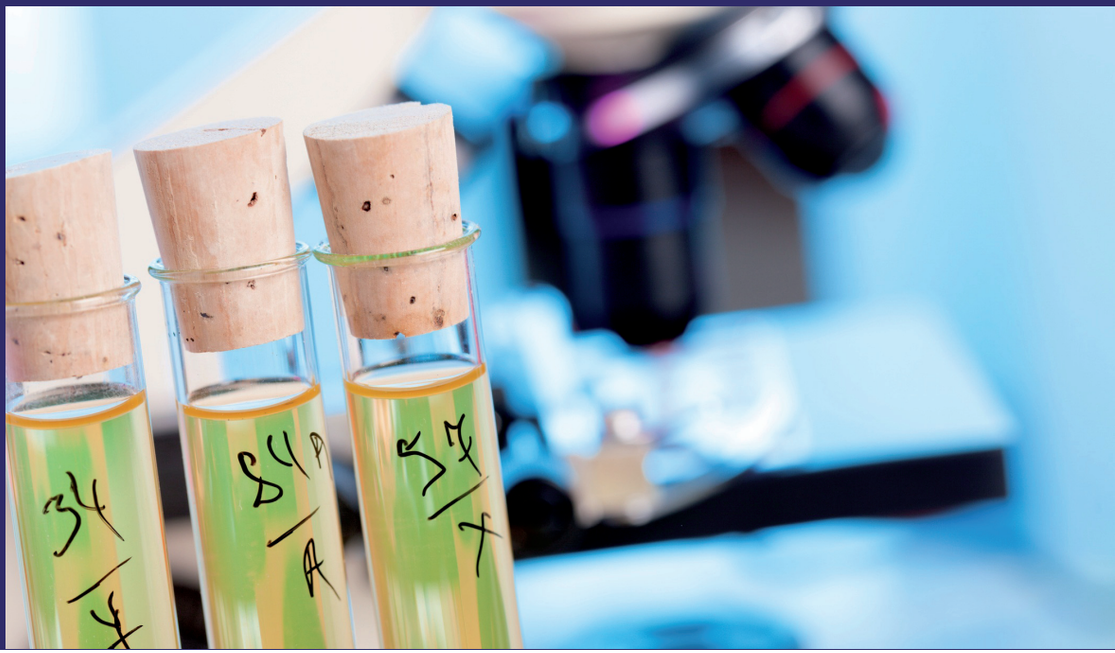
The silver-staining technique for nucleolar organizer regions (AgNOR) of Ploton et al, as popularized by Crocker et al, is being widely used for evaluation of nucleolar function, especially in neoplasia. This work is carried out to evaluate the usefulness of AgNOR technique in the differentiation between benign and malignant lesions in urine cytology, and to evauate the correlation of AgNOR count and morphology with tumor grade and patients' sex. Urine samples from sixty-four patients were centrifuged, deposits smeared on slides and stained with Hematoxylin and Eosin (H&E) and AgNOR stain. The diagnosis of each patient had been established depending on histological analysis of bladder biopsies and the diseases were classified into three groups: non-neoplastic lesions (n=34), low grade carcinoma (n=12) and high grade carcinoma (n=18). AgNOR counts, variation in size and distribution of AgNOR dots in smears were graded and compared in the three groups. The correlation between AgNORs and patients' sex was also analyzed.



Haider Ghazi Hussein

Dr. Haider Ghazi Hussein, FICMS (pathology), MIAC. Graduated as a physician from College of Medicine-University of Baghdad at 1998 & received a Fellowship Degree in the field of Pathology & a Membership at the International Academy of Cytopathology. Work now as the Head of Cytopathology Department at the Central Public Health Lab. in Baghdad-Iraq.

978-3-8443-8987-6

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Impressum / Imprint
Bibliografische Information der Deutschen Nationalbibliothek: Die Deutsche Nationalbibliothek verzeichnet diese Publikation in der Deutschen Nationalbibliografie; detaillierte bibliografische Daten sind im Internet über http://dnb.d-nb.de abrufbar.

Bibliographic information published by the Deutsche Nationalbibliothek: The Deutsche Nationalbibliothek lists this publication in the Deutsche Nationalbibliografie; detailed bibliographic data are available in the Internet at http://dnb.d-nb.de.

Coverbild / Cover image: www.ingimage.com

Verlag / Publisher:
LAP LAMBERT Academic Publishing
ist ein Imprint der / is a trademark of
AV Akademikerverlag GmbH & Co. KG
Heinrich-Böcking-Str. 6-8, 66121 Saarbrücken, Deutschland / Germany
Email: info@lap-publishing.com

Herstellung: siehe letzte Seite /
Printed at: see last page
ISBN: 978-3-8443-8987-6

Zugl. / Approved by: Baghdad, Al-Nahrain university, Diss., 2007

Table of Contents:

List of Tables:

List of Figures:

List of Abbreviations:

AgNOR	Argyrophilic Nucleolar Organizer Region
BrdU	BromodeoxyUridine
CIS	Carcinoma in Situ
DNA	Deoxyribonucleic Acid
H&E	Hematoxylin and Eosin
ISUP	International Society of Urological Pathology
kD	Kilo Dalton
mAgNOR	Mean number of Argyrophilic Nucleolar Organizer Region
ml.	Milliliter
No.	Number
NOR	Nucleolar Organizer Region
pAgNOR	Proliferation index of Argyrophilic Nucleolar Organizer Region
PCNA	Proliferating Cell Nuclear Antigens
PCR	Polymerase Chain Reaction
rDNA	Ribosomal Deoxyribonucleic Acid
RNA	Ribonucleic Acid
rpm	Round per minute
rRNA	Ribosomal Ribonucleic Acid
SAPA	Subjective AgNOR Pattern Assessment
SD	Standard Deviation
WHO	World Health Organization

Acknowledgement

I would like to present my sincere appreciation and gratitude to my teacher and supervisor ***Professor Dr. Hussam Hassun Ali*** for his help, guidance, encouragement and moral support throughout the study.

I am also greatly indebted to the Chairman of the Scientific Council of Pathology ***Professor Dr. Raji Al-Hadithi*** for his fatherhood spirit and for giving me the opportunity to continue my postgraduate study.

I would like to express my gratitude to ***Assistant Professor Dr. Alaa Ghani***, Head of Pathology Department; College of Medicine; Al-Nahrain University for his valuable scientific support and encouragement.

Special thanks to the skillful technicians at the Cytology Laboratory at Al-Kadhimiya Teaching Hospital for their cooperation and assistance, and to the technicians of the Cytology Laboratory of Baghdad Teaching Hospital in the Medical City.

This Work is Dedicated to My Wife And Children for their great help, moral support and care

Chapter One: Introduction & Review of Literatures

Introduction:

Urinary cytology is recognized as a reliable method for the diagnosis of urothelial lesions and follow up of bladder tumor treatment, reducing the number of cystoscopies. [1]

Urine cytology is primarily used for diagnosis of symptomatic patients, detection of cancer in high-risk patients (e.g., those exposed to industrial chemicals and metals, cigarette smokers, and those with schistosomiasis), and follow-up of patients with history of urinary tract neoplasia. Lesions may be detected cytologically before they can be seen cystoscopically. Urinary cytological examination is capable of detecting small or hidden lesions (e.g. in diverticuli, ureters, renal pelvis, prostatic ducts, residual urethras). [2, 3]

Urinary cytology can detect most aggressive neoplasms and carcinoma in situ. Patients with low-grade non-invasive tumours can be followed up cytologically. Patients with negative cytological findings have a very low risk of recurrence, while high-grade cytological abnormalities predict an aggressive tumour course. Urine cytology is also a better indicator of the presence of concomitant urothelial atypia than pre-selected mucosal biopsies. [4, 5]

Unfortunately, the diagnosis of urinary tract specimens is less perfect even among experts. A review of 17 published series showed that at their worst, the false-negative rates were more than 50% for primary bladder cancer and averaged nearly 75% for papillomas. An important diagnostic principle is that the higher the grade of the tumour, the more accurate the diagnosis. [6, 7, 8, 9, 10]

These problems have motivated the development of new techniques to augment routine methods and to improve the accuracy and reproducibility of prognostication. Of the various newer techniques used for assessing tumor tissue based on nuclear studies, staining of the nucleolar organizing regions by silver

compound (AgNOR) has become popular for its simplicity, ease of use, low cost and its good correlation with other proliferative markers, as their frequency within nucleus are significantly higher in malignant cells than in normal, reactive or benign neoplastic cell. [11, 12]

Nucleolar organizer regions [NORs] are loops of ribosomal DNA (rDNA) occurring in the nucleoli of cells. [13] In the human genome NORs are located on short arm, of five acrocentric chromosomes (13, 14, 15, 21 and 22); and they can be visualized by staining with silver nitrate solution under prescribed conditions. The number of discernible and therefore countable dots (AgNOR) depends upon several factors. [13]

The correlation between AgNOR numbers and cell proliferation has been widely investigated in tumors by comparing AgNOR values with kinetic data obtained by applying a panel of proliferation markers. [11]

Therefore the AgNOR parameter can reflect the neoplastic nature of cells and represents a promising prognostic indicator in tumor pathology. [11, 12]

Aims of the Study:

The aims of the present study were:

1. To investigate whether the argyrophilic nucleolar organizer region (AgNOR) technique applied on urine cytology could be helpful in the diagnosis of neoplastic and non-neoplastic lesions of the bladder, using AgNOR count and morphology as parameters for evaluation.

