Show a significant positive correlation between miR-٩٦ and miR-١٢٦ relative expression in the studied samples.

Table (٣٦): Cutoff value, sensitivity, specificity, positive predictive value, negative predictive value, accuracy and AUC of miR-٩٦.

Marker	Cutoff value	Sensitivity	Specificity	PPV	NPV	Accuracy	AUC	P-value
miR-٩٦ FRU (log)	>٤,٨٤	٦٨,٠٠٪	٩٥,٠٠٪	٩٧,١	٥٤,٣	٨٤,٢٪	٠,٨٤٢	<٠,٠٠١

**AUC: Area under the curve.*

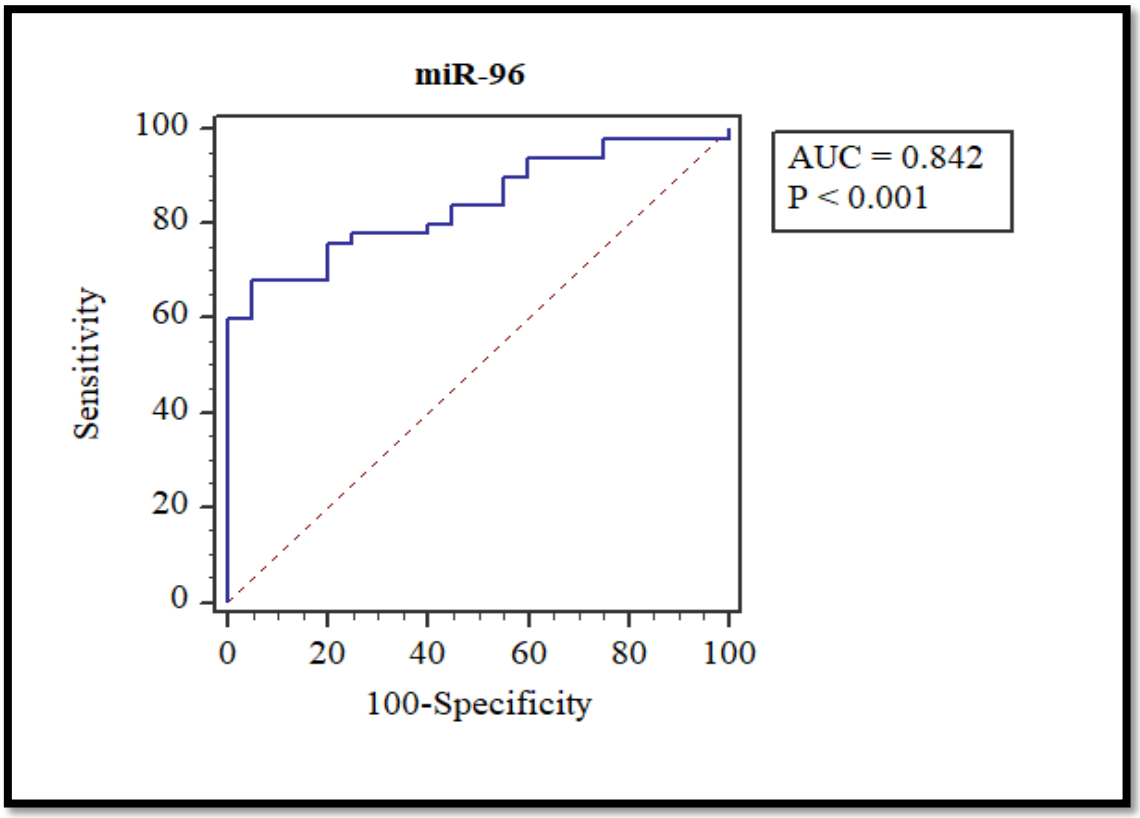


Figure (٣٤): *ROC curve for Performance of miR-٩٦ in early detection of BC.*

Table (٣٦) & Figure (٣٤): Show that miR-٩٦ (the blue line) had high specificity (٩٥٪) with sensitivity (٦٨٪) in detection of early BC.

Table (٣٧): Cutoff value, sensitivity, specificity, positive predictive value, negative predictive value, accuracy and AUC of miR-١٢٦.

Marker	Cutoff value	Sensitivity	Specificity	PPV	NPV	Accuracy	AUC	P-value
miR-١٢٦ FRU (log)	>٥,٠٩	٥٤,٠٠٪	٨٥,٠٠٪	٩٠	٤٢	٧٠,٤٪	٠,٧	٠,٠٠١

