Micronucleus index in epithelial exfoliated cells of urothelium and buccal mucosa, and peripheral blood lymphocytes of patients with papillary urothelial carcinoma

Podrimaj-Bytyqi, Arjeta

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This doctoral thesis was conducted in the Faculty of Natural Sciences- Department of Biology, University of Pristina (FNS-UP) and Institute of Pathology and Urology Clinic of University Clinical Centre of Kosovo (UCCK), Medical Faculty, University of Pristina.

Mentors:

- Prof. dr. Isa Elezaj, PhD - Professor at the Faculty of Natural Sciences, University of Pristina.
- Dr. Ana Borovecki, MD, PhD, Research Associate at the Faculty of Medicine, University of Zagreb and Pathologist at the Institute of Pathology "Enge", Zürich.

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TABLE OF CONTENTS

ABBREVIATIONS

AJCC - American Joint Committee on Cancer

APC/C - anaphase promoting complex/cyclosome

ASR - age-standardized rate

ARF - transcription factor

BEC - buccal exfoliated cells

BFB - breakage- fusion- bridge

BN - binucleated cells

CA - chromosomal aberrations

CBMN - cytokinesis-block Micronucleus Assay

CEA - carcinoembryonic antigen

CGH - comparative genomic hybridization

COLO230DM- colorectal cancer cell line

CIS - carcinoma in situ

CK7 - cytoskeletal protein encoded from KRT7 gene, expressed in epithelial cells.

CK20 - type I cytokeratin, encoded from KRT20 gene, expressed in enterocytes, goblet cells, found especially in intestinal and gastric mucosa

CpG - cytosine-guanine-dinucleotide island

DBC1 - deleted in Bladder Cancer gene, located within chromosome 9 (9q32-33)

DM - duble minutes

DNA - deoxyribonucleic acid

DNMT (1, 3A, 3B)- DNA methyltranspherase (1, 3A, 3B)

DPX - slide mounting medium

DDBs - double stranded breaks

EDTA - ethylene-diamine-tetra-acetic acid

FGFR3 - fibroblast growth factor 3

FISH - fluorescent in situ hybridization

HeLa - immortal cell line used in scientific research

HCl - hydrochloric acid

HRAS - proto-oncogene located in chromosome 11

HUMN - the International Human Micronucleus Project

HUMNxL - the International Human Micronucleus Project in exfoliated cells

IARC - International Agency for Research in Cancer

ImmunoCyt - multiplex immune-cyto-fluorescence bladder cancer detection assay

INK4A - tumour suppressor locus

Ki67 - cellular marker of proliferation

k-MT - kinetochore microtubule

LDQ10 - monoclonal antibody that detects cancer related mucine (MUC2)

MIB-1 - gene that encodes the enzyme E3 ubiquitin-protein ligase MIB1

MIBC - muscle invasive bladder cancer

MIUC - muscle-invasive urothelial carcinoma

M344 - synthesized histone deacetylase inhibitor

MNed - micronucleated

MNi - micronuclei

NE - nuclear envelope

NBUDs - nuclear buds

NHEJ - non-homologous end joint

NPBs - nucleoplasmic bridges

NPCs - nuclear pore complexes

NMIBC - non-muscle invasive bladder cancer

NMIUC - non-muscle invasive urothelial carcinoma

NaCl - sodium Chloride

PBL - peripheral blood lymphocytes

PBmax - lymphocyte cultivating medium

PI3KCA - phosphatidylinositol 3-kinase oncogene

PN - primary nucleus

PTEN - phosphatase and tensin homolog

PTCH - protein patched homolog

PUNLMP - papillary urothelial neoplasia of low malignant potential

RAS-MAPK - RAS-MAP kinase

RB - retinoblastoma Gene

ROS - reactive oxygen species

Rpm - round per minute

SAC - spindle assembly checkpoint

SNP - single nucleotide polymorphism

TP53 - tumour suppressor gene

TSC1 - tuberous sclerosis gene 1

TTF1 - transcription termination factor 1

UCC - urothelial cell carcinoma

UCCK - University Clinical Centre of Kosova

UCs - urothelial cells

UCIS - urothelial carcinoma in situ

UEC - urothelial exfoliated cells

UICC - International Union Against Cancer

uFISH - fluorescent in situ hybridization in urothelial cells

WHO - World Health Organization

1. INTRODUCTION

1.1. Bladder anatomy and histology

The adult bladder is located in the anterior pelvis and is enveloped by extraperitoneal fat and connective tissue. It is separated from the pubic symphysis by an anterior prevesical space known as the space of Retzius or retropubic space. The dome of the bladder is covered by peritoneum, and the bladder neck is fixed to neighbouring structures by reflections of the pelvic fascia and by true ligaments of the pelvis. The body of the bladder receives inferior support from the pelvic diaphragm in females or prostate in males and lateral support from the obturator internus and levator ani muscles. At its apex, the medial umbilical ligament, or the urachal remnant, courses along the anterior abdominal wall to the umbilicus (1).

The renal pelvis, ureters, bladder, and urethra (except the terminal portion) are lined by a special form of transitional epithelium called urothelium. Urothelium is composed of five to six layers of cells with oval nuclei, often with linear nuclear grooves, and a surface layer consisting of large, flattened "umbrella cells" with abundant cytoplasm, that cover several underlying cells. The umbrella cells have a trilaminar asymmetric unit membrane and possess apical plaques composed of specific proteins called uroplakins. Toward the basal layer, the cells become smaller or more cylindrical (particularly in the contracted bladder), but they are capable of some flattening when the underlying wall is stretched. This epithelium rests on a well-developed basement membrane, beneath which is a lamina propria. The lamina propria in the urinary bladder contains wisps of smooth muscle that form discontinuous muscularis mucosae. It is important to differentiate the muscularis mucosae from the deeper well-defined larger muscle bundles of the detrusor muscle (muscularis propria) since bladder cancers are staged on the basis of invasion of the latter. If urine flow is obstructed and intravesical pressures rise, the bladder musculature undergoes hypertrophy (2).

Several variants of the normal epithelial patterns may be encountered. Nests of urothelium or in-budding of the surface epithelium may be found occasionally in the mucosa lamina propria; these are referred to as Brunn nests (2).