2. To study the correlation of AgNOR count and morphology with tumor grade and patients' sex.

Urinary Cytology:

The urinary tract is lined by transitional epithelium and historically this epithelium was so named by Friedrich Henle, a 19th century German pathologist, because he thought that the urinary epithelium was 'transitional' between squamous and glandular. [9] It is now recognized as a specific type in its own right. Therefore, it is also called as 'urothelium'. However, it is interesting that some tumours of the transitional epithelium do indeed express the ability to differentiate along squamous or glandular lines and that patches of squamous and glandular mucosa are commonly interspersed in benign transitional epithelium. Urine cytology is useful in diagnosing diseases that involve this mucosal surface and the urinary collecting system lined by transitional epithelium. [2]

Urinary cytology is an outstanding diagnostic tool for primary tumors as well as for recurrent neoplasm. [14]

Since more than 95% of all bladder cancer evolves from urothelial cells, flakes of neoplastic cells can be detected in the urine after specific processing. Depending on the grade of the tumor the sensitivity of this method lies between 65% and 90%. Since the Carcinoma in Situ (CIS) is frequently overlooked in cystoscopy, urinary cytology is especially valuable for its detection. Urinary cytology might be combined with the use of tumor markers and fluorescence cytology to enhance its sensitivity and specificity. [14]

The importance of urine cytology for the detection of urological malignancy is well established. [15, 16] With the increasing incidence of carcinoma of the urinary bladder in industrialized countries the use of cytology has become more important. [17]

Urine cytology is often used as an initial step in bladder cancer diagnosis. While urine cytology has a high sensitivity for high-grade tumors, lower-grade neoplasms

may be difficult to identify, also the invasiveness of a tumor cannot be assessed on urine cytology. [18]

Cytological examination of a urine specimen is a simple, safe, and inexpensive method that may uncover a hidden urothelial cancer. [8, 9]

Tumours of the urinary tract are relatively inaccessible to direct biopsy, and the tumours are often multifocal. Since the entire mucosal surface, including the farthest reaches of the urinary tract, is bathed in this easily obtained fluid, in theory, urine is the perfect specimen to examine for evidence of tumour. Cytology may become positive long before the cystoscopy or biopsy. But lesions cannot be anatomically localized with urine cytology alone. [2]

It must be appreciated that the urothelium may undergo changes as the result of the systematic administration of drugs. The use of cytotoxic drugs may lead to epithelial alterations almost indistinguishable from carcinoma. [19]

It is therefore imperative that all specimens of urine for cytodiagnosis should be accompanied by an adequate clinical history which includes data on treatment with drugs and radiation. [19]

Indications for Urine Cytology Examination:

Urothelial carcinogenesis is of particular significance for certain high-risk groups of the population, namely those who work in certain chemical industries, the heavy smoker and the patient with schistosomiasis. Aniline dyes, benzidine, B-naphthylamine and other chemicals, when absorbed, may lead, after an interval of many years, to urothelial changes which may be present many years before the tumor manifests itself. In some of these workers the first lesion may be a carcinoma in situ, which can be detected by routine screening of the exposed workers. Patients with schistosomiasis also frequently develop urothelial neoplasia, usually of squamous cell

type. A direct etiological relationship between schistosomiasis and urothelial neoplasia has, however, not been established with certainty. [19]

The well known indications for urine cytology examination are: [9]

1. Tumour detection and diagnosis of aggressive neoplasms or their follow-up, carcinoma in situ, small or inaccessible lesion as in ureters, pelvis, diverticuli etc.
2. Screening of high-risk asymptomatic patients, as with industrial chemical or metal exposure, those with schistosomiasis or smokers etc.
3. Monitor tumours and therapy, for example low-grade non-invasive tumours, carcinoma in-situ.

It is therefore suggested that the following signs and clinical histories are indications for urine cytodiagnosis: [19]

(1) Haematuria and microhaematuria.
(2) Persistent urological symptoms (chronic infection).
(3) High-risk groups (industrial workers in chemical and other industries).
(4) Schistosomiasis.
(5) Follow-up after treatment for urinary tract tumors.

Technique for Collecting Specimens:

There are three basic types of exfoliated urinary tract specimens: (1) voided urine, (2) catheterized urine, and (3) brushing/washing specimens. [2] Specimens obtained from bladder irrigation are superior to those resulting from voided urine. [20]

Controversy exists on whether the patient should be hydrated or dehydrated before the collection of the specimen. When the patient is dehydrated, the urine is more concentrated with cells, and a small volume is satisfactory. With hydration, although diluted, the amount of urine and the number of cells is increased. [21]

These specimens should be processed immediately or refrigerated and processed as soon as possible. If a delay is anticipated, immediate fixation with 50% ethanol may preserve the specimen for several days. In the cytology laboratory the 'urinary fluid' is treated as other body fluids and is centrifuged. The processed specimen is used to prepare smears. [2]

Benign Cells in Urine Specimens:

Urine is an acellular liquid product of renal excretory function. As the liquid passes through the excretory renal tubules, renal pelvis, ureter, bladder, and urethra, it picks up desquamating cells derived from the epithelia of these organs. [22]

Normal Cells:

Normal bladder urine contains very few epithelial cells and no bacteria, erythrocytes or inflammatory cells. The urothelial cells have pale cyanophilic cytoplasm, distinct cytoplasmic borders and round nuclei with fine granular chromatin. There is a marked variation of their size (10-80µ) and their shape is often triangular. The nuclei are central or eccentric and the nuclear/ cytoplasmic ratio is approximately 1/ 3. The cells are comparable in size with the parabasal cells of squamous epithelium and may have long extensions and irregularities in shape. A few eosinophilic or orangeophilic squamous cells from the urethral meatus are seen in voided specimens from males. Non-catheterized specimens from females always contain vaginal squamous and inflammatory cells and organisms. For this reason, definitive cytological study in women with urinary diagnostic problems is best carried out on catheterized specimens or specimens obtained by transpubic puncture. [19, 21]

The urothelium in the base of the bladder is influenced to some extent by estrogenic substances to differentiate along squamous lines. This occurs to a variable degree in women during the menstrual cycle or as the result of estrogenic therapy in

men or women. If it is desirable to study this change for any reason, accurate evaluation requires catheterized bladder urine specimens from both sexes. [19]

Renal tubular epithelial cells are seen singly or in groups of 4 to 5 cells in renal parenchymal disease or circulatory disturbances resulting in loss of nephrons. They are round or cuboidal, with indistinct cytoplasmic borders and rather dense faintly granular, grey or reddish-brown cytoplasm and round nuclei often showing degenerative changes. Tubular casts of various types often accompany these. [19]

Special caution is necessary in interpreting cellular changes if the patient has been subjected to radiation therapy to the pelvis or abdomen or is receiving cytotoxic drugs. Unusually large nuclei and variation in cell size occur in these circumstances. Even the common analgesic drugs have been shown to cause excessive desquamation of renal tubular epithelial cells which are rather large, pleomorphic and sometimes binucleated. Traumatic evulsion of normal urothelium by ureteral catheter produces many pleomorphic urothelial cells and groups of cells indistinguishable from those of low-grade papillary carcinoma. Such cells found in ureter catheter specimens should be considered significant only if they have been observed in previous bladder urine specimens. [19]

Traumatically exfoliated transitional cells may shed singly, in clusters, or in large sheets. The cell size (15±5μ) and shape are uniform. They are elongated or triangular when single or polyhedral when in sheets. [22]

A further caution is warranted with respect to contamination of urinary or prostate specimens with seminal fluid. Large round or cuboidal dusky grey cells, at times containing brown pigment, from the epithelium lining the seminal vesicles are often found. Such cells have large hyperchromatic nuclei and may be mistaken for carcinoma cells. They do not have prominent nucleoli, however, and the presence of spermatozoa is the clue to their identity. [19]

Occasionally, intact Brunn's nests with a central lumen containing secretion may be seen. (19)

Metaplastic Cells:

Squamous metaplasia of the urothelium or prostatic duct epithelium not uncommon and may be focal or diffuse, non-keratinizing or keratinizing. Small foci are sometimes related to the presence of kidney, bladder or prostatic stones or chronic prostatitis. The non-keratinized metaplastic cells develop a more angular, flattened shape with cytoplasmic staining qualities ranging from clear cyanophilia to pale eosinophilia. The nuclei are central and vesicular. In the extreme degree of keratinizing metaplasia, large, flat, angular cells are densely eosinophilic or orangeophilic. They may be anuclear or have central pyknotic nuclei. (19)

In acute cystitis there may be scattered neutrophils and erythrocytes and small clumps of urothelial cells. Cytoplasmic vacuoles may be present. The nuclei are somewhat enlarged and contain sparse coarse chromatin granules and small eosinophilic nucleoli. (19)

Malakoplakia:

Malakoplakia of the bladder may be represented in the sediment large macrophages containing Michaelis-Gutmann bodies. (19)

Inflammation, ***Bacteria and Red Blood Cells:***

Polymorphonuclears, monocytes, and histiocytes are always present in a normal urine, but in small amount, especially in specimens from females. (23)

The inflammatory cellular alterations should be considered as a spectrum encompassing inflammatory exudates, secondary reactions of normal cells, degeneration, and accompanying repair changes. (22)

Chronic infection of the bladder, urethra, ureter or renal pelvis gives rise to urothelial cell changes deceptively similar to those of malignant cells. In specimens from patients with a history of chronic urinary infection and in specimens with a background containing many bacteria and inflammatory cells, the presence of urothelial cells showing nuclear enlargement, binucleation, eosinophilic nucleoli and sharply granular chromatin must be interpreted with caution, and the diagnosis of cancer made only on strong evidence of intranuclear abnormality. [19]

Chronic cystitis cystica may reveal itself in the urinary sediment by the appearance of spherical nests of uniform urothelial cells, the outer members of which tend to encircle the group. [19]

Bacteria are scanty in fresh specimens and abundant in the urine in which processing has been delayed. [21]

Freni (in 1977), using a careful collection technique, documented that a few erythrocytes may be observed in virtually all healthy adults. [22]

Bladder Neoplasms:

About 95% of bladder tumors are of epithelial origin, the remainder being mesenchymal tumors. Most epithelial tumors are composed of urothelial (transitional) type cells and are thus interchangeably called urothelial or transitional tumors, but squamous and glandular carcinomas also occur. [24]

Urothelial (Transitional Cell) Tumors:

These represent about 90% of all bladder tumors and run the gamut from small, benign lesions that might never recur to aggressive cancers associated with a high risk of death. [24, 25]

Classification of Urothelial Tumors:

Several classification systems of bladder transitional cell carcinoma have been proposed over the years. These represent attempts at grading the increasing degrees of architectural and particularly ***cytologic*** disarrays of a single tumor type**.** [20]

Table (1-1) lists two of many systems of grading these tumors. [24, 26, 27, 28, 29] The World Health Organization (WHO) 1973 classification grades tumors into a rare totally benign papilloma and three grades of transitional cell carcinoma (grades I, II, and III). A more recent classification, based on a consensus reached at a conference by the International Society of Urological Pathology (ISUP) in 1998, recognizes a rare benign papilloma, a group of papillary urothelial neoplasms of low malignant potential, and two grades of carcinoma (low and high grade). This system was proved to be more valid (correlates well with tumor stage and prognosis) than the previous classification systems and was adopted by the WHO in 2004. [24, 30]

The criteria used to place the tumors into these categories are listed in Table (1-2). [20]

Table 1-1: Grading of Urothelial (Transitional Cell) Tumors [24]

WHO/ISUP Grades*
Urothelial papilloma
Urothelial neoplasm of low malignant potential
Papillary urothelial carcinoma, low grade
Papillary urothelial carcinoma, high grade
WHO Grades†
Urothelial papilloma
Papillary urothelial carcinoma, Grade 1
Papillary urothelial carcinoma, Grade 2
Papillary urothelial carcinoma, Grade 3

*Adopted as the WHO System in 2004.
†The 1973 WHO grades.
WHO, World Health Organization; ISUP, International Society of Urological Pathology.