**AUC: Area under the curve.*

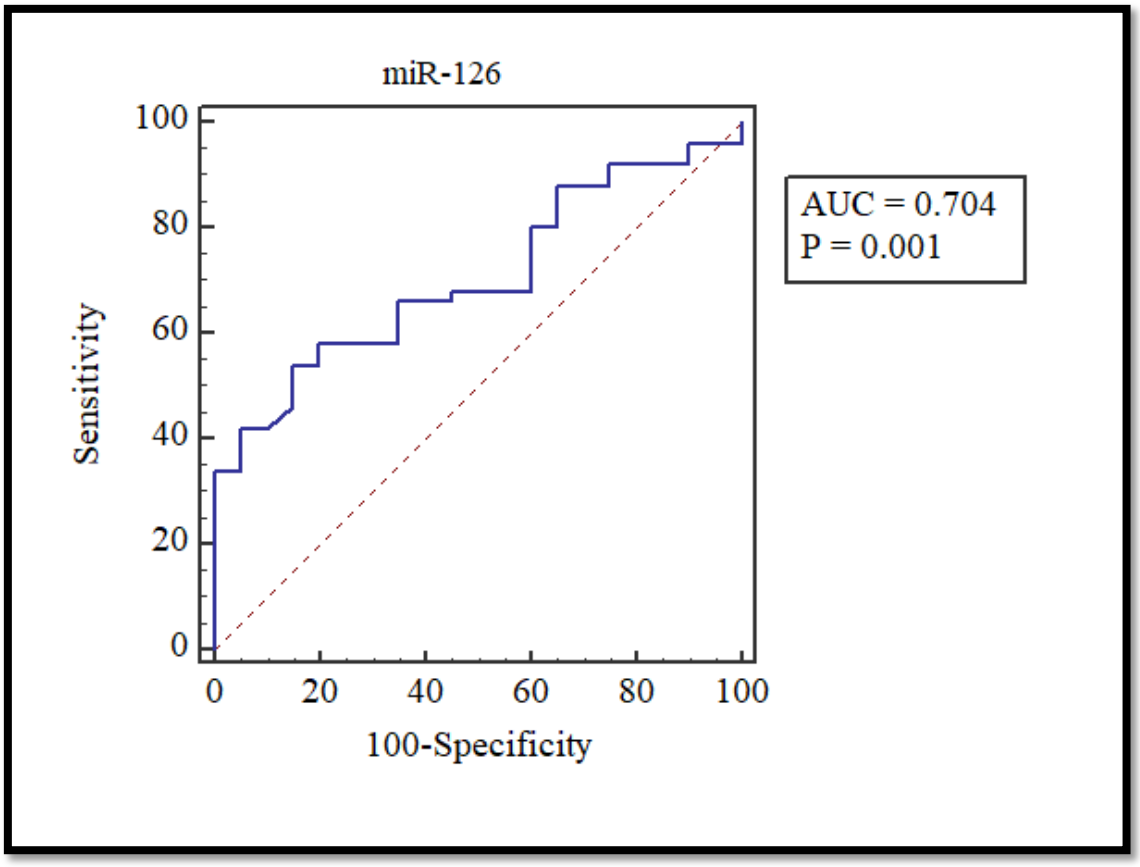


Figure (٣٥): *ROC curve for Performance of miR-١٢٦ in early detection of BC.*

Figure (٣٧) & Table (٣٥): Show that miR-١٢٦ (the blue line) had high specificity (٨٥٪) with sensitivity (٥٤٪) in detection of early BC.

Table (٣٨): Cutoff, sensitivity, specificity, positive predictive value, negative predictive value, accuracy and AUC of combined miR-٩٦ and miR-١٢٦.

Marker	Cutoff value	Sensitivity	Specificity	PPV	NPV	Accuracy	AUC	P-value
miR-٩٦ + miR-١٢٦	>٧.١٤٦	٧٦٪	١٠٠٪	١٠٠	٦٢,٥	٨٨٪	٠.٨٨٢	<٠,٠٠١

**AUC: Area under the curve.*

Table (٣٨) & Figure (٣٦& ٣٧): Show that miR-٩٦ + miR-١٢٦ had higher specificity (١٠٠%) with sensitivity (٧٦%) in detection of early BC.

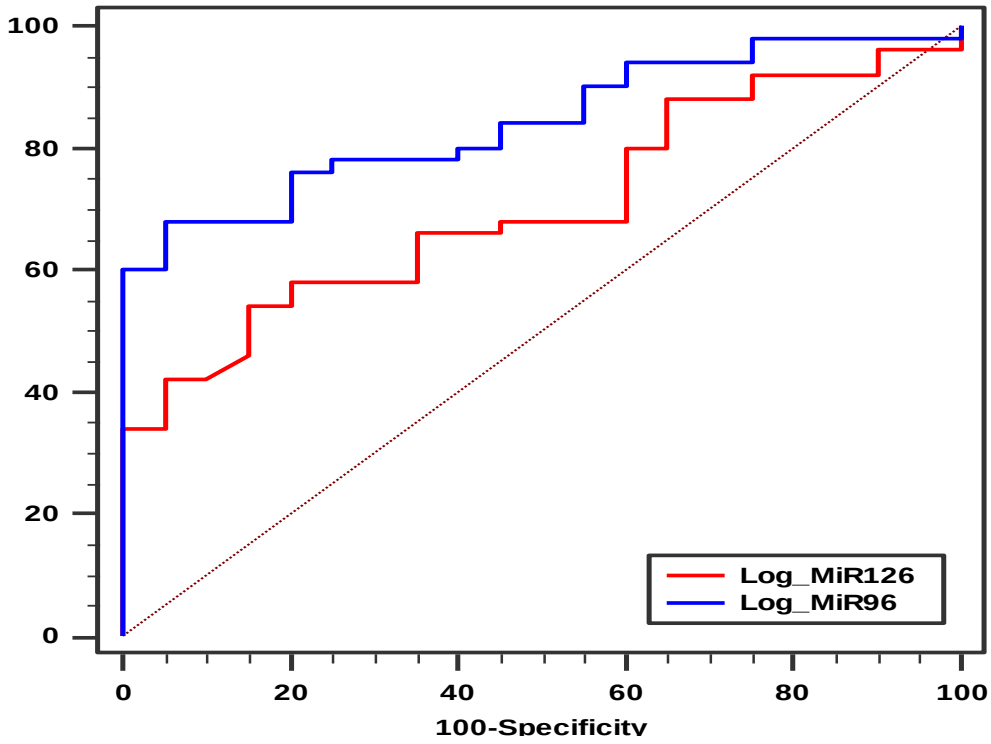


Figure (٣٦): *ROC curve for Performance of both miR-٩٦ and miR-١٢٦ in early detection of BC.*