1.2. Bladder embryology and development

The human urinary bladder derives from the urogenital sinus, and it is initially continuous with the allantois. The upper and lower parts of the bladder develop separately and join together around the middle part of development. At this time the ureters move from the mesonephric ducts to the trigone (3). In male, the base of the bladder lies between the rectum and the pubic symphysis. It is superior to the prostate and separated from the rectum by the recto-vesical pouch. In females, the bladder sits inferior to the uterus and anterior to the vagina; thus, its maximum capacity is lower than in males. It is separated from the uterus by the vesico-uterine pouch. In infants and young children, the urinary bladder is in the abdomen even when empty (4).

1.3. Bladder pathology

The bladder is an organ that is prone to different diseases. An important part of all bladder pathology which constitutes an important source of clinical signs and symptoms are bladder inflammations. While bladder tumours are an important source of both morbidity and mortality (2).

Bladder non-tumoral pathology extends from congenital anomalies (bladder diverticula, extrophy, vesicoureteral reflux, congenital fistulas, urachal cysts, etc), inflammations (acute and chronic cystitis, interstitial cystitis, malacoplakia, polypoid cystitis) and metaplastic lesions (cystitis glandularis, cystitis cystica, squamous metaplasia, nephrogenic metaplasia) (2).

Bladder tumours can be either benign or malignant tumours.

1.3.1. Benign tumours

Neoplasms of the bladder pose biologic and clinical challenges. Despite significant inroads into their origins and improved methods of diagnosis and treatment, they continue to exact a high toll in morbidity and mortality (5).

Urothelial papilloma. It is an exophytic tumour composed of a delicate fibrovascular core covered by urothelium indistinguishable from that of the normal urothelium. The incidence is low, usually 1-4%, with a male to female ratio from 1.9:1, and they tend to occur in younger patients. The lesion is characterized by discrete papillary fronds, with occasional branching, but without fusion. Cytokeratin 20 expression is identical to that of normal urothelium- in superficial umbrella cells only. Alteration of p53 is not seen (5).

Inverted papilloma. A benign urothelial tumour that has an inverted growth pattern with normal to minimal cytologic atypia of the neoplastic cells. It comprises about 1% of urothelial neoplasms, being more 4-5-fold more frequent in male, with a pick frequency in 6^{th} and 7^{th} decade. Haematuria is the most common symptom. If located in the low bladder neck or the ureter it can produce also signs of obstruction. It appears as a smooth-surfaced pedunculated or sessile polypoid lesion, covered by histologically and cytologically normal epithelium with randomly scattered endophytic cords (5).

1.3.2. Bladder cancer

1.3.2.1. Epidemiology

Urinary bladder cancer has become a common cancer globally, with an estimated 430,000 new cases diagnosed, and 165,000 bladder cancer deaths occurred worldwide in 2012, with 75% of the total burden occurring in men. It ranks as the ninth most frequently diagnosed cancer worldwide, with the highest incidence rates observed in men in Southern and Western Europe, as well as in

certain countries in North America and Western Asia. Incidence rates are consistently lower in women than men, although sex differences vary greatly between countries (6). The highest incidence rates in male were recorded in Southern Europe, particularly in Spain (age-standardized rate (ASR) =36.7 per 100,000) and Italy (ASR=33.2 per 100,000). Incidence rates are also very high among men in Nordic countries (e.g.: Denmark ASR=27.4 per 100,000). In females, the highest incidence rates are observed in Denmark (ASR=8.4 per 100,000), Norway (ASR=6.4 per 100,000) and Switzerland (ASR=6.3 per 100,000) (6).

The most common type of bladder cancer in developed countries is urothelial carcinoma, derived from the uroepithelium, which constitutes more than 90% of bladder cancer cases in the USA, France or Italy. However, in other regions (e.g. Eastern and Northern Europe, Africa, Asia) the relative frequency of urothelial carcinoma of the bladder is lower. In general, among all registries included into the 8th volume of "Cancer Incidence in Five Continents" urothelial carcinoma constitutes 84% of bladder cancer in males and 79% in females. Other types of bladder cancer, i.e. squamous cell carcinoma and adenocarcinoma have a much lower relative frequency. In all "Cancer Incidence in Five Continents" registries squamous cell carcinoma accounts for 1.1% and 2.8% of all bladder cancers in men and women respectively. Adenocarcinoma of the bladder constitutes respectively 1.5% and 1.9% of all bladder tumours worldwide (5, 7). As with most of the carcinomas, its development seems to depend on a combination of genetic and environmental factors (8).

1.3.2.2. Risk factors

There are several known and potential risk factors for bladder cancer. Tobacco smoking and occupational exposure to aromatic amines are the most important among them (9). It is estimated that the risk of bladder cancer attributed to tobacco smoking is 66% for men and 30% for women. The risk of bladder cancer in smokers is a 2-6-fold that of non-smokers. The risk increases with increasing duration of smoking and with increasing intensity of smoking (number of cigarettes per day). The risk of bladder cancer goes down after stopping smoking, and 15 years after cessation tends to be approximately that of non-smokers (5).

The association of bladder cancer and occupational exposure was first observed in 1895 by Rehn, who reported high rates of bladder cancer among men employed in the aniline dye industry. It was estimated that contact with occupational carcinogens (benzidine, 2-naphtylamine, 1-naphtylamine, etc.) causes up to 25% of all bladder tumours (5).

The bladder tumours are more frequent in industrial areas, where people are exposed to different environment pollutants (especially in those associated with petrochemicals) (10). In general, the prevalence of bladder tumours in developed countries is approximately 6-times higher compared with that in developing countries (5).

Several epidemiological studies indicate that chronic abuse of analgesics containing phenacetin greatly enhance the risk of developing urothelial cancer of renal pelvis, ureter and bladder. The relative risk has been estimated to be 2.4 to more than 6. Other medicinal drugs like clornaphazine and cyclophosphamide, are associated with the development of bladder cancer (5).

Another important factor in the pathogenesis of bladder carcinoma is infestation with Schistosoma haematobium, which is thought to be pathogenically linked to urothelial and squamous cell carcinoma of the bladder, making this cancer to have a greater concentration in areas with endemic infestation with this parasite (11-13). The underlying mechanism may lead to chronic irritation of the bladder epithelium, which may increase bladder cancer risk (5).