Table 1-2: Histological features used to classify urothelial papillary lesions according to the scheme proposed by the WHO/ISUP [20]

	Papilloma	**Papillary neoplasm of low malignant potential**	**Low-grade papillary carcinoma**	**High-grade papillary carcinoma**
Architecture				
Papillae	Delicate	Delicate: occasionally fused	Fused, branching, and delicate	Fused, branching, and delicate
Organization of cells	Identical to normal	Polarity identical to normal; any thickness; cohesive	Predominantly ordered, yet minimal crowding and minimal loss of polarity; any thickness; cohesive	Predominantly disordered with frequent loss of polarity; any thickness; often dyscohesive
Cytology				
Nuclear size	Identical to normal	May be uniformly enlarged	Enlarged with variation in size	Enlarged with variation in size
Nuclear shape	Identical to normal	Elongated, round-oval, uniform	Round-oval; slight variation in shape and contour	Moderate-marked pleomorphism
Nuclear chromatin	Fine	Fine	Mild variation within and between cells	Moderate-marked variation both within and between cells with hyperchromasia
Nucleoli	Absent	Absent to inconspicuous	Usually inconspicuous*	Multiple prominent nucleoli may be present
Mitoses	Absent	Rare, basal	Occasional, at any level	Usually frequent at any level
Umbrella cells	Uniformly present	Present	Usually present	May be absent

* If present, small and regular and not accompanied by other features of high-grade carcinoma.
(From Epstein JI, Amin MB, Reuter VR, Mostofi FK, and the Bladder Consensus Conference Committee. The WHO/ISUP Consensus Classification of Urothelial (Transitional cell) Neoplasms of the Urinary Bladder. Am J Surg Pathol 1998, **22**: 1435-1448)

Other Types of Carcinoma:

Squamous cell carcinomas represent about 3% to 7% of bladder cancers in the United States, but in countries endemic for urinary schistosomiasis, they occur much more frequently. [6, 24, 31] Pure squamous cell carcinomas are nearly always associated with chronic bladder irritation and infection. **Mixed urothelial cell carcinomas with areas of squamous carcinoma** are more frequent than pure squamous cell carcinomas. Most are invasive, fungating tumors or infiltrative and ulcerative. True papillary patterns are almost never seen. The level of cytological differentiation varies widely, from the highly differentiated lesions producing abundant keratohyaline pearls to anaplastic giant cell tumors showing little evidence of squamous differentiation. They often cover large areas of the bladder and are deeply invasive by the time of discovery. [24]

Adenocarcinomas of the bladder are rare and they are histologically identical to adenocarcinomas seen in the gastrointestinal tract. [24, 32, 33] Some arise from urachal remnants or in association with extensive intestinal metaplasia (discussed earlier). Urachal tumors occur in the dome or anterior wall, arise within the wall rather than from the mucosa, and extend out of the bladder towards the umbilicus. Rare variants of adenocarcinoma are the highly malignant **signet-ring cell carcinoma, and mixed adenocarcinoma and urothelial cell carcinomas**. [24]

Cytology of Urothelial (Transitional Cell) Tumors:

Because of the small size of transitional papillomas and the good cohesion of the nearly normal cells, exfoliation is scant. Because of good differentiation and minimal

deviation from normal of the epithelial cells of papilloma, there are no specific findings that permit a cytological diagnosis. [22]

The diagnostic accuracy of urinary cytology is closely related to the histological grade of the tumor and the pretreatment or post treatment status and only minimally to the specific therapy mode. [20] Well differentiated (grade I and some grade II) lesions are more likely to be overlooked because the cells are very similar to those of normal bladder mucosa. [20]

The most distinctive cytological features of transitional cell carcinoma (which can be useful for their recognition at a metastatic site) are the spindle, pyramidal, and racquet-like shape of the tumor cells, the eccentric nuclei, and the cytoplasmic evidence of both squamous and glandular differentiation (including endo-ectoplasmic interfaces and intracytoplasmic vacuoles). [20]

Cercariform cells recently have been described as a significant clue to the cytological diagnosis of transitional cell carcinoma. The cercariform cell is a single tumor cell with a nucleated globular body and a unipolar cytoplasmic process with a nontapering, flattened, or bulbous end. [34]

Koss summarized the criteria of diagnosis for malignant cells as follows: [22]

- **Nuclear changes**: Including nuclear enlargement, pleomorphism, fragmentation, hyperchromatism, course clumping of chromatin, chromatin condensation at nuclear membrane, and prominent nucleoli. As summarized in Table (1-3).
- **Cytoplasmic changes**: Include basophilia, vacuolation, endocytoplasmic inclusions and cellular pleomorphism.

Papillary transitional cell carcinomas grade I, differ only slightly from transitional cell papillomas. They are usually somewhat longer and have a slightly greater degree of cytological atypia, urine samples are more cellular than normal, the cell clusters

found tend to be irregular and have ragged borders, in contrast to the cohesive, ball shaped papillary clusters with smooth borders that are found after instrumentation. [35, 36]

Papillary transitional cell carcinoma grade II, are usually identifiable by cytological examination, the urine samples are even more cellular, the neoplastic cells show more striking nuclear abnormalities and still are frequently arranged in clusters. [8, 37]

Papillary transitional cell carcinoma grade III with highly cellular urine and the nuclei are more than twice the size of normal nuclei with an irregular chromatin pattern and nuclear pleomorphism. Many of these tumors are partially necrotic or ulcerated and red blood cells, leukocytes and necrotic debris are abundant. [8, 35]

Table 1-3: Nuclear abnormalities in the papillary tumors of the bladder [22]

	Enlargement	**Hyperchromasia**
Papilloma	Not significant	Absent
Papillary tumors grade I	Slight to moderate	Slight in occasional cells
Papillary tumors grade II	Moderate to marked	Slight to moderate in 25-50% of cells
Papillary tumors grade III	Marked; extreme variability of size	Marked in 50% or more of cells

Carcinoma in Situ Cells in Urine Specimens:

Carcinoma in situ (CIS) is defined by the presence of any cytologically malignant cells within a flat urothelium. CIS may range from full-thickness cytologic atypia to

scattered malignant cells in an otherwise normal urothelium, the latter termed pagetoid spread. (24)

The cytology of carcinoma in situ (CIS) is characterized by the presence of numerous abnormal cells that are moderately larger than normal transitional cells and have hyperchromatic, enlarged, and irregularly outlined nuclei. The nucleo-cytoplasmic ratio is high with large and prominent nucleoli. (22)

The pattern is relatively monotonous. Many of these cells exfoliate singly, but a few occur in small fragments or clusters. (7)

In many cases there is no ulceration or inflammation, and the background is usually clean. Because of extensive exfoliation from these lesions, the cytological accuracy in diagnosis is high. (38)

Cytology of Other Types of Carcinoma:

Cytological presentation of squamous cell carcinoma of urothelium closely resembles similar lesions of the uterine cervix and bronchus. The tumors shed squamous cancer cells with eosinophilic, often markedly keratinized cytoplasm. The nuclei are pyknotic and occasionally may be totally submerged by keratin formation, with resulting formation of ghost cells. (22)

Primary adenocarcinomas of the urothelium are predominantly of the enteric type, although occasionally a tumor of clear cell type resembling vaginal or endometrial carcinoma may be observed. (22)

Nucleolar Organizer Regions:

The practice of histopathology involves direct or, more usually, indirect assessment of cellular proliferation (and related phenomena such as differentiation) in many situations. (39)

The proliferative capacity of neoplastic cells is an important feature of growing tumors. Assessment of cell proliferation may provide both pathologists and clinicians with more objective prognostic information. [40, 41]

Several methods have been proposed to be of value in assessment of tumor proliferation such as mitotic count, Thymidine or Bromodeoxyuridine labeling, flow cytometry, and immunohistochemical markers such as Ki-67 and PCNA. [41]

Nucleolar organizer region (NOR) evaluation is another indicator of cell proliferation, although of a very different kind. [20] The existence of NORs has been well-known to cytogeneticists for many years; this NOR-DNA was visualized by in situ hybridization making use of radio-labeled rRNA, a reliable but time consuming method. With the use of silver colloid impregnation, described by Goodpasture and Bloom in 1975 and modified by Ploton et al in 1986, NORs can be identified much easier. [42, 43]

By using the silver nucleolar organizer region (AgNOR) impregnation technique the number, size and shape of NORs can be studied in a fast and simple way, not only in fresh frozen tissue specimens but also in formalin fixed paraffin embedded material. The amount of silver deposit in a cell, reflecting the amount of NORs that are involved in protein-synthesis, is thought to be related to the proliferative capacity of that cell. The exact relationship between proliferation, protein-synthesis and expression of AgNORs is, however, not yet well understood. But the expression of AgNOR is either causally or indirectly coupled to DNA-synthesis and thus AgNOR can be considered as a cell proliferation marker. [44]

Pathologist's interest in AgNOR proteins increased greatly around the end of 1980s following the observation that malignant cells frequently exhibit a greater AgNOR protein amount as compared with the corresponding benign or normal cells. Subsequent investigations also demonstrated that the AgNOR protein quantity represents a valuable parameter of cell kinetics, being significantly associated with

rapidity of cell duplication. Over the past 12 years, the "AgNOR method" has been applied in tumor pathology for both diagnostic and prognostic purposes. (45)

In order to achieve definitive standardization of the AgNOR method and produce comparable data in all laboratories, the "International Committee on AgNOR Quantitation" was founded, and during the first Workshop "AgNORs in Oncology" held in Berlin in 1993 guidelines for AgNOR protein evaluation were first defined. (45)

Definition of the NORs:

An early definition of nucleolar organizer regions (NORs) described them as the morphological sites around which the nucleoli develop at the end of mitosis (McClintock 1934). (46)

First described as weakly staining chromatinic regions around which nucleoli reorganize during telophase, NORs are now known to contain ribosomal genes (as shown by in situ hybridization) and a number of acidic proteins that have a high affinity for silver (AgNOR proteins). The latter feature has been effectively used for the rapid identification of NORs in light microscopic sections using a simple one-step silver technique. NORs appear as black dots of metallic silver, about 0.5 to 1μ m in diameter, localized within secondary constrictions of metaphase chromosomes or within nuclei. (20)

Nucleolar organizing regions (NORs) are loops of rDNA placed in the nucleolus which have an important role in the synthesis of ribosomes and gene proteins. In human karyotype, NORs are located on each of the short arms of acrocentric chromosomes 13, 14, 15, 21, and 22.These areas are the sites which hybridize with rRNA and are of importance with respect to the ultimate synthesis of protein. NOR-DNA, the associated proteins and rRNA are located in the nucleolus of the cell,

where also the nucleolar chromosomes rest in interphase. In simple terms: NORs are parts of DNA in the nucleolus that encode for rRNA; this rRNA forms the ribosomes, the "protein-factories" of the cell. [44, 47]