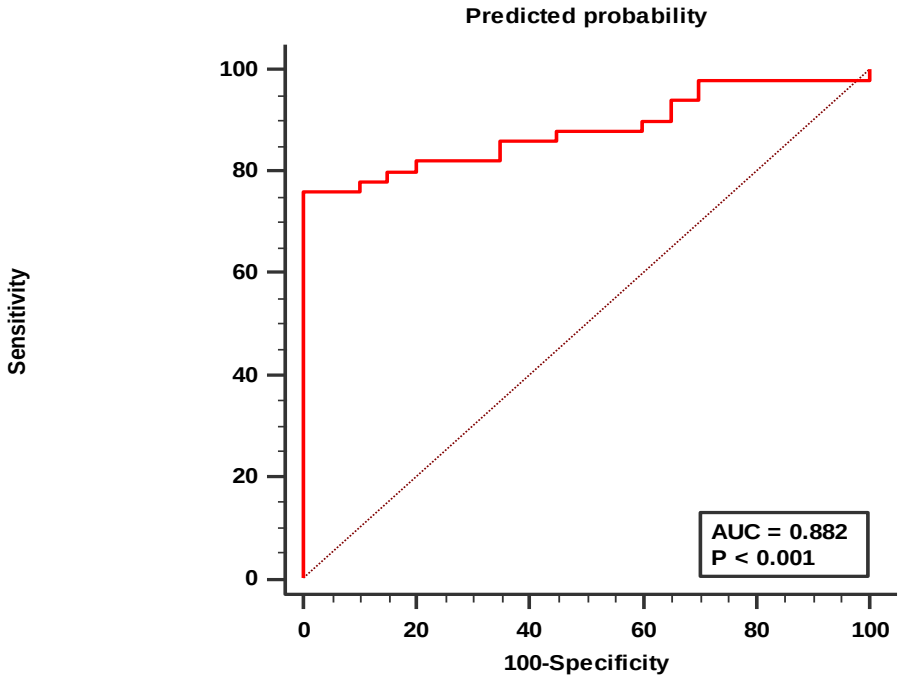


Figure (٣٧): *ROC curve for Performance of combined miR-٩٦ and miR-١٢٦ in early detection of BC.*