Several studies showed that the use of drinking water containing chlorination by-products or contaminated by arsenic may increase the risk of bladder cancer (14, 15). An IARC Monographs Working Group reviewed in 2004 the relevant epidemiological studies and concluded that arsenic in drinking water is carcinogenic to humans (Group 1) and that there is sufficient evidence that it causes bladder cancer (5).

It is believed that there is a slightly increased risk of bladder carcinomas development in patients who have undergone radiation therapy for prostate cancer (16).

Most cases of urothelial carcinoma are seemingly present at patients over the age of 50 years, but they can also occur in younger adults and children (17, 18).

1.3.2.3. Clinical manifestations

The urothelial carcinoma clinically is manifested by painless and intermittent haematuria, changes in the bladder's habits or symptoms of irritations, etc. The type and severity of clinical signs and symptoms of infiltrating urothelial carcinoma depend on the extent and location of the tumour. Most patients with urothelial tumours present with at least microscopic haematuria. The most common presenting symptom of bladder cancer is painless gross haematuria which occurs in 85% of patients. Subsequent clotting and painful micturition may occur. In the case of large tumours, bladder capacity may be reduced resulting in frequency. Tumours located at the bladder neck or covering a large area of the bladder may lead to irritative symptoms, i.e. dysuria, urgency and frequency. Similar symptoms may be present in the case of extensive carcinoma in situ. Tumours infiltrating the ureteral orifice may lead to hydronephrosis, which is considered a poor prognostic sign. Rarely, patients with extensive disease present with a palpable pelvic mass or lower extremity oedema. In the case of advanced disease weight loss or abdominal or bone pain may be present due to metastases (19).

1.3.2.4. Morphologic features

The urothelium lines the inner surfaces of almost the entire urinary tract, including the renal pelvis, ureter, bladder and proximal urethra. Because it is the permeability barrier between urine and blood, the urothelium is constantly exposed to an assortment of potential carcinogens. It is of no great surprise then that urothelial carcinoma is one of the most common cancers worldwide (20). Urothelial tumours can arise anywhere in the bladder. In a series of 1,000 cases, the location was listed as follows: lateral walls, 37%; posterior wall 18%; trigone, 12%; ureteric orifices, 10%; dome 8%; and anterior wall, 4%. Synchronous or metachronous multicentricity is common. Most clinical and molecular genetic evidence suggests that in the large majority of cases the tumours arise from a common clone and that therefore they are the consequence of intramucosal seeding of a single tumour rather than true multicentric neoplasms (19).

The pattern of growth may be exophytic or endophytic, or a combination of both. When exophytic, the tumour may adopt a papillary or a villo-glandular configuration (with central fibrovascular

cores) or a solid (nodular) appearance (19). Carcinoma devoid of papillary structures is called carcinoma in situ (CIS) and is, by definition, high grade. Papillary tumours are also quite varied, including reactive proliferations and papilloma as well as the papillary urothelial proliferation of low malignant potential (PUNLMP) and low- and high- grade papillary carcinoma (21). These carcinomas are graded as low and high grade depending upon the degree of nuclear anaplasia and some architectural abnormalities (5).

Urothelial tumours may present as non-invasive or invasive patterns. Non-invasive tumours can be divided into two categories: flat or papillary. Stromal invasion by urothelial carcinoma proceeds in two stages: invasion of the lamina propria and invasion of the muscle layer (19). The most important element in the pathologic evaluation of urothelial cancer is the recognition of the presence and extent of invasion (5).

Approximately 70% of bladder urothelial carcinomas are non-muscle invasive (Ta/T1), papillary tumours that are usually morphologically categorized as low-grade urothelial carcinoma. They have a good prognosis, which results in relatively long survival, but may be associated with recurrence and 'progression' to high-grade urothelial cell carcinoma (UCC) in approximately 10–15% of cases. The remaining 30% are muscle-invasive (≥ T2) tumours, which are histologically categorized as high grade, are associated with worse overall survival than low-grade UCC and with a mortality rate of approximately 60% (22, 23).

Detection of muscle invasion is of great consequence because of its influence on therapy and prognosis. This feature can be misinterpreted in case of inconsistent, but prominent fascicles of muscularis mucosae (particularly common in women) as belonging to the muscularis propria. Lymphovascular invasion may be present, but immunohistochemical studies for endothelial markers suggest that this feature can be closely simulated by retraction artefact. It is also important not to misinterpret the mature adipose tissue commonly present in the lamina propria or muscularis propria as perivesical soft tissue, in order to avoid a tumour adjacent to fat in a biopsy specimen being badly over-staged (19).

Histologic variants. Urothelial carcinoma has a propensity for divergent differentiation with the most common being squamous followed by glandular. Virtually the whole spectrum of bladder cancer variants may be seen in variable proportions accompanying otherwise typical urothelial carcinoma. Histological variants may be as follows (5):

1. Urothelial carcinoma with squamous differentiation

2. Urothelial carcinoma with glandular differentiation
3. Nested variant
4. Microcystic variant
5. Micropapillary variant
6. Lymphoepithelioma-like carcinoma
7. Lymphoma-like and plasmacytoid variants
8. Sarcomatoid variants
9. Urothelial carcinoma with giant cells
10. Urothelial carcinoma with trophoblastic differentiation
11. Clear cell variant
12. Lipid-cell variant
13. Undifferentiated carcinoma.

When small cell differentiation is present, even focally, it portends a poor prognosis and has different therapeutic ramifications, and hence should be diagnosed as small cell carcinoma (5)

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1.3.2.5. Molecular genetic features

Most epithelial tumours are thought to progress along a single pathway from benign to malignant by successive mutations in crucial genes that regulate growth, survival, apoptosis and cell-cell interactions. Urothelial carcinomas present as a heterogeneous group of diseases that consist of two main phenotypic variants with drastically different biological behaviours and prognoses. The low-grade papillary variant (a classification that encompasses the terms urothelial papilloma, papillary urothelial neoplasm of low malignant potential, and non-invasive low-grade papillary urothelial carcinoma) accounts for about 80% of urothelial carcinomas and is often multifocal and recurrent, with limited potential to become muscle invasive (20). These tumours are thought to have originated from simple and nodular urothelial hyperplasia and, if treated early by surgical resection and intravesical (within the bladder) immunotherapy, the 5-year survival rate approaches 90%. The second main variant, which accounts for about 20% of urothelial carcinomas, presents as an invasive tumour and the patient usually has no previous history of low-grade non-invasive papillary tumours. These tumours seem to arise de novo or derive from flat, high-grade carcinoma in situ

(CIS) lesions. Despite radical cystectomy and debilitating systemic therapy, at least 50% of patients with invasive urothelial carcinomas die from metastases within 2 years of diagnosis. Treatment fails in 95% of patients with advanced disease, and the 5-year survival rate for metastatic bladder cancer is just 6% (20).