NORs can be detected by staining with silver nitrate and the structures thus demonstrated are termed AgNORs. [47]

Proteins of the NOR:

The NOR-associated proteins bind very well to silver. This argyrophilia is associated to the step of phosphorylation of the protein **nucleolin (protein C23)**, by which this protein is activated, and the transcription of rDNA is made possible. Nucleolin is the major silver staining protein in this process. Nucleolin is a 92kD nucleolar protein, which is thought to control rDNA transcription. The phosphorylation of the protein nucleolin is probably performed by p34cdc2 kinase, which is a subunit of M phase kinase, an enzyme involved in bringing cells into mitosis. [48]

Another important NOR-associated protein beside nucleolin is **nucleophosmin (protein B23)**. There exists a good correlation between mean AgNOR area count and amounts of nucleolin and protein B23 inside the same cell. [49] However, stimulation of rRNA synthesis, and thus of proteins associated to rRNA, does not necessarily give a quantitative increase in amounts of nucleolin and protein B23. This means that increased rRNA synthesis may occur without increase in NORs. [50]

The two argyrophilic proteins which are associated with rRNA transcription and processing (namely nucleolin and nucleophosmin) are argyrophilic and are easily stained by silver stains. [51]

The major AgNOR proteins in nucleoli during interphase are not the same as those associated with ribosomal gene during mitosis. So mitotic AgNOR proteins associated with the ribosomal genes constitute the basal level of AgNOR staining and

that modulation during interphase in nucleoli is due to additional proteins (nucleolin and nucleophosmin). [52]

Problems in the AgNOR Technique:

The silver-staining technique for nucleolar organizer regions (AgNOR) of Ploton *et al*., as popularized by Crocker, is being widely used for evaluation of nucleolar function, especially in neoplasia, and the staining protocol that Crocker described has been used by most workers in the field. [43, 53, 54]

This procedure suffers from certain problems such as background staining, precipitates, and fading of the sections. Several authors have proposed variations in the original procedure to reduce these problems, such as experimenting variations in the solution concentration or the solution temperature. [53]

In addition, the staining intensity varies considerably with slight variations in staining time; this either obscures the individually clustered AgNORs within nucleoli if over-stained or renders them faint and not assessable if under-stained. [54]

Major problems with technique as applied to cytology include clumping of cells into three-dimensional aggregates and the subjectivity in resolving individually clustered AgNORs within silver-binding nucleoli, thereby making reproducible assessment difficult. [55]

Quantitation of AgNORs:

For scoring the amount of silver deposit after staining of a tissue slice or a cytological preparation, many different methods are being used. In older studies the number of intranuclear “silver-dots” was hand-counted, making use of a light-microscope. In most studies this method is still used and AgNOR staining is expressed as mean

number of silver dots or AgNORs per cell or per nucleus or per nucleolus. A major problem is caused by the clustering of small deposits to one larger dot, which makes it difficult to establish the total number of small dots. To overcome this problem the total surface area of the silver deposit can be measured, which has the advantage that an automated computer assisted analysis can be performed. The disadvantage of the latter technique is that also extranuclear precipitated silver particles will be counted. (56, 57)

Counting the number of AgNORs is subject to intra- and interobserver variability, which is often regarded as a limitation to the reliability of the results. However, when the interobserver variation was tested, it appeared that there was a statistical significant correlation between the results found by two observers, (56, 57) but the correlation coefficient was higher in counting AgNOR-areas than in AgNOR numbers. (58, 59) To make the counting more specific, the silver-deposit only inside the nucleolus instead of the whole nucleus can be regarded. In one study this led to the conclusion that measurement of the whole nucleolar size has the same relevance as AgNOR scoring in these nucleoli. (60) Another study found a positive correlation between AgNOR number and nuclear size (61) but both had no value in predicting survival of the patient. These results were contradicted by Tosi et al. who found that the number of AgNORs had a significant predictive value in patient outcome, but form, shape and size of the nucleus had not. (62)

The mean number of AgNOR dots per cell, or per nucleus, is often referred to as mAgNOR. The mAgNOR scores in cells with slow proliferation may be in the range between 0.5 and 1.5; however there are only very few reports giving "normal-values" of mAgNOR for different types of human tissues. The mAgNOR scores are higher in fast proliferating tissues. Most AgNOR studies focus on the difference in AgNOR counts among tumors of different pathological grades and tissues in different stages of neoplasia, i.e. dysplasia, in situ carcinoma or invasive carcinoma. (60)

The pAgNOR refers to the percentage of cells in a tumor or tissue slice that harbors more than a certain number of AgNORs per cell (mostly more than 5); this is also called the AgNOR distribution score and sometimes the AgNOR proliferation index. Some studies, correlating the results of flow cytometry and BrdU-labeling with AgNOR staining, demonstrated that pAgNOR correlates with percentage of cells in S-phase of the cell cycle, or with proliferative activity, whereas mAgNOR correlates with ploidy. [63, 64]

Many investigators have regarded the size and some of them also the shape and the localization of the AgNOR-dots. An inverse relationship between number of AgNORs and AgNOR size is often reported. [65, 66, 67] However, in contradiction to this also an increase in AgNOR size together with increasing malignancy is reported. [68, 69, 70] In general it is assumed that malignant cells, showing more AgNORs per nucleus, have smaller AgNOR dots than benign cells, showing less and larger AgNORs. While individual AgNOR dots become smaller as their number increases with increasing malignancy, total AgNOR area per cell increases together with increasing malignancy. [71, 72] AgNOR expression is also related to cell maturation; aging cells from the anterior lobe of rat-hypophysis show that the number of AgNORs and the total AgNOR area decrease, whereas the size of the individual AgNOR particles increase. [73, 74, 75] Furthermore, AgNOR expression depends on the level of cell-differentiation, resulting in a decrease of AgNOR number and size with increasing differentiation. This decrease of AgNOR number and size during aging, maturation and cell-differentiation may reflect suppression of rDNA transcription. [76]

AgNOR scores are modified in different ways in various reports. These modifications mostly try to incorporate not only the AgNOR number, but also the AgNOR size. This has led to AgNOR scores being expressed as the ratio of mean AgNOR-number and mean AgNOR size [100], the ratio of AgNOR-area and nuclear-area [77] or the ratio of number of small AgNORs (< 3 microns) and number of large

AgNORs (> 3 microns) [62]. Also the localization of the AgNOR inside the nucleus might be related to cell behavior; with increasing malignancy in certain tumors, AgNORs moved from central to peripheral inside the nucleus. [70]

With the improvement of the AgNOR-staining technique over the years and the use of automated computer-assisted surface-area measurement of the precipitated silver-colloid inside the nucleus, the AgNOR staining has become much more standardized and reliable. However it should be realized that the number of detectable NORs depends on several factors: the level of transcriptional activity, the number of NOR-bearing chromosomes in the karyotype and the stage of the cell-cycle in which they are sought. [78]

Prognostic Value of AgNORs:

Accurate histopathological typing, grading and staging of tumors are of proven value in the clinical management of cancer. In many cases, however, histopathological assessment does not correlate accurately with clinical outcome and may not reveal all possible markers of prognostic importance. [13]

Investigation of biological behavior of tumors has been intensive and has resulted in the identification of new parameters which can be used in diagnosis, prognosis, and treatment choices. [79]

Of the various new techniques used for assessing tumor behavior, is the AgNOR method. Nucleolar organizer regions represent DNA segments associated with nonhistonic argyrophilic protein. The number of these regions in the nucleus is directly proportional to protein synthesis and reflects the intensity of cell activity and cell proliferation. As the AgNOR count shows the intensity of cell proliferation, this method can be used in differentiating between benign and malignant tumors, as well as between tumors with high and low malignancy. [79]

The number and size of NORs reflect cell activity, proliferation and transformation and help to distinguish benign from malignant cells. [80] Evaluation of the quantitative distribution of AgNORs has been applied in tumor pathology both for diagnostic and prognostic purposes. A number of studies carried out in different tumor types demonstrated that malignant cells frequently present a greater AgNOR count than corresponding non-malignant cells. [81, 82]

Many studies have shown that AgNOR values can serve as a useful prognostic parameter and a marker for tumor progression in certain tumors. [83, 84]

AgNOR in the Iraqi Studies:

AgNOR has got very much interest from pathologists in our county, and many studies had been published about this interesting subject; for example:

- Hassan R. K. submitted a comparative study about benign and malignant liver diseases. [85]
- Al-Rawi F. A. evaluated the AgNOR in prostatic tumors. [86]
- Hussein A. G. evaluated NOR in lung tumors. [87]
- Al-Zaidy A. evaluated NOR in nervous system tumors. [88]
- Al-Shama'a Z. evaluated NOR in trophoblastic tumors. [89]
- Alash I. evaluated the benefit of AgNOR in differentiating benign and malignant cells in human effusions. [90]
- Al-Amery R. T. evaluated AgNOR in acute leukemia. [91]

Chapter Two: Patients, Materials & Methods

Patients:

A total of 91 samples of urine were collected from 91 patients. The first set consisted of 57 samples of urine that were taken from patients complaining of signs and symptoms suggestive of bladder carcinoma and they were candidates for cystoscopical examination. Depending on the histopathological report of the biopsy material, 49 cases were proved to be of urothelial carcinoma and 30 of these samples were positive for malignant cells cytologically with adequate cellularity for interpretation, and these were included in the study.

The other set represents 34 urine samples that were obtained from patients complaining of other urological diseases (such as vesical stone and urinary tract infection) as a control.

So, the final number of urinary samples included in the study was 64 samples.

The samples were collected from patients attending Medical City and Al-Kadhimiya Teaching Hospitals during the period between May 2006 and October 2006.

A case sheet including the history of the present illness and past history for each patient has been prepared as follows:

Case Sheet

- Case No.
- Name:
- Age:
- Sex:
- Occupation:
- Smoking habits:
- Chief complaint:
- History of present illness:
- Past medical history:
- Past surgical history:
- Urine examination:
  - Naked eye examination:
  - Cytomorphological findings:

Materials:

- **Equipments and instruments used:**

1. Plastic disposable sterile tubes (5 ml)
2. Glass slides.
3. Centrifuge (ordinary) graded (1000-5000) rpm.
4. Incubator set at 37°C.
5. Humid chamber.
6. Jars.
7. Racks.
8. Beakers.
9. Washing bottle.
10. Graduated cylinders.
11. Micropipette.
12. Wooden sticks.
13. Slide tray.
14. Tweezers.
15. Filter papers.
16. Cover slips.
17. Plastic gloves.
18. Light microscope.

- **Chemicals and solutions used:**

1. Gelatin powder.
2. Formic acid solution (1%).
3. Silver nitrate powder.

4. Sodium thiosulfate powder.
5. Ethyl alcohol (with a concentration of 30%, 50%, 70%, 90% and 99%).
6. Xylene.
7. Distilled water.
8. Egg albumin, glycerin and thymol.
9. DPX mount.