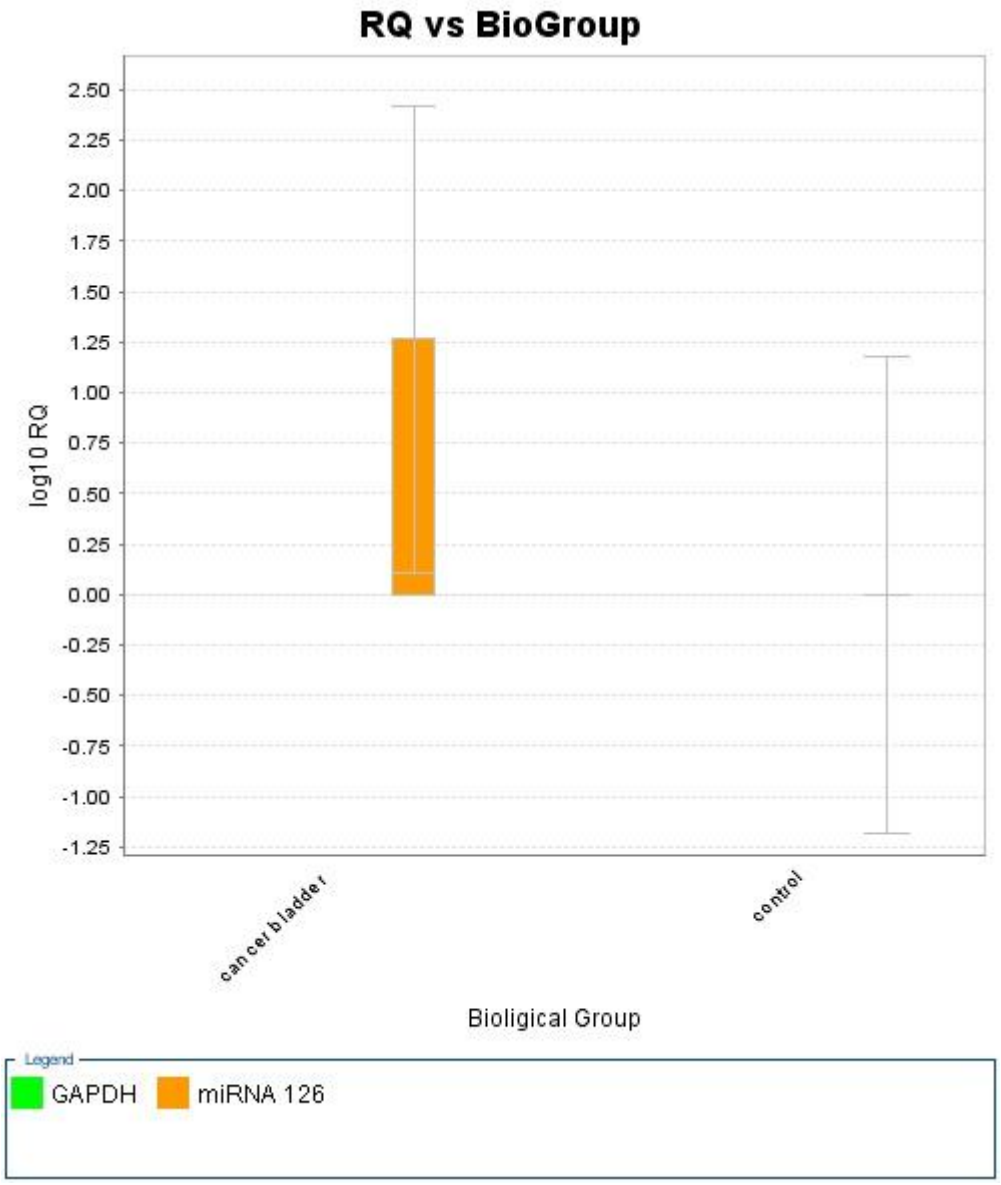


Figure (٣٨): *Fold expression changes of the expression of miR-١٢٦*

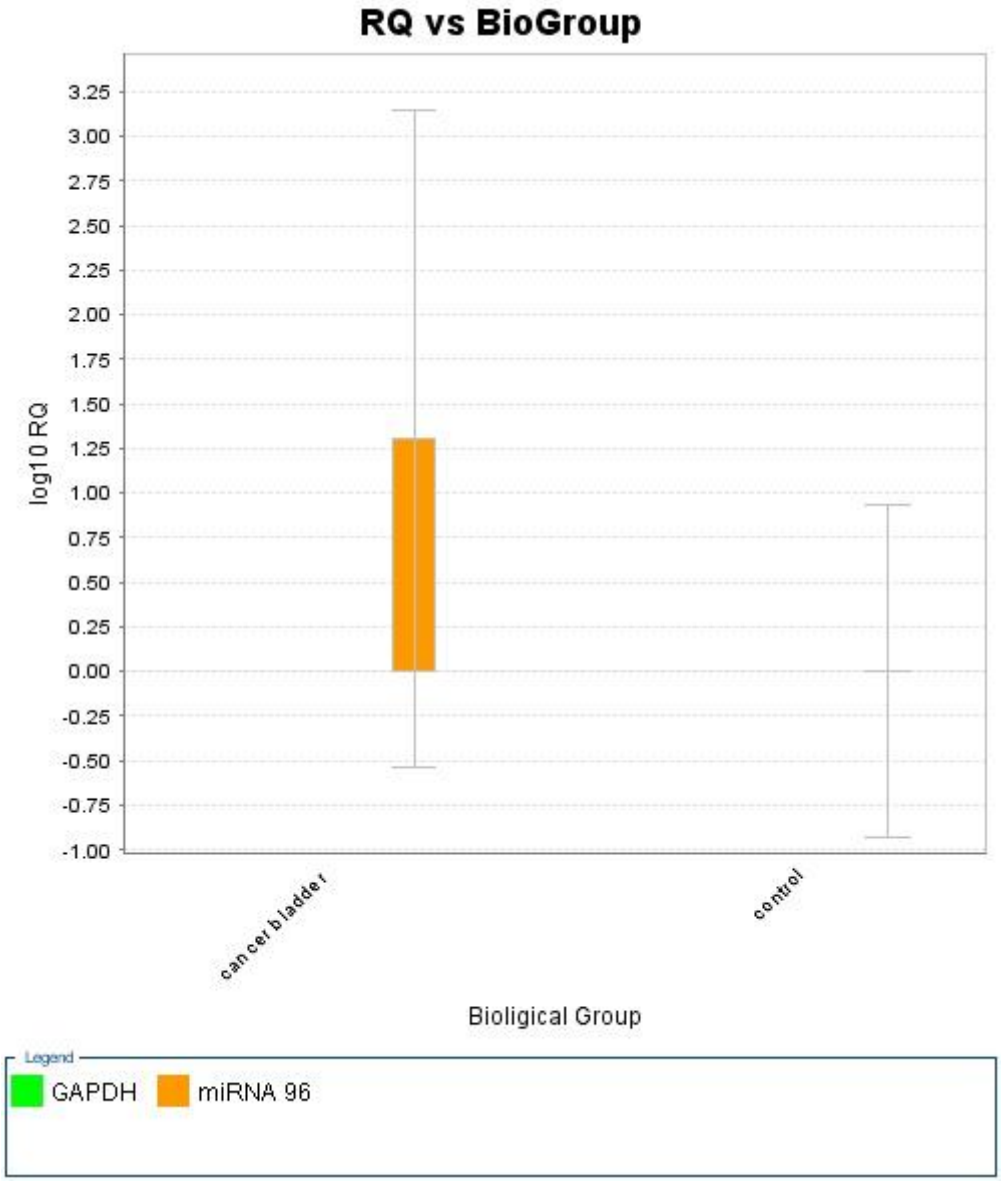


Figure (٣٩): *Fold expression changes of the expression of the miR-* ٩٦

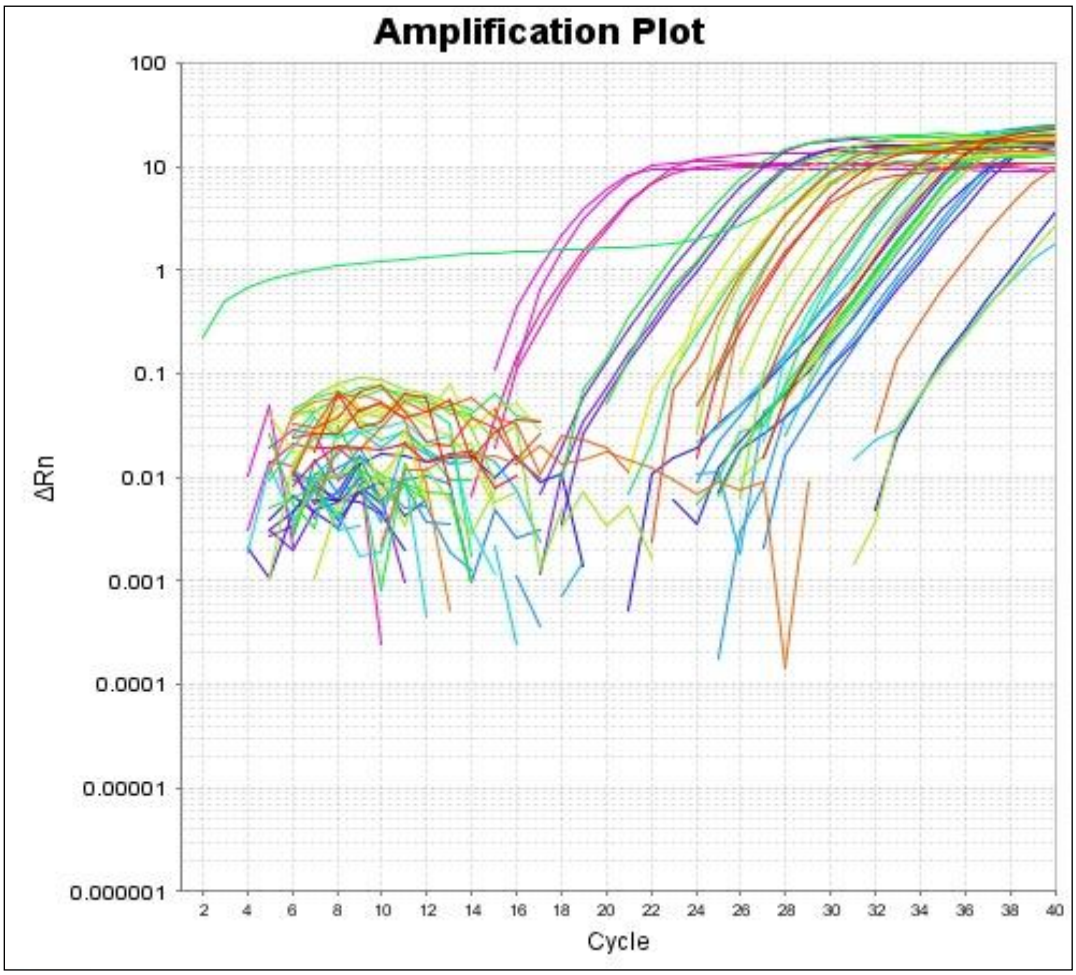


Figure (٤٠): *Typical amplification plot of miR ٩٦ and GAPDH as reference gene.*

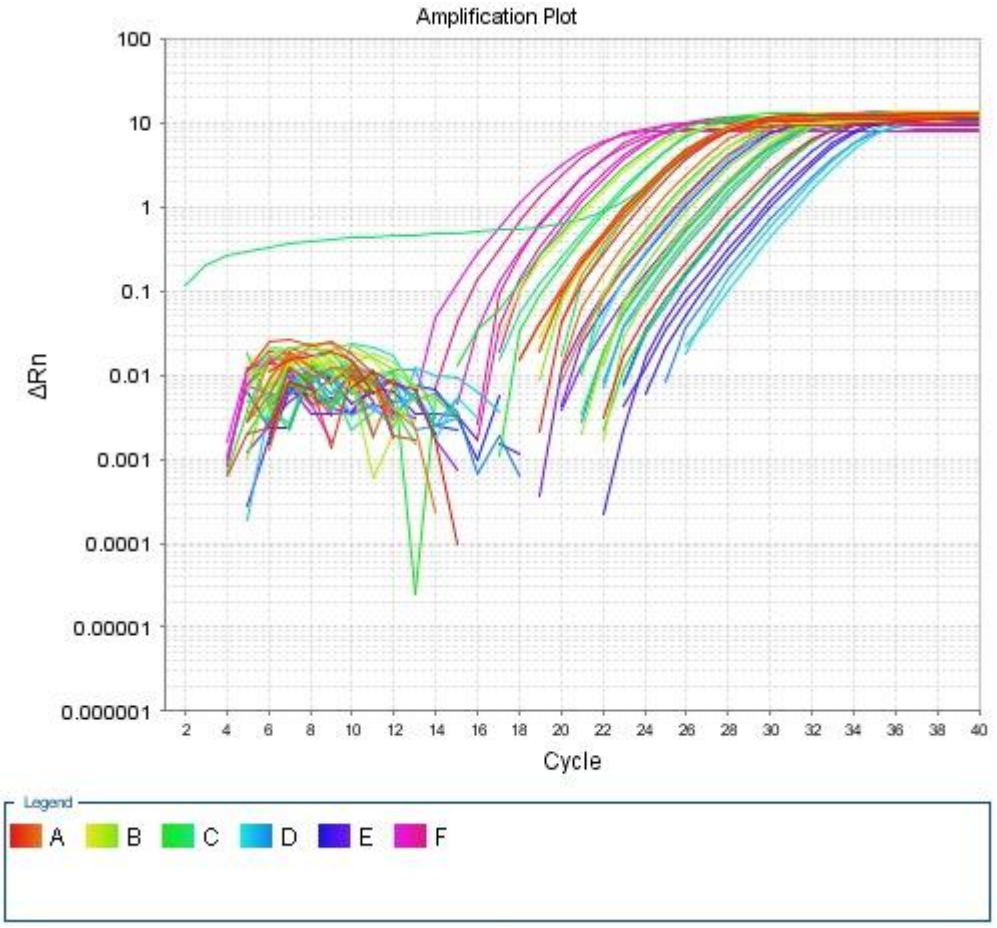


Figure (٤١): *Typical amplification plot of miR-١٢٦ and GAPDH as reference gene.*

Discussion

Bladder cancer is still the most common malignant tumor among males in Egypt, some African and Middle East countries ***(Khaled, ٢٠١٣)***. According to the National Cancer Institute in Cairo, Egypt, it constitutes ٣٠,٣٪ of all cancers ***(Eissa et al., ٢٠١٠)***. The annual death rate from this disease is significant and every year there is an increase in its incidence globally ***(Khochikar, ٢٠١١)***.

Bladder cancer patients at an early stage could be treated by radical surgery, which may improve the patients' life quality and prognosis. However, those patients at advanced stage may only be treated by chemotherapy, radiotherapy and targeted therapy, and the clinical efficacy is quite different among different individuals. Thus, the early diagnosis of bladder cancer is closely correlated with the clinical outcome, indicating that the validation of effective biomarkers for early diagnosis of bladder cancer is vital to the clinical management of such patients ***(Fendler et al., ٢٠١٦).***