1.3.2.6. Genetic and epigenetic alterations

Numerous molecular factors are involved in determining UCC phenotype, genotype, biological behaviour, and clinical outcomes. Recent researches on urothelial carcinoma tumorigenesis have shown that urothelial carcinomas contain numerous genetic and epigenetic abnormalities, which could be categorized into four different levels: 1. Chromosomal level alterations that include numerical and/or structural anomalies of chromosomes, 2. Gene-level alterations that include genetic mutation, fragment deletions, amplification, or abnormal epigenetic changes, 3. Expression alterations and 4. Protein-level alterations that include up- or down-regulated protein expression, tumour-associated proteins, and fusion proteins (24).

Numerical chromosomal alterations. Numerical chromosome aberrations represent changes of copy numbers of various genetic regions. The most frequent changes were observed on chromosomes 1, 8, 9, 10, 11, 13 and 14 (25).

Structural chromosomal aberrations. Structural alterations involve gains, losses, translocations, or more complicated rearrangements of chromosomes, resulting in the alteration of DNA copy number at the chromosome level. Gains of material were detected on long arms of chromosomes 1, 8 and 13 and losses of material on short arms of chromosomes 8 and 11 and on long arms of chromosomes 9, 10, 11 and 14. These alterations can be assessed by FISH, microsatellite analysis, SNP and CGH. Chromosome 9p21 deletion is frequently observed in the early stages of urothelial carcinogenesis (24, 25). Subsequent studies found both 9q and 9p losses in low-grade non-invasive papillary tumours and urothelial hyperplasia. Strikingly, even the normal-appearing urothelium that is adjacent to the tumour lesion harbours this chromosomal abnormality (26). These deleted regions harbour tumour-suppressor genes. For instance, the cyclin-dependent kinase inhibitor 2A

(CDKN2A) gene resides on 9p21 and encodes two alternatively spliced products, INK4A and ARF53, which induce cell-cycle arrest through the retinoblastoma protein (RB) and p53 signalling pathways, respectively (20). Other chromosomal anomalies such as a loss of 8p, which occurs in 25–30% of urothelial carcinomas and is primarily associated with high-grade and late-stage tumours (27).

DNA- sequence aberrations. DNA-sequence aberrations (mutations) are alterations in the DNA sequence of the genome that lead to various abnormalities such as the production of non-functional or truncated proteins, or destruction/generation of DNA methylation sites. Gene mutations frequently associated with TCC include loss of function mutations of TP53 and activation mutations of FGFR3, which characterize alternative genetic pathways in the pathogenesis of

UCC (28).

Major UCC-related molecular pathways. FGFR3 and TP53 mutations have been recognized as key genetic pathways in the carcinogenesis of transitional cell carcinoma. The first pathway is represented by FGFR3; activating mutations in the fibroblast growth factor receptor 3 (FGFR3) signalling pathway consequently activates the RAS-MAP kinase (RAS-MAPK) pathway and phospholipase Cγ (PLCγ), leading to uncontrolled cell proliferation (29). This appears to be the most frequently mutated oncogene in transitional cell carcinoma (60-80%). Its mutation is strongly associated with low tumour grade, early stage, and low recurrence rate, which confer a better overall prognosis. There are several other genes implicated in this pathway like Chromosome 9q deletion, HRAS, TSC1, PTCH, DBC1, PI3KCA etc. (19, 30).

In contrast, the second pathway tumours, represented by TP53 mutations, are associated with higher tumour grade, more advanced stage, and more frequent tumour recurrences. Here also belongs the urothelial carcinoma in situ, which commonly shows mutations or deletions in the tumour suppressor genes TP53, RB and PTEN (24). The factors p53 (transcription factor) and RB (retinoblastoma) are the 2 key factors in the cell cycle regulation pathway, which is altered in 93% of bladder cancer cases (31). Factor p53, which is encoded by TP53, is the most famous and well-studied tumour-suppressor and is inactivated by somatic mutations in approximately 50% of all human cancers (31, 32). *TP53* mutations are highly frequent (>40%) in MIBCs, in contrast to the NMIBCs, of which only about 8% possess *TP53* mutations (33). These molecular markers offer the potential to characterize individual urothelial neoplasms more completely than is possible by

histologic evaluation alone. Assessment of key genetic pathways and expression profiles could ultimately establish a set of molecular markers to predict the biological nature of tumours and to establish new standards for molecular tumour grading, classification, and prognostication (24).

Epigenetic alterations. Unlike genetic mutations and copy number variation, epigenetic events regulate gene expression outcome without changing the underlying DNA sequence. Epigenetic regulation includes DNA methylation, histone modifications, microRNA regulation and nucleosome positioning, all of which are distorted in every form of human cancer (32). DNA methylation has been recognized to be important in developmental biology and cancer aetiology in general (34). DNA methylation of cytosine-guanine dinucleotide (CpG) islands involves the addition of a methyl moiety to the cytosine-5 position at a gene promoter region. This process alters gene function without changing the base sequence of DNA and has been recognized as a common alternative mechanism for gene inactivation, especially during the early stages of tumour development. There is growing evidence that methylation plays a pivotal role in the function of key gene promoters, blocking transcriptional activation (35). Cancer cells, including bladder cancer, show overexpression of DNMT1, DNMT3A, and 3B, which in turn results in DNA hypermethylation of promoter regions, and the possible subsequent silencing of tumour suppressor genes (36). Many tumour suppressor genes contain CpG islands and show evidence of methylation-specific gene silencing associated with neoplasia. Hypermethylation of CpG islands is associated with transcriptional repression, whereas hypomethylation may lead to increased potential for gene activity or chromosomal instability (37). Mutations in the form of C→T transitions at methylated CpG sites are the hallmark of hydrolytic deamination of 5-methylcytosine and commonly produce mutations in tumour suppressor genes such as TP53 (38).