Methods:

Sample Collection and Preparation:

From each patients, 30 ml of mid stream freshly voided urine samples were collected excluding the early morning samples. [17] The samples were processed within less than 2 hours from collection.

Urine samples were centrifuged at 3000 rpm for 15 minutes. The supernatants were decanted and the sediments were smeared on pre-albumenized slides (to assist adherence of cells to the slides during processing) using micropipette method for obtaining the sediments. [17] Four slides were prepared for each sample.

The smears were fixed immediately in 95% ethyl alcohol for at least 15 minutes.

Staining the Smears:

At least four slides for each case were stained by routine Hematoxylin and Eosin (H&E).

From these slides, the most representative ones were chosen to be restained with silver stain without prior destaining.

For AgNOR staining; a modified version of the staining technique described by Ploton *et al* was performed as follows:

Method of AgNOR Staining: [43]

Two solutions were needed; to prepare the first one, Gelatin was dissolved in 1% formic acid to make a 2% solution (stored at room temperature).

The second one is a 50% silver nitrate dissolved in distilled deionized water (prepared fresh.)

Working solution was freshly prepared using two parts of silver nitrate solution and one part of Gelatin solution.

To carry out AgNOR staining the smears were brought to water through graded alcohols (with a concentration of 30%, 50%, 70%, 90% and 99%) using distilled water (10 dips in each jar). The AgNOR technique was completed by pouring the final solution on the smears (about 0.5 ml for each slide), lying flat in a humid chamber and incubated at 37°C in dark for 25-30 minutes.

Slides were washed thoroughly with distilled water then passed to 5% sodium thiosulfate solution (for 2 minutes) to improve permanence of the stain, washed thoroughly, and dehydrated. After drying, the slides were cleared in xylene (2 jars with five minutes in each one) and mounted in DPX.

AgNOR Counting:

In silver stained slides, enumeration of AgNOR dots was done using the method suggested by Crocker *et al*. [54]

All urothelial cells had their silver precipitations counted using oil immersion (× 100). The number of cells evaluated per case was 50 (A study that carried out a cumulative mean technique to determine the number of nuclei that needs to be

counted before the mean became stable , have recommended 50 nuclei "range 20-70" for carcinomas). [92] Thus we have also counted 50 nuclei per smear in this study.

By careful focusing, all clear distinguishable black dots within the nucleus were identified and counted. Black dots both smaller and larger (corresponding to clusters) were treated as one AgNOR. The silver precipitations in the nucleus was identified as 'homogeneous' (small to medium sized dot-like precipitations, sometimes angulated but with smooth contour, without aberrations) or 'heterogeneous' (large irregularly shaped precipitations with great variations in size between cells, often with bizarre forms, sometimes finely divided).

Fine focusing was used to eliminate dust particles and deposit debris that interfered with the counting of AgNOR dots.

Inflammatory cells were excluded from counting; however, lymphocytes that showed a single well stained AgNOR dot was used as internal control.

The overall mean AgNOR (mAgNOR) per nucleus was obtained by counting the AgNOR dots in 50 nuclei and the average was calculated.

In malignant samples, the values of mAgNORs were correlated with grade of tumor and statistically analyzed for their significance.

In the non-neoplastic samples, the mAgNOR scores were also calculated and statistically analyzed.

In addition to the AgNOR counts, variations in AgNOR size and distribution were also recorded by using the criteria of Ahsan *et al*. [93]

Size Variation was graded as follows:

0 = More or less uniform in size.

1+ = Two different sizes.

2+ = More than two different sizes (but not those of 3+).

3+ = All grades and sizes, including too minute to be counted.

Distribution of AgNORs in the nuclei was graded as follows:

0 = Limited to nucleoli.

1+ = Occasional distribution outside nucleoli.

2+ = Moderate distribution outside nucleoli.

3+ = Widely distributed throughout the nucleus.

The final diagnosis (gold standard) was determined by the histopathological report of subsequent bladder biopsies.

The diagnoses were grouped in three categories: (I) non-neoplastic lesions; (II) low grade carcinoma; (III) high grade carcinoma; according to the WHO/ISUP classification system. [1, 24]

Statistical Analysis:

The results AgNOR of all nuclei were calculated and expressed as the mean (±standard deviation).

Student's t test was used to compare mean AgNOR counts and Chi-square test was used for evaluating AgNOR size and distribution. P value <0.05 was considered statistically significant.

Chapter Three:

Results

Results:

The study was performed on 64 urinary specimens, consisting of the 30 cases (22 males and 8 females) histopathologically proved to be primary urothelial tumors and were positive for malignant cells cytologically, and 34 cases (25 males and 9 females) with inflammatory reaction or without pathological changes.

The sensitivity of the conventional urine cytology was 61% and the specificity was 100% (there were a 19 false negative cases and there was no false positive cases).

The mean age for the non-neoplastic group was 47.17 (range 30-65 years) and for the malignant group was 63.5 (range 41-82 years).

Table 3-1 summarizes the clinical presentation of cases with urothelial carcinoma in relation to their age and gender.

The largest number of patients in the malignant group was within (50-69) years.

The main clinical presentation for most of the patients with bladder cancer was haematuria (only 2 patients presented with chronic cystitis-like symptoms and 1 patient with Micturition disorders).

Table 3-1: Summary of clinical presentation of the malignant cases with their gender and age distribution

Chief Complaint	Sex		Total	Age Range (years)					Total
	M	F		40-49	50-59	60-69	70-79	>80	
Gross hematuria	21	6	27	3	9	8	5	2	27
Chronic urinary infection	—	2	2	—	—	1	1	—	2
Micturition disorders	1	—	1	—	—	1	—	—	1
Total	*22*	*8*	*30*	*3*	*9*	*10*	*6*	*2*	*30*

The samples were classified into three groups: non-neoplastic lesions (n=34), low grade urothelial carcinoma (n=12) and high grade urothelial carcinoma (n=18) according to the cytomrphological study of the H&E stained slides. The final diagnosis of each patient had been established from the histological reports of bladder biopsies.

Using the silver colloid method for staining, the AgNORs appeared as brown-black dots in a light brown or orange-yellow staining nucleus. The AgNOR dots were arranged in clusters and/or dispersed throughout the nucleus.

Lymphocytes in the smears were characterized by the presence of only one dark dot in the solitary nucleus, the macrophages and neutrophil polymorphs showed one to two dots.

The non-neoplastic group showed low numbers of fairly homogeneous silver precipitations in the urothelial cells (Figures 3-1 to 3-3), while low grade urothelial carcinoma often showed small precipitations arranged in clusters with increased AgNOR numbers when compared with normal cells (Figures 3-4 to 3-7). On the other hand, high grade urothelial carcinoma showed very heterogeneous AgNOR features, with great variations of size and shape and especially peculiar forms with

angulations, sometimes with confluence of individual precipitations (Figures 3-8 to 3-11).

It was observed that AgNOR dots tended to be homogenously staining and regular in the nucleus of normal urothelial cells (Figure 3-2) as well as; to some extent; in low grade urothelial carcinomas (Figure 3-5). As the grade increased, AgNOR dots became irregular and were more widely dispersed in the nucleus. In some cells, large giant dots with satellite of small dots were also noticed especially in high grade urothelial carcinoma (Figure 3-9).

Cercariform cells have been noticed mainly in high grade malignant samples, and by staining with AgNOR they showed high AgNOR count with highly irregular size and shape of the AgNOR dots (Figure 3-12).

The mean number of AgNORs per nucleus was highest in the high grade carcinoma group (Table 3-2), but there were also some cases with relatively low values.

The mean differences were significant between urothelial carcinoma and non-neoplastic cases ($p < 0.05$) (Figure 3-13).

Regarding the histological grade; the mean AgNORs count of cancer cells increases from low grade to high grade tumor but the differences were statistically non-significant ($P>0.05$) (Table 3-2).

Heterogeneous precipitations were observed mainly in neoplastic cells (Figure 3-14).

Table 3-2: Comparison of AgNOR counts in low grade, high grade urothelial carcinoma cells & non-neoplastic urothelial cells

Groups	*No. of cases*	*Mean AgNOR Counts / Cell*		
		Range	*Mean*	*± SD*
Non-neoplastic cases	34	1.9-4.2	2.82*	± 0.73
Low grade urothelial carcinoma	12	2.7-8.9	6.32†	± 1.87
High grade urothelial carcinoma	18	4.1-14.2	7.94	±3.51

*p< 0.05 (Significantly lower as compared to low grade and the high grade carcinoma groups).
†p>0.05 (Not significant as compared to high grade carcinoma group).

By comparing the AgNOR size variation and distribution within the nuclei there was a significant difference ($p<0.05$) among the three histopathological categories (Tables 3-3 & 3-4 respectively).

Table 3-3: Comparison of AgNOR size in low grade, high grade urothelial carcinoma cells & non-neoplastic urothelial cells

Groups	*No. of cases*	*AgNOR size 0 to 1+*	*AgNOR size 2+ to 3+*
Non-neoplastic cases*	34	28	6
Low grade urothelial carcinoma†	12	5	7
High grade urothelial carcinoma	18	3	15
Total	64	36	28

*p< 0.05 (Significant as compared to low grade and the high grade carcinoma groups).
†p< 0.05 (Significant as compared to high grade carcinoma group).

Table 3-4: Comparison of AgNOR distribution in low grade, high grade urothelial carcinoma cells & non-neoplastic urothelial cells

Groups	*No. of cases*	*AgNOR distribution 0 to 1+*	*AgNOR distribution 2+ to 3+*
Non-neoplastic cases*	34	30	4
Low grade urothelial carcinoma†	12	5	7
High grade urothelial carcinoma	18	4	14
Total	64	39	25

*p< 0.05 (Significant as compared to low grade and the high grade carcinoma groups).
†p< 0.05 (Significant as compared to high grade carcinoma group).

The mean AgNOR counts and the size and distribution of AgNORs in the nucleus were not related to patients' sex as shown in Tables 3-5, 3-6 and 3-7 respectively.

Table 3-5: Mean numbers of AgNOR scores in malignant urothelial cells as related to patients' sex

Sex	*No. of cases*	*Mean AgNOR Counts / Cell*		
		Range	*Mean*	*± SD*
Males	22	2.8-14.2	6.30*	± 3.69
Females	8	2.7-13.1	5.91	± 3.65

*p> 0.05 (Not significant as compared to female cases).

Table 3-6: Comparison of AgNOR size in malignant urothelial cells as related to patients' sex

sex	*No. of cases*	*AgNOR size 0 to 1+*	*AgNOR size 2+ to 3+*
Males*	22	5	17
Females	8	3	5
Total	30	8	22

*p> 0.05 (Not significant as compared to female cases).