The pathogenesis of cancer is a complex process; various aspects affect its genesis and development. A commonly held view is that the imbalanced expression of oncogenes and tumor suppressor genes contributes to tumor cell proliferation, and invasion ***(Zhou et al., ٢٠١٧)***.

The current methods of bladder cancer diagnosis are urine cytology and cystoscopy. Urine cytology is a procedure with ٩٥٪ specificity but low sensitivity, especially in low-grade tumors. Cystoscopy is the current gold-standard method for bladder cancer detection, but it is an invasive and expensive procedure with low specificity and sensitivity in detecting flat CIS tumors ***(Zhu et al., ٢٠١٩)***.

Therefore, there have been lot of efforts in the field to find sensitive, and specific molecular markers for bladder cancer ***(Schmitz-Dräger et al., ٢٠١٥)***. It has been suggested that miRNAs are useful in this respect ***(Dip et al., ٢٠١٢)***. In the past few years, accumulative research has shown the important roles of miRNAs in the development of BC ***(Wang et al., ٢٠٢٠)***.

miRNAs are a class of highly conserved small RNAs that bind the ٣'-UTR region of its target gene and regulate the expression of target genes. The involvement of miRNAs in gene regulatory processes and their implication in several diseases, including cancer, makes them very attractive for diagnosis, prognosis, and treatment in clinical application ***(Armstrong et al., ٢٠١٧)***. The increasing number of studies investigating miRNA expression profiles specific to bladder cancer indicate the growing interest in searching for specific miRNAs to function as diagnostic biomarkers ***(Schmitz-Dräger et al., ٢٠١٥)***.