In addition, a significant number of genetic mutations of epigenetic regulator genes occur in virtually every cancer type, thereby disturbing the epigenome patterns. These include somatic mutations of genes that encode for DNA methyltransferases, chromatin modifiers, and chromatin re-modelers. A substantial portion (76%) of all primary bladder tumours displays mutations in at least one chromatin regulatory gene (31).

MicroRNAs (miRNAs) are short noncoding RNA molecules that modulate messenger RNA (mRNA) at the posttranscriptional level. They act as tumour suppressors or oncogenes and appear to be involved in cancer development and progression (24). The alterations of miRNA processing

genes/proteins and miRNA gene promoter hypermethylation are partly responsible for miRNA downregulation (39). Recent evidence suggests that alterations of miRNA expression contribute to urothelial carcinogenesis.

1.3.2.7. Diagnosis

In patients with signs suspicious for bladder cancer (e.g., haematuria), direct cystoscopic visualization of the bladder is the key diagnostic assessment followed by urine cytology and TUR (transurethral resection) with biopsy. The new technology including optical coherence tomography and confocal laser endomicroscopy may improve the sensitivity and specificity of identifying bladder tumours while also providing pathologic information (40).

Histopathology remains the gold standard for bladder cancer diagnosis and it is the most important prognostic factor to predict clinical behaviour. Immunohistochemistry is very helpful in differentiating benign lesions from the malignant ones, as well as infiltrative focuses, using protein markers like P53, CK7/CK20, Ki67, CEA, TTF1, GATA3, MIB-1, etc. (19, 23).

Urine cytology is a non-invasive method for detecting bladder cancer by identifying abnormal urothelial cells in the voided urine or bladder washes. Urine cytology has high specificity but relatively low sensitivity, particularly in well-differentiated low-grade bladder tumours. In order to improve the diagnostic sensitivity of urine cytology, the immunostaining of urothelial cells was developed to be applied to urine specimens.

ImmunoCyt (DiagnoCure, Inc., Quebec, Canada) is a multiplex immune-cyto-fluorescence bladder cancer detection assay that combines fluorescently labelled monoclonal antibodies for M344, LDQ10, 19A211, and a glycosylated form of the carcinoembryonic antigen (CEA). A minimum evaluation of 500 epithelial cells is required, and the presence of one fluorescent cell is considered as positive.

Fluorescent in situ hybridization is used to find molecular alterations in bladder cancer cells from the urine specimens. The UroVysion test (Abbott Molecular, Inc., Des Plaines, IL, USA) is a 4-colour FISH assay designed to detect aneuploidy of chromosome 3, 7 and 17, as well as loss of the 9p21 locus, using urine specimens from patients with haematuria. It consists of fluorescently

labelled DNA probes to the pericentromeric regions of chromosomes 3 (red), 7 (green), and 17 (aqua) and to the 9p21 band (gold) location of the P16 tumour suppressor gene. The criteria for detecting bladder cancer by UroVysion are >4 urothelial cells with a gain of >2 chromosome 3, 7, or 17 or >12 cells with loss of the 9p21 locus. In addition, >10 urothelial cells showing a gain for a single chromosome 3, 7, or 17; or >10 cells with tetrasomy or near tetrasomy for chromosomes 3, 7 and 17 are also considered abnormal (22). The advantage of uFISH test related to cytology is increased sensitivity for detection of all stages and grades tumours, especially in recurrent bladder cancer (41).

These methods, except urine cytology, although having a high detection rate, are expensive, time-consuming, invasive and uncomfortable (42). Patients with non-muscle invasive bladder cancer are regularly monitored for tumour recurrence and progression with cystoscopy and urine cytology. Therefore, there is a critical need for the identification of biomarkers to diagnose bladder cancer at an early stage, monitor recurrence, refine prognostic estimates, and predict response to treatment in patients with bladder cancer (42).

1.3.2.8. TNM classification and staging

The estimation of tumour stage is based on the TNM system which was created and is updated by the American Joint Committee on Cancer (AJCC) and the International Union Against Cancer (UICC) WHO (5).

T – Primary tumour

TX: Primary tumour cannot be assessed

T0: No evidence of primary tumour

Ta: Non-invasive papillary carcinoma

Tis: Carcinoma in situ: "flat tumour"

T1: Tumour invades subepithelial connective tissue

T2: Tumour invades muscle

T2a: Tumour invades superficial muscle (inner half)

T2b: Tumour invades deep muscle (outer half)

T3: Tumour invades perivesical tissue:

T3a: Microscopically

T3b: Macroscopically (extravesical mass)

T4: Tumour invades any of the following: prostate, uterus, vagina, pelvic wall, abdominal wall

T4a: Tumour invades prostate, uterus or vagina

T4b: Tumour invades pelvic wall or abdominal wall

N – Regional lymph nodes

Nx: Regional lymph nodes cannot be assessed

N0: No regional lymph node metastasis

N1: Metastasis in a single lymph node 2 cm or less in greatest dimension

N2: Metastasis in a single lymph node more than 2 cm but not more than 5 cm in greatest dimension, or multiple lymph nodes, none more than 5 cm in greatest dimension

N3: Metastasis in a lymph node more than 5 cm in greatest dimension

M – Distant metastasis

Mx: Distant metastasis cannot be assessed

M0: No distant metastasis

M1: Distant metastasis

Stage Grouping

Stage 0a	Ta	N0	M0
Stage 0is	Tis	N0	M0
Stage I	T1	N0	M0
Stage II	T2a, b	N0	M0
Stage III	T3a, b	N0	M0
	T4a	N0	M0
Stage IV	T4b	N0	M0
Any	T	N1, N2, N3	M0
Any	T	Any N	M1

1.4. Cancer, genomic instability and micronucleus formation

Cancers are multifactorial diseases having a genetic basis, arising due to inherited mutations or de novo gained mutations in somatic tissues. Human cancers may be studied and examined at various levels of investigation. At the clinical level, cancers are usually recognized by clinical symptoms, such as abnormal tissue function and size, and can be visualized by conventional radioscopic methods. At the tissue level, after histological examination of sections from tumour biopsies, cancers are characterized by altered tissue morphology, structure and growth, metastasis, angiogenesis and lymph-angiogenesis. At the systemic level, changes in leukocyte levels may be monitored, and circulating factors may be associated with tumours. At the cellular level, for instance, cancer cells can be distinguished by abnormal centrosome numbers and conspicuous morphological changes (43). Furthermore, many common traits are found also at the protein level, especially in pathways that regulate replicative lifespan of cells. Remarkably, most cancer cells have either telomerase activity (90%), inactivated p53 and pRB pathways (50%), or abrogation of the INK4aARF locus (30%). Most importantly, the final diagnosis for cancer lies at the nuclear

level. Cytogenetic analysis of cultured tumour cells reveals that most solid tumours have aneuploid karyotypes, i.e. aberrant nuclei with altered chromosome numbers and structure (43).