Table 3-7: Comparison of AgNOR distribution in malignant urothelial cells as related to patients' sex

sex	*No. of cases*	*AgNOR distribution 0 to 1+*	*AgNOR distribution 2+ to 3+*
Males*	22	8	14
Females	8	3	5
Total	30	11	19

*p> 0.05 (Not significant as compared to female cases).

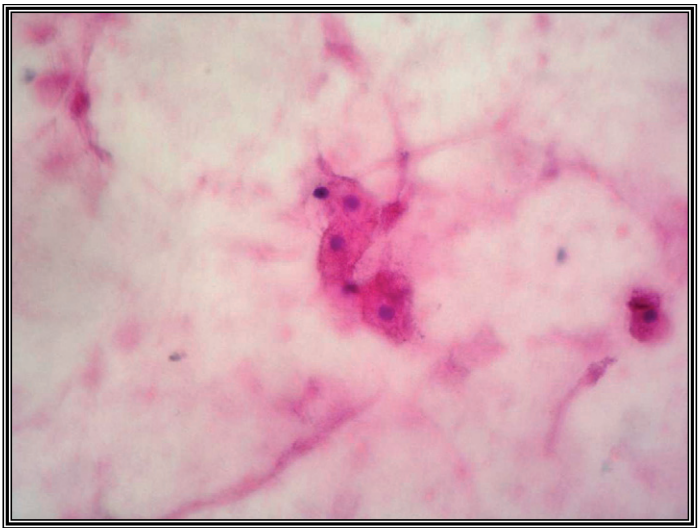

Figure 3-1: Urine cytology showing small uniform nuclei with fine chromatin and absent nucleoli in normal urothelial cells. (H&E stain × 1000)

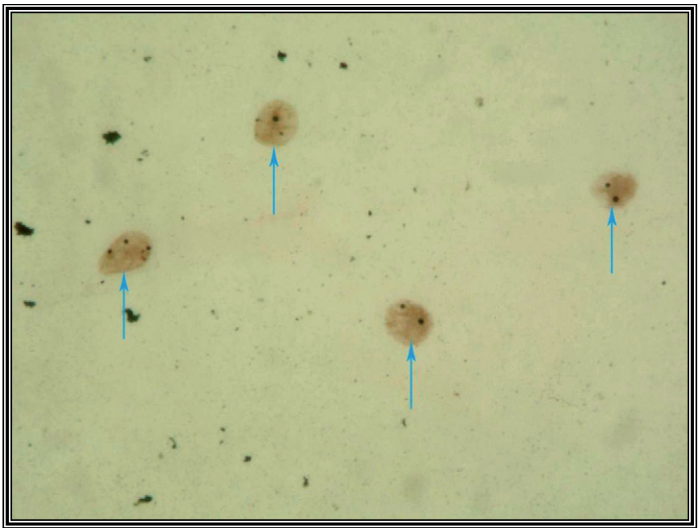

Figure 3-2: Urine cytology showing regular AgNOR black dots, often one to two per nucleus, in normal urothelial cells (homogeneous pattern). (AgNOR stain × 1000)

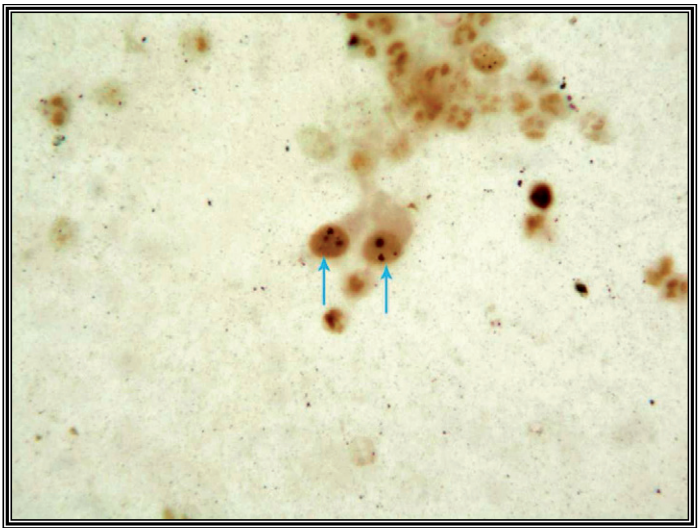

Figure 3-3: Urine cytology showing low number of AgNOR dots, uniform in shape and regularly distributed within the nuclei of benign urothelial cells (arrows) in a case of chronic urinary tract infection. (AgNOR stain × 1000)

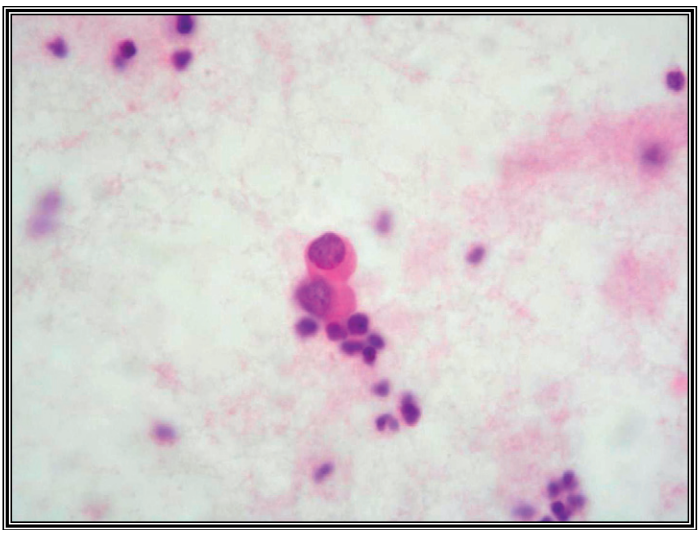

Figure 3-4: Urine cytology showing low grade urothelial carcinoma cells with enlarged nuclei, moderate pleomorphism and inconspicuous nucleoli. (H&E stain × 1000)

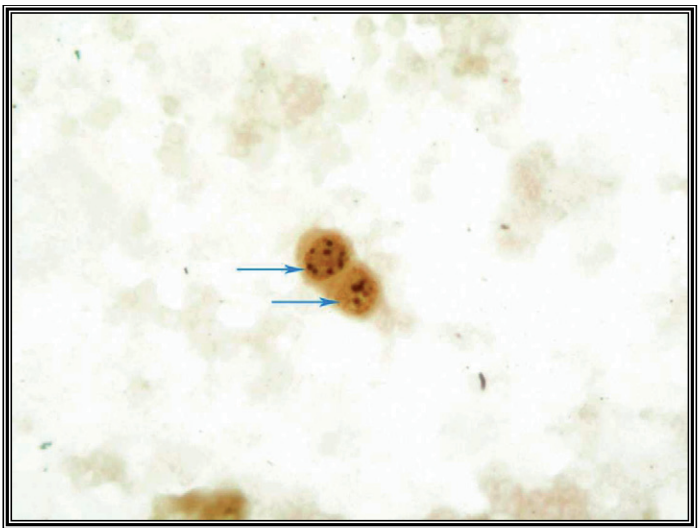

Figure 3-5: Urine cytology showing low grade urothelial carcinoma cells with small and regular black clustered dots (homogeneous pattern) (arrows). (AgNOR stain × 1000)

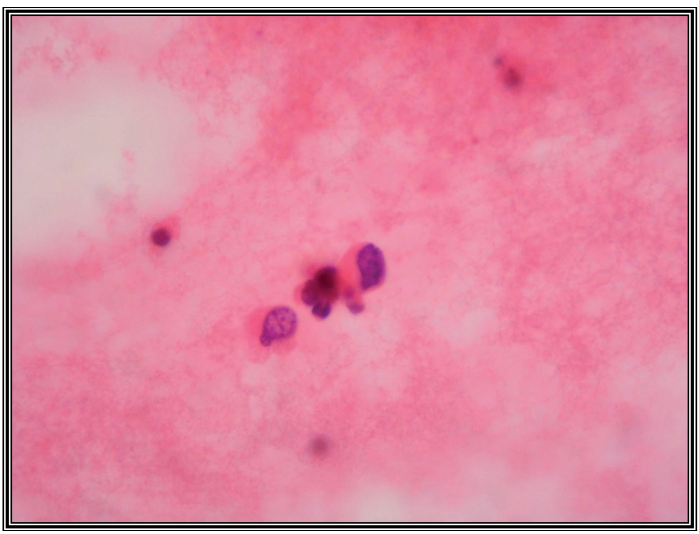

Figure 3-6: Urine cytology showing low grade urothelial carcinoma cells with enlarged round to oval nuclei and inconspicuous nucleoli. (H&E stain × 1000)

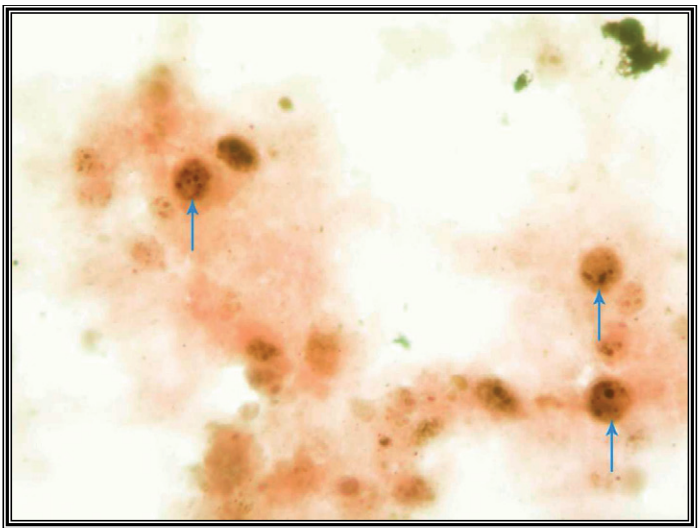

Figure 3-7: Urine cytology showing low grade urothelial carcinoma cells note that despite the relatively large number of AgNOR dots, they look fairly regular in size and shape (homogenous) (arrows). (AgNOR stain × 1000)

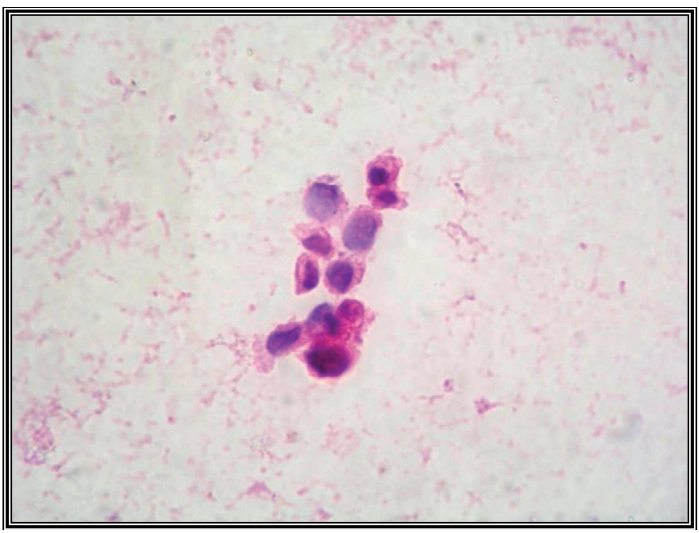

Figure 3-8: Urine cytology showing high grade urothelial carcinoma cells with enlarged nuclei, marked variation in size and shape and prominent hyperchromasia. (H&E stain × 1000)