Recent studies showed that miR-٩٦ was frequently increased in several human cancers. MiR-٩٦ has been reported to exert an oncogenic effect in non-small cell lung cancer, esophageal cancer, hepatocellular carcinoma, breast cancer ***(Yang et al., ٢٠١٩)***. The target genes of miR-٩٦ include the tumor suppressor genes *FOXO١* and *FOXO٣a* in breast cancer ***(Gao & Wang, ٢٠١٥)*** and other validated targets of miR-٩٦ include *RECK* in esophageal cancer ***(Xia et al., ٢٠١٥)***, *EphrinA٥* in Hepatocellular carcinoma ***(Wang et al., ٢٠١٦)***, *SAMD٩* in non-small cell lung cancer (NSCLC) ***(Wu et al., ٢٠١٦).***

On the other hand, miR-٩٦ has been reported to function as a tumor suppressor in several cancers. For instance, miR-٩٦ directly targeted the GTPase Kras (KRAS) oncogene in pancreatic cancer cells and ectopic expression of miR-٩٦ through a synthetic miRNA precursor inhibited KRAS and triggered apoptosis in cells ***(Feng et al., ٢٠١٤).***

To study the expression pattern of both miR-٩٦ and miR-١٢٦ in bladder cancer, voided urine samples were collected from BC patients as well as normal volunteers.

Our study found that the expression level of miR-٩٦ was significantly increased in BC urinary samples compared with that in control samples (BC vs. Control: ٥,٢٢ ± ٠,٨٧ vs. ٤,١٥ ± ٠,٥٣, P < ٠,٠٠١), offering an initial evidence that miR-٩٦ is a promising diagnostic biomarker for BC.

Our findings were in line with the previous genome-wide miRNA expression profile reported by ***Yoshino et al. (٢٠١٣)*** and **Eissa *et al., (٢٠١٥)*** who identified miR-٩٦ as one of upregulated miRNAs in clinical BC tissues compared to normal bladder tissues. **Kriebel *et al. (٢٠١٥)*** reported that the expression of plasma miR-٩٦ in patients with bladder cancer was significantly increased.

In addition, ***Yamada et al., ٢٠١١*** observed that miR-٩٦ was significantly higher expressed in urine of ١٠٠ bladder cancer group than in healthy controls (miR-٩٦, P = ٠,٠٠٥٩) and significantly correlated with tumor grade and stage.

Also, ***Liu et al., ٢٠١٤*** found that miR-١٨٣/٩٦/١٨٢ cluster might play oncogenic roles in BC and ***Wu et al., ٢٠١٥*** concluded that miR-٩٦ may function as an onco-miRNA in BC.

While ***Wang et al.,*** ٢٠١٢ reported that miR-٩٦ was expressed at higher levels in human bladder urothelial carcinomas compared to normal tissues and found that miR-٩٦ increased invasion and differentiation of human bladder T٢٤ cells and promoted cell growth.

Scheffer et al. (٢٠١٤) confirmed that the expression of ٢٢ miRNAs in bladder cancer tissues was upregulated, including miR-٩٦.

Xu et al., ٢٠١٨ applied the gene expression microarray to validate that miR-٩٦ expression was upregulated in bladder cancer tissue.

On the other hand, ***Guo et al.,*** ٢٠١٠ identified that miR- ٩٦ was downregulated in transitional cell carcinoma tissues compared to normal bladder tissues, and regulated FOXO١-mediated cancer cell apoptosis. In this study, urinary miR-٩٦ was found to be higher in low grade BC patients in comparison to high grade tumors.

Upregulation of miR-٩٦ in TCC tumorigenesis is one of the mechanisms of repression of transcription factors (FOXO) of Forkhead Box O subfamily, which is a tumor suppressor gene causing G١ cell cycle arrest and cell death ***(Myatt et al., ٢٠١٠).*** Another mechanism is that hsa-miR-٩٦ by upregulating MAP٤K١ and insulin receptor substrate ١ (IRS١) levels may affect the growth of bladder cancer cells ***(Wang et al., ٢٠١٢).***

Zhang et al. (٢٠١٨) found that miR-٩٦ that regulates the BC progression could be driven by transforming growth factor (TGF-β١), which indicates that miR-٩٦ is a key factor in BC in response to TGF-β signaling. **XU *et al.,*** ٢٠١٧ demonstrated that miR-٩٦ expression was upregulated in bladder cancer cell lines.

In addition, expression of this miRNA decreased significantly after radical surgery, suggesting that it can be used also as a prognostic molecular marker of cancer recurrence ***(Yamada et al., ٢٠١١).***

Moreover, miR-٩٦ expression in the superficial bladder tumors was lower than in invasive tumors and significantly related to the clinical stages of bladder carcinoma and the pathological types. These results revealed that miR-٩٦ maybe plays a role in the process of development, occurrence and infiltration of bladder carcinomas ***(Wang et al., ٢٠١٢).***

As regards, miRNA-١٢٦, our study revealed overexpression of miR-١٢٦ in urine of bladder cancer patients compared of that in control (BC vs. Control: ٤,٨٢± ١,٢٣ vs. ٤,٠٤± ١,٠٥, P = ٠,٠١٥), indicating the feasibility and effectiveness of the urine detection of miR-١٢٦ level in the early diagnosis of bladder cancer.

This finding is in consistent with that obtained by ***Xu et al., (٢٠١٢)*** who reported increase in urine miR-١٢٦ expression level in bladder cancer patients compared to that in non-bladder cancer patients (P=٠,٠٣).

Also, ***Hanke et al. (٢٠١٠)*** reported that miR-١٢٦ was clearly overrepresented in the urine of BC patients in comparison to control groups (healthy donors and patients with urinary tract infections) with a mean fold change of ١٨,٠.

Xiao et al. (٢٠١٦) reported that overexpression of miR-١٢٦ negatively regulated the target gene *phosphatidylinositol ٣-kinase regulatory subunit beta* (*PIK٣R٢*) and further inhibited the phosphatidylinositol ٣ kinase (PI٣K)-protein kinase B (Akt) (PI٣K/Akt) signaling pathway, thereby inhibiting proliferation, migration, and

invasion and promoting apoptosis in human bladder transitional cell carcinoma cell line (BLS) cells.