It is commonly accepted that malignant transformation is a lengthy multi-step process and arises through an accumulation of mutations at various genetic loci. Most tumours arise through clonal selection and waves of expansion of a somatic cell that has acquired genetic alterations in essential genes either controlling cell death or cell proliferation (43).

Several hundred genes, representing 1% of the human genome, have been implicated in tumorigenesis and progression, that means that tumour cells probably require only a small number of essential genes for cell proliferation (44).

The integrity or completeness of genomic information is one of the fundamental prerequisites for life. To maintain this integrity, cells have many mechanisms, many of which have been the subject of recent studies. The relationship between genome stability and human health becomes more obvious in diseases typically characterized by progressive deterioration of specific tissues, susceptibility to cancer, chromosomal rearrangement, and hypersensitivity to genotoxic agents (45). In human tumour cells, some part of one, or more, of these mechanisms is disrupted, thus genomic integrity is easily broken down. This critically contributes to the malignant transformation of cells and cancer development (46). Furthermore, the stability of the genome in cancer cells becomes precarious and compromised because several cancer-predisposing mutations affect genes that are responsible for maintaining the integrity and number of chromosomes during cell division (43).

Cancer cells typically harbour chromosomal alterations, such as aneuploidy or polyploidy and abnormal chromosome structures (47). A hallmark cause of these events is the chromosomal instability (CIN), which is observed in most solid tumour cells and is often associated with the missegregation of chromosomes that results from improper kinetochore-microtubule attachments and the consequent presence of lagging chromosomes during anaphase (48-50). One form of chromosome instability (CIN), the recurrent missegregation of whole chromosomes during cell division (W-CIN), leads to aneuploidy, a hallmark of most solid tumours. Tumour cells acquire W-CIN through three major mechanisms: mitotic checkpoint defects, centrosome overduplication or faulty sister chromatid cohesion. It promotes gains of extra copies of oncogenes or losses of tumour-suppressor genes, allowing the selection of karyotypes that thrive in certain environments. W-CIN leads to aneuploidy and the formation of micronuclei and

binucleate cells (51). The genomic instability due to changes in chromosomal structure named as structural CIN (S-CIN), in form of translocations, deletions, inversions and chromosomal fragmentation, as a result of poor repair of damaged DNA (52). Genetic instability is a transient or a persistent state that causes a series of mutational events leading to gross genetic alterations. It is now clear that most cancers have altered genomes. Genetic instability has been found in many types of cancer (43).

Cells continuously encounter DNA damage either through endogenous sources (including radical species as by-products of cellular metabolism) or through exogenous sources (such as ultraviolet rays in sunlight). In order to maintain genetic stability while being under constant assault, evolutionary conserved pathways exist that can detect and repair DNA damage which are collectively termed the DNA damage response (DDR)(53). A specifically toxic type of DNA damage are double strand breaks, which can be repaired using two mutually exclusive types of DNA repair; non-homologous end-joining (NHEJ) which ligates two ends of the broken template and it is inherently prone to generating mutations, or homologous recombination (HR) which usually uses the DNA of the sister chromatid as a template to repair the damaged DNA, and is not prone of generating mutations (52). Even though cells are equipped with several repair systems and cell cycle checkpoints that prevent cells with damaged DNA from undergoing mitosis, cells can end up in mitosis through several paths in the presence of DNA breaks, including checkpoint failure and mitotic processing of replication aberrancies (52). Various errors in DNA replication, DNA repair and chromosome segregation during mitosis and cell cycle checkpoint defects can lead to the physical isolation of chromosomes or fragments of chromosomes - which could induce the formation of micronuclei (MNi) (54), to defective separation of sister chromatids - which could result with nucleoplasmic bridges (NPBs) formation, and DNA amplification - which elimination could result in nuclear buds (NBUDs) formation (55). Chromosome breakage, loss and rearrangement are important initiating events in cancer; however, they also play an important role during the evolution of cancer when a genome instability phenotype is established (56). The use of biomarkers of chromosomal damage due to genetic instability in order to predict the risk of cancer as well as to identify high-risk individuals is both valuable and imperative (57). In the last decades, they are extensively used for early detection of biological effects of DNA-damaging agents and genome instability (58-60).

1.4.1. Mechanisms of micronuclei, nucleoplasmic bridges and nuclear buds formation

1.4.1.1. Micronucleus formation

Micronuclei are defined as small chromatinic bodies that appear in the cell cytoplasm, lagging behind the cell division (61). MN, also known as Howell–Jolly bodies, were originally identified and described in erythrocytes by the haematologists William Howell and Justin Jolly and they were later found to be associated with deficiencies in vitamins such as folate and vitamin B12 (62).