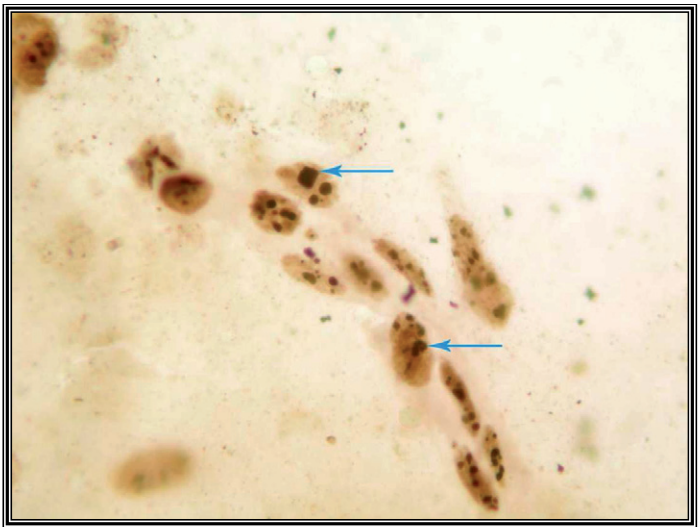

Figure 3-9: Urine cytology showing high grade urothelial carcinoma cells presenting different size and shape of AgNORs (arrows), with angulations and coarse clustering (heterogeneous pattern). (AgNOR stain × 1000)

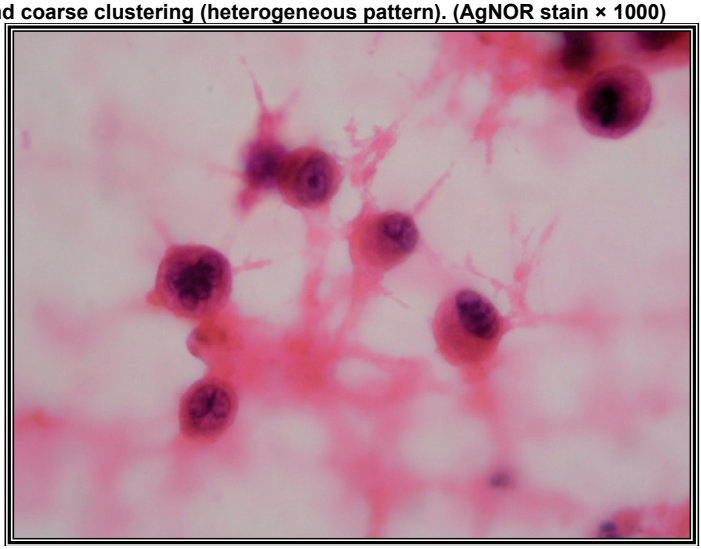

Figure 3-10: Urine cytology showing high grade urothelial carcinoma cells with high nuclear/ cytoplasmic ratio, marked pleomorphism and prominent nucleoli. (H&E stain × 1000)

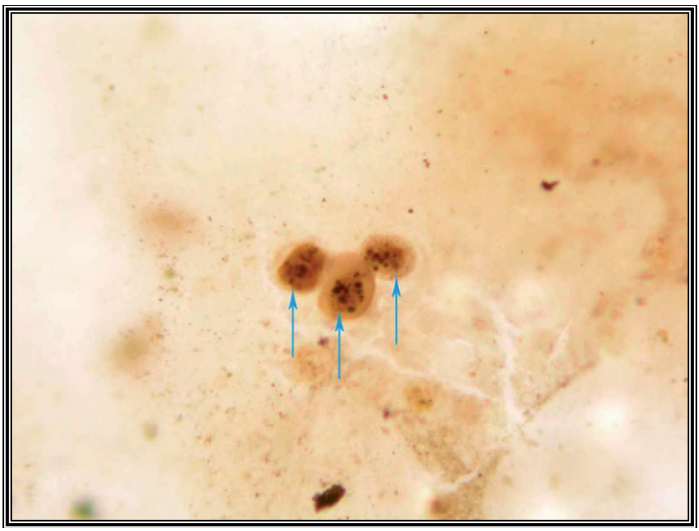

Figure 3-11: Urine cytology showing high grade urothelial carcinoma cells. There are more than two different sizes of AgNORs (arrows) with moderate distribution (size 2+, distribution 2+).(AgNOR stain × 1000)

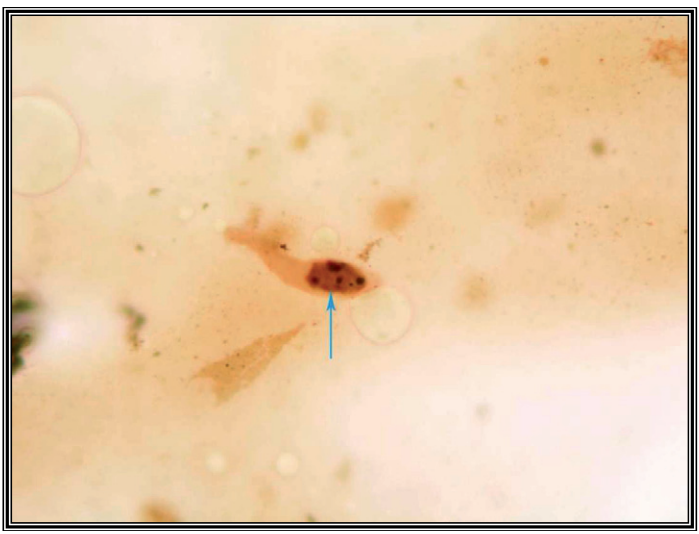

Figure 3-12: Urine cytology showing cercariform cell from a case of high grade urothelial carcinoma presenting heterogeneous pattern of AgNOR dots (arrow). (AgNOR stain × 1000)

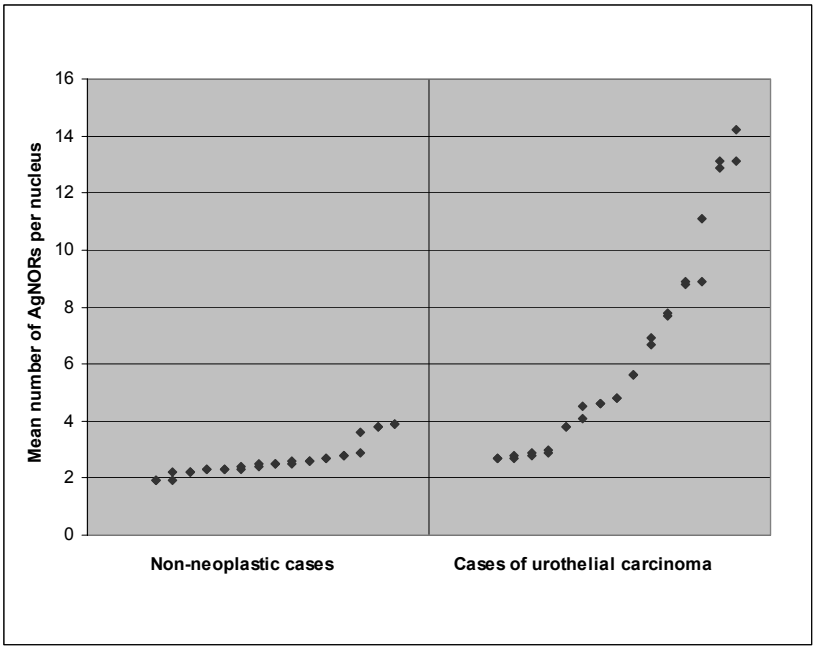

Figure 3-13: Scatter chart of the mean number of AgNORs in the nuclei of non-neoplastic and malignant urothelial cells.

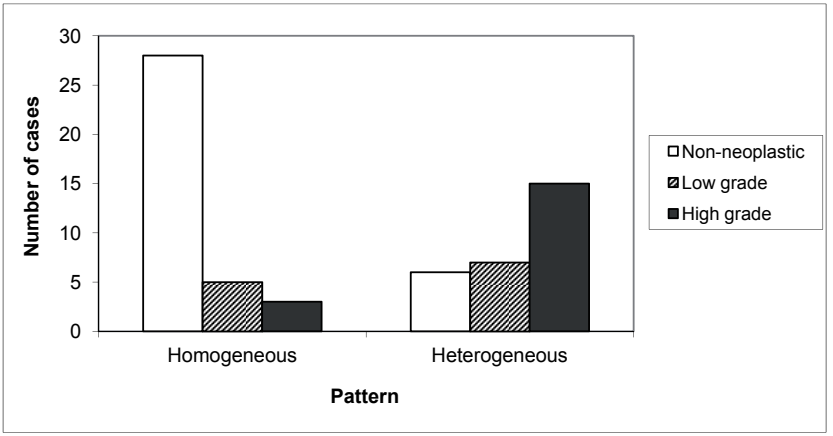

Figure 3-14: Diagram of AgNOR pattern distribution. Note that the heterogeneous pattern is mainly observed in neoplastic lesions.