Both in vitro and in vivo experiments have demonstrated that miR-١٢٦ could enhance tumor cell proliferation, invasion and migration, but suppress tumor cell apoptosis in bladder cancer, suggesting that miR-١٢٦ could play an oncogenic role in the development and progression of bladder cancer ***(Liu et al., ٢٠١٧).***

Low miR-١٢٦ expression has been associated with an invasive phenotype in many tumors, such as ductal invasive tumors of the breast ***(Tavazoie et al, ٢٠٠٨)***, pancreatic adenocarcinoma **(Hamada et al, ٢٠١٢)**, and liver hepatocellular carcinoma ***(Chen et al, ٢٠١٣)***. In addition, low serum levels of miR-١٢٦ in a three-miRNA plasma signature served as a significant prognostic biomarker for tumor progression in lung adenocarcinoma ***(Sanfiorenzo et al, ٢٠١٣)***. Moreover, reduced miR-١٢٦ expression was a marker of tumor progression and nodal metastasis in oral squamous cell carcinoma ***(Sasahira et al, ٢٠١٢)***.

Jia et al. (٢٠١٤) reported down regulation of miR-١٢٦ in BC tissue compared with that in normal tissue. ***Tian et al. (٢٠١٦)*** assumed that low expression of miR-١٢٦ in transitional cell carcinoma of the bladder (BTCC) promoted the expression of VEGF and the microvascular formation in BTCC. It suggested that as a tumor suppressor factor, miR-١٢٦ might play an important role in the pathogenesis and progression and be a potential marker for the metastasis of BTCC.

On the other hand, ***Snowdon et al. (٢٠١٣)*** revealed a moderate sensitivity of ٨٠٪ and a high specificity of ١٠٠٪ for the diagnostic accuracy of miR-١٢٥b and miR-١٢٦.

Hanke et al., ٢٠١٠ examined the specificity and sensitivity of miR-١٢٦: miR-١٥٢ and reported a moderate sensitivity of ٧٢٪ and a high specificity of ٨٢٪ for the diagnostic accuracy of miR-١٢٥b and miR-١٢٦.

Conclusions

It could be concluded that the significant increase in urinary miR-٩٦ and miR-١٢٦ expression level in bladder cancer patients compared to the controls suggests their role as tumor diagnostic non- invasive markers. the results of this study revealed that miRNA-٩٦ expression level in urine sample is a potentially useful urinary biomarker for early diagnosis of bladder cancer.

Recommendation

The followings are recommended:

- ✓ Further studies using wider scale with large study sample are needed.
- ✓ Using miR-٩٦ in applicable way in assessment the prognosis of bladder cancer.
- ✓ The expression of miR-٩٦ and miR-١٢٦ in bladder cancer are attractive as potential biomarkers and new targets for bladder cancer therapy
- ✓ Study of the expression level of miR-٩٦ and miR-١٢٦ as biomarkers
  in the follow up of bladder cancer.

Summary

Bladder cancer is the tenth most common malignant disease and a common cause of cancer death worldwide in both developed and developing countries such as Egypt. It is known to affect men more than women and disease prevalence increases with age. The main type of bladder cancer is urothelial cell carcinoma with frequent recurrence and/ or metastasis.

Cystoscopy, often in combination with cytology, is the gold standard for the diagnosis of bladder cancer. It is however an invasive, painful, operator dependent and costly procedure, which places a substantial burden on patients and healthcare resources.

In addition to cystoscopy and cytology many noninvasive urinary markers have been developed for detection of bladder cancer. However, up till now no urinary-based tumor markers have demonstrated sufficient sensitivity and specificity to replace cystoscopy in the detection of bladder cancer.

Numerous studies have addressed the potential role of microRNAs as diagnostic biomarkers based on their implication in various types of cancers as either oncogenes or tumor suppressors.

The current work aimed to study the role of miR-٩٦ and miR-١٢٦ in
bladder cancer by determining their expression in relation to various clinico-pathological parameters. Also to determine the relationship between the expression of miR-٩٦ and miR-١٢٦.

٧٠ subjects of both sexes were selected from Urology Department, Faculty of Medicine, Benha University Hospital. Their ages ranged from ٣٤- ٨٤ years with mean value ٦١,٠± ٨,٩ years. The subjects were categorized into ٢ groups:

A. Malignant lesion group: Included ٥٠ patients, they were diagnosed as bladder cancer patients by clinical, radiological, cystoscopic and histopathological examinations.

B. Control group: Comprised ٢٠ persons, age and sex matched, with
histopathologically normal urothelium.

All patients were subjected to:

١. Full history taking.

٢. General and local urological examinations.

٣. Routine preoperative laboratory investigations including: urine analysis, complete blood count (CBC), fasting blood sugar, liver function tests, kidney function tests and coagulation profile.

٤. Radiological investigations including: abdominopelvic plain X ray, ultrasonography and CT.

٥. ECG (if indicated for preoperative assessment).

٦. Diagnostic cystoscopy and biopsy for histopathology.

٧. Molecular biology investigations: Quantitative reverse transcription polymerase chain reaction (qRT-PCR) for detection of miR-١٤٥ and Oct٤ gene expression levels.

Voided urine (٣٠–٦٠ mL) samples were obtained from all individuals. Urine samples were collected using sterile urine collection cups, sealed immediately and placed on ice stored at -٨٠^{o}c and centrifuged at ٢،٥٠٠–٤،٠٠٠ xg for ١٥–٢٠ min. the samples were used for estimation of miR-٩٦ and miR-١٢٦ gene expression levels by qRT-PCR.

The results of the current study showed that miR-٩٦ expression level significantly increased in bladder cancer group compared to the control group. Also, miR-١٢٦ showed significant increase in bladder cancer group compared to control group.

The results showed that the urine expression of miR-٩٦ was related to tumor stage and tumor grade. The higher the tumor stage, the higher the expression of miR-٩٦ in urine. The expression of miR-٩٦ in urine of patients with tumor grade I was significantly lower than that of patients with tumor grade II ($P < .٠٠١$) that was significantly lower than that of patients with tumor grade III. However, the expression of miR-٩٦ in urine of patients with bladder cancer was not related to age, sex, smoking and malignant type ($P > .٠٥$). It is suggested that the expression of miR-٩٦ in urinary sediment of patients with bladder cancer is related to the progression of tumor.