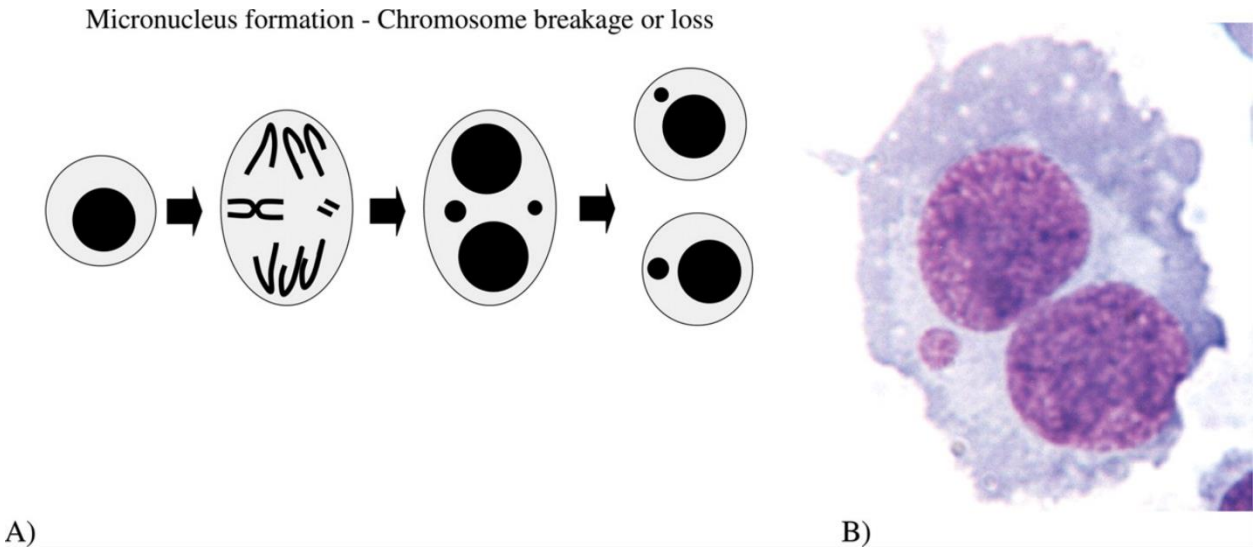

Figure 1. (**A**) Schematic diagram showing the origin of MN from either a lagging chromosome fragment or a whole chromosome. (**B**) A photomicrograph of a mitogen-stimulated, cytokinesis-blocked lymphocyte containing one MN. Adopted from Bonassi S et al (63).

It is now well-established that MN mainly originates from acentric chromosome fragments, acentric chromatid fragments or whole chromosomes that fail to be included in the daughter nuclei

at the completion of telophase during mitosis because they did not attach properly with the spindle during the segregation process in anaphase. These displaced chromosomes or chromosome fragments are eventually enclosed by a nuclear membrane and, except for their smaller size, are morphologically similar to nuclei after conventional nuclear staining (55).

As MNi derive from chromosomal fragments and whole chromosomes lagging behind in anaphase, the MN assay can be used to show both clastogenic and aneugenic effects. The distinction between these phenomena is important since the exposure studied often induces only one type of MN. MN harbouring chromosomes can be distinguished from those harbouring acentric fragments by the presence of a centromere. The proportion of centromere-positive MN in human lymphocytes increases with age, which primarily reflects an age-dependent micronucleation of the X and Y chromosomes. The X chromosome especially tends to lag behind in female lymphocyte anaphase, being micronucleated (MNed) more efficiently than autosomes. Sex chromosomes contribute the majority of chromosome loss events with increasing age, and the X chromosome can account for up to 72% of the observed MN in female (64). The possible mechanism includes hypomethylation of cytosine in centromeric and pericentromeric repeat sequences such as classical satellite repeats at pericentromeric regions and higher-order repeats of satellite DNA in centromeric DNA (65). There is some evidence for an enhanced prevalence of fragments from chromosome 9 in spontaneous human lymphocyte MN and from chromosomes 1, 9 or 16 in MN induced in vitro by some clastogens; the breakage appears to occur in the heterochromatic block of these chromosomes. Understanding the mechanistic origin and contents of MN is essential for the proper use of this cytogenetic end-point in biomarker studies, genotoxicity testing and risk assessment (64).

As research on the MN advances, extremely varied origins of MN and MN-like structures are emerging. It is thought that MN are not only caused by chromosome segregation failure in mitosis itself, but also can result from a wide variety of stresses occurring during any of the previous stages of the cell cycle. The underlying pathways that drive MN are complex and heterogeneous. Based on the time when the first hit of micronucleation occurs, the origins of MN can be divided into four groups: mitotic, pre-mitotic, post-mitotic and interphase origins (Figure 2) (54).

Mitotic origin of MN. Mitosis is a key process related to micronucleus formation. Supported by direct live-cell imaging of young, middle-aged and old-aged primary human dermal fibroblasts, a

recent study finds that MN increases with ageing due to general dysfunction of the mitotic machinery (66). Accurate chromosome segregation during cell division ensures that each daughter cell inherits a complete and identical copy of the genome. Chromosome segregation is driven by the centromeres and mitotic spindle, a self-organizing micro machine composed of microtubules and associated proteins. Kinetochore, the protein complex assembled at each centromere on each chromosome, serves as the attachment site for spindle microtubules. Faithful chromosome segregation is ensured by the bi-oriented (amphitelic) kinetochore–microtubule (k–MT) attachments (67). Since microtubules capture kinetochores randomly, erroneous k–MT attachments can form. These include monotelic attachments (only one kinetochore in the pair binds to microtubules) and syntelic attachment (both kinetochores are attached to microtubules emanating from the same centrosome). In normal cases, the incorrectly attached kinetochores generate the "waiting signal" for the spindle assembly checkpoint (SAC), which delays the activation of the anaphase promoting complex/cyclosome (APC/C). APC/C is a ubiquitin-protein ligase that promotes the proteasomal destruction of securin, an inhibitor of separase, and the cyclin B (68). When the SAC deficiency exists, cells with monotelically attached or unattached chromosomes are formed, and they may progress to anaphase (69) and the laggards are prone to be subsequently incorporated into MN (48). Thus, SAC is the major cellular regulatory system acting during mitosis to prevent MN formation, so SAC dysfunction: insufficiency and overexpression of SAC proteins, are linked to micronucleation (54).

Pre-mitotic MN. Besides the dysfunction of the mitotic apparatus, chromosome breakage is also a factor leading to the formation of MN. Acentric chromosome fragments are formed pre-mitotically, as a result of unrepaired DNA double-strand breaks (DSBs) caused by pre-mitotic DNA replication stress or as a result of mis-repair of DNA breaks (70), (71). Acentric chromosome fragments are particularly prone to micronucleation because they are incapable of forming normal attachments with the mitotic spindle (72). During metaphase, acentrics localize to the outer edge of the metaphase plate, separated from the main mass of chromosomes. On entry into anaphase, they remain in this position while the main mass of sister chromosomes separates. During the late anaphase, some of the acentrics segregate to correct poles (73). The fragments that fail to segregate are generally encapsulated by the reformed nucleoplasmic envelope to generate MN (74).