Chapter Four: Discussion, Conclusions & Recommendations

Discussion:

Urinary bladder cancer is a common disease; it is the 9th most frequent cancer worldwide. (94) It is the fourth most common cancer in men in the UK. (95)

The incidence of bladder epithelial tumors in the United States has been steadily increasing during the past years and is now more than 57,000 new cases annually. (96)

In Iraq, it represents 12.3% of cancer cases among men and 4.9% of cancer cases among women. (94)

The importance of urine cytology for the detection of urological malignancy is well established. As the incidence of urothelial carcinoma increases, so too does the demand for urine cytology. (97, 98)

Urine is an inhospitable environment for cells; consequently degenerative changes that make diagnosis difficult are common. Exfoliated cells in a urine sample, especially single cells, deteriorate rapidly and degenerative changes are seen within one hour. Reactive changes caused by stones, inflammation, infection, therapy among many others, as well as 'papillary clusters', are responsible for most false diagnoses. In addition, transitional cells can normally show marked variation in size and shape, can be multinucleated, and can frequently exhibit nuclear and cytoplasmic degenerative changes that can mimic malignancy. The paradox of urine cytology is that pleomorphic cells with enlarged hyperchromatic nuclei containing prominent nucleoli can be benign while cancer can be composed of nearly normal-appearing monomorphic cells with bland nuclei. (2, 7)

The importance of urinary cytology relates mainly to the early detection of bladder cancer, but renal and lower urinary tract diseases may also be detected. The sensitivity of urinary cytology for the former lesions improves with increasing grade of the tumor, the challenge being to improve the diagnosis of low grade lesions, which include papillomas and grade I urothelial tumors. (10)

In addition to the routine examination of cytology samples, additional methods have been used to increase the diagnostic accuracy for detection of malignant cells. In this regard, interest has been focused on the assessment of cell kinetics or oncogenes when evaluating the biologic aggressiveness of the tumor and a wide variety of methods can be used to estimate the proliferative activity of the cells. [99]

A number of sophisticated tests including DNA flow cytometry, restriction enzyme fragment length polymorphism, PCR sequencing, monoclonal and polyclonal antibodies have been employed to distinguish the benign from malignant cells. A comparatively simple technique of staining argyrophilic nucleolar organizer regions (AgNORs) has also been used in tumor pathology to assess the proliferative activity of the cells. [80, 100]

Many studies for the evaluation of AgNORs have been conducted on different benign and malignant tissues of the human body, demonstrating that malignant cells frequently present a greater AgNOR number than benign cells. [101, 102]

Furthermore, a great deal of attention has been focused on AgNOR count in histological sections of bladder carcinoma and different methods have been devised to evaluate it. [103]

In contrast to AgNOR studies on histological sections of bladder mucosa, there are very few publications on AgNOR staining of urinary cytology. [1, 104, 105]

Although some authors quantify all AgNOR precipitations indiscriminately, many study groups stress the importance of additional parameters such as size, shape, area, density and distribution of the precipitations. [93]

Many attempts have been made to find new methods of evaluating AgNORs. Hansen *et al* [106] described three types of AgNORs in benign and malignant prostate lesions. However, their method of typing was not found to be of practical use as all the three types described by them were found in different areas of the same sections. [93]

Khanna *et al* introduced a subjective AgNOR pattern assessment (SAPA) score. With this scoring system, statistically significantly higher SAPA score was observed in malignant skin tumors as compared with benign tumors. [107]

Another parameter is the AgNOR proliferative Index (pAgNOR). It denotes percent of cells with 5 or more AgNOR dots. This parameter shows proliferative activity and has been correlated with flow cytometric studies. [64]

However, the most widely used method, besides AgNOR counts, is the AgNOR size imaging by electron microscopy. AgNOR size has been measured and it has been found in a number of studies that increased AgNOR size correlate well with proliferative activity, malignant potential and prognosis. [108, 109]

Ahsan *et al* devised a simpler technique than the above mentioned procedure for the scoring of AgNOR size and distribution in the nuclei using the conventional light microscope. [93] This scoring of AgNOR parameters was first described by Ahsan *et al* [93] in their study of prostatic lesions and has subsequently been used in diseases of the liver [110] and effusions. [111]

In this study, AgNORs were generally present in non-neoplastic cells as black dots, regular, spherical and isolated, but in cells from high grade carcinomas they were larger and irregular in size and shape, giving a heterogeneous appearance (Figure 3-14).

This observation (recently shown to be useful for the examination of colorectal and gastric mucosa), [112] prompted us to introduce the size and distribution of AgNORs in the nucleus as additional parameters.

The results of this study show that using the "mean number of AgNOR precipitations per nucleus" as a single parameter permits a clear distinction to be made between malignant and benign urothelial cells (Figure 3-13) (benign urothelial cells have a lesser number of AgNORs as compared to malignant cells). On the other hand, there was no significant difference between the mean number of AgNOR of

low grade and high grade urothelial carcinoma cells (Table 3-2). But the evaluation of AgNORs has been further elaborated by taking into account their size and distribution in the nucleus (Tables 3-3 and 3-4).

In this study, variation in AgNOR size and distribution was graded following the criteria devised by Ahsan *et al*, [93] we found that the AgNORs in malignant cells belonging to the high grade carcinoma category were greater in number, irregularly distributed throughout the nucleus and markedly heterogeneous in size, while in the low grade carcinoma cells; although the number of AgNOR dots was relatively high; they were more uniform in size and; to some extent; regularly distributed as compared to the high grade urothelial carcinoma group. On the other hand, non-neoplastic urothelial cells were characterized by a lesser number of small, homogenously sized, regular, and isolated AgNORs. The difference was statistically significant in both of the parameters (Tables 3-3 and 3-4). Both of these parameters are easy to use and give reproducible results.

These results show that the "mean number of AgNORs per nucleus" is an important variable for the separation between the non-neoplastic urothelium and low grade carcinoma, but is not very efficient in separating low and high grade neoplasia. This observation might reflect a slight difference in proliferation states between the different grades of urothelial carcinoma and to some extent might be explained by the subjectiveness of grading as a qualitative method, with considerable intraobserver and interobserver variations. The addition of other parameters (namely size and distribution of AgNORs within the nucleus) allows a better separation between low and high grade carcinomas.

These results are in agreement with the study conducted by Trevisan *et al* [1] who investigated the usefulness of the AgNOR technique in urine cytology taken into account the morphology of the AgNORs as an additional parameter besides the mean number of the dots and reported that the use of a combination of various parameters

regarding the AgNOR dots might be helpful for differentiating bladder mucosa lesions.

Another study investigating the value of argyrophilic proteins of the nucleolar organizer region (AgNORs) in urine cytology was done by Masuda *et al*, [104] using the mean number of AgNOR dots as the only parameter disregarding the pattern of these dots and they found a significant difference between non-neoplastic urothelial cells and benign cells however they could not find a significant difference between the different grades of urothelial carcinoma, which is similar to the results of this study.

The results of these studies assist the opinion that asserts the importance of additional parameters such as size, shape, area, density and distribution of the precipitations in the evaluation of AgNORs.

Other techniques, alternative to visual assessment, like back scattered electron imaging have also been utilized to study a number of parameters pertaining to AgNORs, including calculation of the total AgNOR area. [113]

This technique also yielded a significant difference between the benign and malignant cells, with the mean AgNOR area of malignant cells being greater than that of benign cells. [113] It was adopted by Takeuchi *et al* [105] to study the application of AgNOR to urinary cytology and they found a stepwise increase from non neoplastic cases through low grade to high grade transitional cell carcinoma and concluded that this technique could offer an objective index for the cytological assessment of urinary bladder carcinoma. [105]

However; it was seen that the conventional AgNOR staining and visual counting proved to be a much simpler technique than the above mentioned procedures and was adopted in a number of studies. [114]

Regarding the relation with the patients' sex, it is known that bladder cancer is nearly three times more common among men than women, a fact that might be

explained by differences in genetic mechanisms, hormonal status, or an anatomical predisposition to urinary retention and exposure to industrial and environmental carcinogens in men. [115, 116]

In this study, there was no relation between the AgNOR parameters (namely number, size and distribution) and the patients' sex (Tables 3-5, 3-6 and 3-7 respectively). This disagrees with the study done by Korneyev *et al* [103] who observed a higher proliferation potential of bladder neoplasms in men using AgNOR score and a lower rate of tumor related survival in men than in women and suggested a higher cellular activity in men. [103]

This disagreement might be explained by the limited number of cases in this study as compared to the one done by Korneyev *et al* [103] and also it might be due to their use of histological sections of bladder carcinoma rather than urinary cytology smears.

Limitations of AgNOR technique:

AgNOR technique is a rapid, reproducible method but it suffers from certain limitations:

- Effects of temperature. Although the literature on AgNOR staining of chromosomes notes that staining is more rapid at elevated temperatures, the room temperature procedure has been usual for tissue specimens because of better control and less precipitates and background staining. [117] We observed that variations in room temperature produced large differences in NOR staining density and better reproducibility was obtained by staining in an incubator set at 37˚C for 25-30 minutes.
- Reduction of Precipitates. Black precipitates scattered over the slide is one of the problems that can cause difficulty in interpretation of the results and limit the routine use of the technique. [117] In order to minimize such precipitates the following points was taken into account:

a. Frequent changing of the solutions.
b. Cleanliness of the glassware
c. Slides was processed and stained as soon as possible since old slides might be contaminated with argyrophilic bacteria that stain with silver making slide interpretation difficult or even impossible.

- Problems related to urine samples. There are certain limitations in handling urine sample were taken into account:
  a. Urine is an inhospitable environment for cells and they deteriorate rapidly (within one hour) after collection especially in hot weather, [(2)] so urine samples had to be transferred immediately to lab.
  b. Urine is generally hypocellular so we chose to stain NORs in previously stained slides because pre-staining with H&E or Papanicolaou stains can help us to choose cellular slides to be stained for AgNOR and not to waste the materials on hypocellular slides. Most stains based on organic dyes, notably H&E and Papanicolaou stains, and will be completely removed during the strongly acidic silver staining step without prior destaining. The presence of these dyes did not affect the NOR staining. [(117)]
- AgNOR counting. The following point are related to the limitations in the conventional visual counting technique:
  a. Counting AgNORs in itself is a difficult and time-consuming procedure.
  b. Quantitative analysis of the AgNORs needs to be performed under high magnification with careful focusing.
  c. Counting the number of AgNORs is subject to intra- and interobserver variability.

Conclusions:

1. Typing of AgNOR size and distribution was found to be an easy and reproducible in addition to traditional AgNOR counts for differentiating malignant from non-malignant urothelial cells and low grade from high grade urothelial carcinoma cells.
2. AgNOR staining technique is a rapid, cost effective, and easily applied method to cytological specimens. However, it needs a lot of dedication, standardization and meticulous bench work to achieve good results.
3. AgNOR technique can definitely be used as a supportive tool to routinely performed Hematoxylin and Eosin or Papanicolaou staining and may be employed as an additional diagnostic tool for use in urinary cytology samples when the cytological diagnosis poses a problem, especially in the diagnosis of low grade urothelial tumors.

Recommendations:

1. In order to avoid technical difficulties, the AgNOR staining method must be meticulously established as regards the duration of staining, temperature, purity of water, reagents, etc. (all these parameters had a great influence on the final result) before it is adapted as routine procedure.
2. Further studies might be done to evaluate the prognostic value of AgNOR, this requires clinical follow up to establish whether a correlation exists between AgNOR scores and the duration of tumor-free period in patients with recurrent urothelial carcinoma of the urinary bladder and with tumor related survival.
3. In the evaluation of AgNOR score; it is important to take other parameters into account mainly the size and distribution of AgNOR dots and clusters because it adds to the accuracy of the technique. However, typing of AgNOR size and distribution as described in this article needs to be further studied on a large number of cases of different tissues.
4. Moreover, these parameters should be correlated with already established but costly techniques of AgNOR size imaging by electron microscopy and flow cytometry. If a direct correlation is found, then these simple light microscopic methods will become very useful and less expensive alternatives.

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