Regarding expression of miR-١٢٦ in urine, the results showed that the urine expression of miR-١٢٦ was related to tumor type (P= ٠,٠١٧) as it was significantly higher in Small cell carcinoma (SCC) compared to Transitional cell carcinoma (TCC) but not related to age, sex, smoking, tumor stage and tumor grade.

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الملخص العربى

سرطان المثانة هو عاشر أكثر الأمراض الخبيثة شيوعاً وسبب شائع للوفاة بالسرطان في جميع أنحاء العالم في كل من البلدان المتقدمة والنامية مثل مصر.ومن المعروف سرطان المثانة يعد شائعا فى الرجال أكثر من النساء و يزداد مع التقدم في السن.

تنظير المثانة، في كثير من الأحيان بالاشتراك مع علم الخلايا، هو المعيار الذهبي لتشخيص سرطان المثانة.ومع ذلك ، فهي عملية جراحية ومؤلمة ومعتمدة على المشغل ومكلفة ، مما يلقي بعبء كبير على المرضى وموارد الرعاية الصحية.

بالإضافة إلى تنظير المثانة وعلم الخلايا تم تطوير العديد من علامات البولية غير الباضعة للكشف عن سرطان المثانة.ومع ذلك، حتى الآن لم تظهر علامات الورم البولية الحساسية الكافية وخصوصية لاستبدال تنظير المثانة في الكشف عن سرطان المثانة.

وقد تناولت العديد من الدراسات الدور المحتمل للاحماض النووية الريبوزية متناهية الصغر كعلامات بيولوجية للتشخيص حيث اثبتت العديد من الدراسات تأثيرها في أنواع مختلفة من السرطانات كمثبط للسرطان وانواع اخرى ثبت ان هذه الاحماض النووية لها دور فى زيادة خطورة الاصابة بالسرطان.

ويهدف العمل الحالي إلى دراسة دور الأحماض النووية الريبوزية المتناهية الصغر (٩٦ و ١٢٦) في تشخيص سرطان المثانة عن طريق تحديد تعبيرهما فيما يتعلق بمختلف العلامات المرضية السريرية.أيضا لتحديد العلاقة بين التعبيرالجينى لكل الأحماض النووية الريبوزية المتناهية الصغر (٩٦ و ١٢٦).

تم اختيار ٧٠ مادة من الجنسين من قسم المسالك البولية، كلية الطب، مستشفى جامعة بنها.وتراوحت أعمارهم بين ٣٤ و٨٤ سنة مع متوسط قيمة ٦١,٠ ± ٨,٩ سنة.تم تصنيف الموضوعات إلى مجموعتين:

١ .مجموعة مرضى سرطان المثانة: شملت ٥٠ مريضا، تم تشخيصهم على أنهم مرضى سرطان المثانة عن طريق الفحوص السريرية والإشعاعية والمنظار الكيسي باثولوجيا الأنسجة.

٢. المجموعة الضابطة: تتألف من ٢٠ شخصا، متقاربين فى السن والجنس مع مجموعة المرضى، وتم التكد من سلامة انسجة المثانة فيهم

وقد تم اخذ الآتى من جميع المرضى:

١. التاريخ المرضى للحالات
٢. فحوصات المسالك البولية العامة والمحلية
٣. اختبارات الدم الروتينية قبل الجراحة بما في ذلك: تحليل البول، تعداد الدم الكامل ،قياس نسبة السكر في الدم ، اختبارات وظائف الكبد، اختبارات وظائف الكلى وملف التخثر
٤. الأشعة التشخيصية بما في ذلك: الأشعة السينية، الموجات فوق الصوتية التشخيصية الأشعة المقطعية على البطن والحوض
٥. تخطيط كهربية القلب (تمت الإشارة إلى التقييم قبل الجراحة)
٦. منظار المثانة التشخيصي
٧. تفاعل البلمرة المتسلسل الكمى لكل من الحمض النووى الريبوى متناهى الصغر ٩٦ و ١٢٦.

أخذ العينات

بعد الحصول على الموافقة من المرضى، تم الحصول على عينات البول (٣٠-٦٠ مل) من جميع الأفراد. تم جمع عينات البول باستخدام أكواب جمع البول المعقمة، وعلى الفور وضعت فى الفريزرعلى درجة الحرارة ^{o}C ٨٠ - وتم وضع العينات فى جهتز الطرد المركزى لمدة ١٥-٢٠ دقيقة.واستخدمت العينات لتقدير مستويات التعبير الجيني لكل من الحمض النووى الريبوى متناهى الصغر ٩٦ و ١٢٦ بواسطة تفاعل البلمرة المتسلسل الكمى.

وأظهرت نتائج الدراسة الحالية أن مستوى التعبير الحمض النووى الريبوى متناهى الصغر ٩٦ زاد بشكل ملحوظ في مجموعة سرطان المثانة مقارنة مع المجموعة الضابطة. أيضا، الحمض النووى الريبوى متناهى الصغر ١٢٦ أظهر زيادة كبيرة في مجموعة سرطان المثانة مقارنة مع مجموعة الضابطة.

الخلاصة

نستخلص من هذه الدراسة أن الزيادة الكبيرة في مستوى التعبير الجينى لكل من الحمض النووى الريبوى متناهى الصغر ٩٦ و ١٢٦ في مرضى سرطان المثانة مقارنة مع الضوابط تشير إلى دورها

كعلامات تشخيص الورم.وكشفت نتائج هذه الدراسة أن مستوى التعبير ميرنا-٩٦ في عينة البول هو علامة حيوية بولية مفيدة للتشخيص المبكر لسرطان المثانة.

دور الأحماض النووية الريبوزية المتناهية الصغر (٩٦ و ١٢٦)
الموجودة بالبول كدلالات حيوية تشخيصية للكشف عن سرطان المثانة

توطئة للحصول على درجة الدكتوراة
فى الكيمياء الحيوية الطبية والبيولوجيا الجزيئية

والمقدمة من

الطبيبة/ هبة الله إمام محمد أحمد

ماجيستير الكيمياء الحيوية الطبية والبيولوجيا الجزيئية
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