Double minutes (DMs) are acentric fragments of extrachromosomal circular DNA (eccDNA) mainly found in human tumours. They are composed of acentric, atelomeric, and autonomously replicating chromatin consisting of a few mega-base pairs in size (75). DMs are formed from amplified DNA and, when occurring in cancers, they usually contain oncogenes or resistance genes that contribute to the over-proliferation potential of cancer cells. The mechanism of DMs formation is initiated by dicentric chromosomes due to the misrepair of DNA breaks by non-homologous end joining (NHEJ) (76). At anaphase, the dicentric chromosome is pulled to opposite poles of the cell. The break of dicentric chromosomes leads to breakage-fusion-bridge (BFB) cycles and DNA amplification, which is a key driver to chromosomal instability. Subsequently, the amplified DNA is eliminated as a mini-circle by a recombinational mechanism (55, 77). In addition, recombination of telomeres with interstitial telomere-related sequences results in a terminally deleted chromosome with a shortened telomere, as well as some DM containing telomeric DNA (78). DMs may stick to normal chromosomes at the time of mitosis, allowing them to be transmitted to daughter cells in a stable manner (79). However, some cells contain aggregates of DMs that are left behind from chromosomes at anaphase, which leads to their micronucleation at telophase when the reconstruction of nuclear membrane proceeds. DM-type MN formed in this way are normally lamin B positive (80). Overall, the physical separation of laggard chromosomes or chromatin fragments from the remaining faithfully segregating ones during anaphase can lead to their exclusion from the primary nucleus and micronucleation in the subsequent G1phase of the cell cycle because of nuclear envelope reassembly happens before the laggards join the mass of segregating chromatids. Laggards are a leading cause of MN, as it accounts for 63.9% of spontaneously generated MN in HeLa cells (81).

Post-mitotic origins of MN. In mammalian cells, NHEJ is a predominant pathway of DSBs repair by searching for neighbouring DNA molecules to re-join the broken end. If this process occurs in G2 and each of the sister chromatid presents a DSB as well, then the two sister chromatids will likely be fused to create a circular or dicentric chromosome. Such fused chromosome is unable to separate in anaphase, and will, therefore, form a chromatin bridge (CB) spanning the spindle midzone (54). In addition to DSBs, CB can be caused by telomere fusion (82), replication stress (83) or persistent chromatid cohesion (84). CBs are highly elastic and rarely break during anaphase (82, 85), ruling out stretching by the elongated mitotic spindle as the cause of breakage. After anaphase, CBs can be severed in various ways. In human colorectal COLO320DM tumour cells,

breakage of the CBs occurs prior to nuclear membrane reformation and the completion of cytokinesis, indicating that mechanical tension rather than cytokinesis is primarily responsible for severing CBs (75). Some of them can persist beyond the completion of mitosis, which may be resolved by the cytoplasmic 3′nuclease TREX1 at 3–20h after anaphase, before the primary nucleus (PN) enter S phase (82). Often the resolution of CBs gives rise to MN in one or both daughter cells at the end of mitosis (83, 86, 87), because intrinsic structural features, make some regions of CBs more easily to stretch and broke (75). It has been estimated that 9.25% of the spontaneously arising MN in HeLa cells can be attributed to CBs breakage (81). MN generated from CB breakage are negative for Lamin B2 (88). Whether the bridged chromosome ends up in the PN or in an MN seems to depend on the extent of the length differential between the two k-fibers bound to the bridged kinetochores (85). It should be mentioned that MN arising from the breakage of telomere fusion-induced CBs is extremely low (82). However, this does not mean telomere fusion is not linked to micronucleation. Alternatively, dicentric chromosomes induced from telomere fusion give risk to MN because they have a high tendency to be lagging during anaphase. This finding suggests that pulling a dicentric chromosome toward opposite poles can generate the necessary force for detaching this chromosome from the microtubules of one or both spindle poles (87).

Observations done in HeLa cells have prescribed an unusual way for MN generation: cell fusion after multipolar mitosis and incomplete cytokinesis (81). The presence of extra centrosomes leads to the formation of a multipolar mitotic spindle, which if fails to cluster their centrosomes prior to anaphase, results in the segregation of chromosomes into more than two daughter cells. Because of the different levels of chromosomal capture and alignment on each pole, multipolar mitosis causes the near-to-stochastic distribution of chromosomes. In extreme cases, one pole might receive only a small amount of chromosomes that resembled the lagging chromosome mass (46). Thus, depending on the nuclei size of the two fused daughter cells, either a binucleated or an MNed cell will arise at the end of cytokinesis. MN formed in this manner may contain more than one chromosome, and are found to be much larger than those formed by other mechanisms (81).

Interphase origins of the MN. Nuclear morphology is an important factor in the proper organization and maintenance of gene expression. Most healthy cells have round or ellipsoid nuclei. Some healthy cell types and some cancer cells exhibit nuclear material protrusion, forming nuclear buds or blebs, which are thought to be sources of MN formed during interphase. The

interphase origin of DM-type MN has been assumed based on the study of the correlation between the frequencies of nuclear buds and MN in fixed interphase cells (89). According to this model, DM are frequently localized at the periphery of the nucleus and can be extruded through nuclear budding and detachment of chromatin from the PN into the cytoplasm (79). About 2–3% of the cells with reduced levels of Lamin B1 showed prominent nuclear blebs, which eventually become nuclear buds and totally separate from the PN and form MN (90). This is a key step in interphase MN generation (91). Lamin B1 depletion is also known to increase to the frequency of lagging chromosomes, suggesting that MN in Lamin B1 depleted cells also have a mitotic origin (92).

Utani et al. revealed that membrane blebbing occurred simultaneously with the extrusion of nuclear content (93). Activation of cytoplasmic membrane blebbing induces nuclear budding, suggesting that blebbing may play a key role in nuclear budding through cytoplasmic membrane dynamics (93).

Nucleoli are recently found to be a new region for MN biogenesis. Nucleoli form at the end of mitosis around the randomly repeated clusters of ribosomal DNA genes and result in a subnuclear compartment that locally concentrates the transcription and processing machinery that are responsible for generating ribosome subunits (94).

Overall, interphase-originated MN is rarely observed and restricted to specific conditions and species. Little is known about their frequency in somatic and cancer cells, as well as for the genetic material inside them (54).

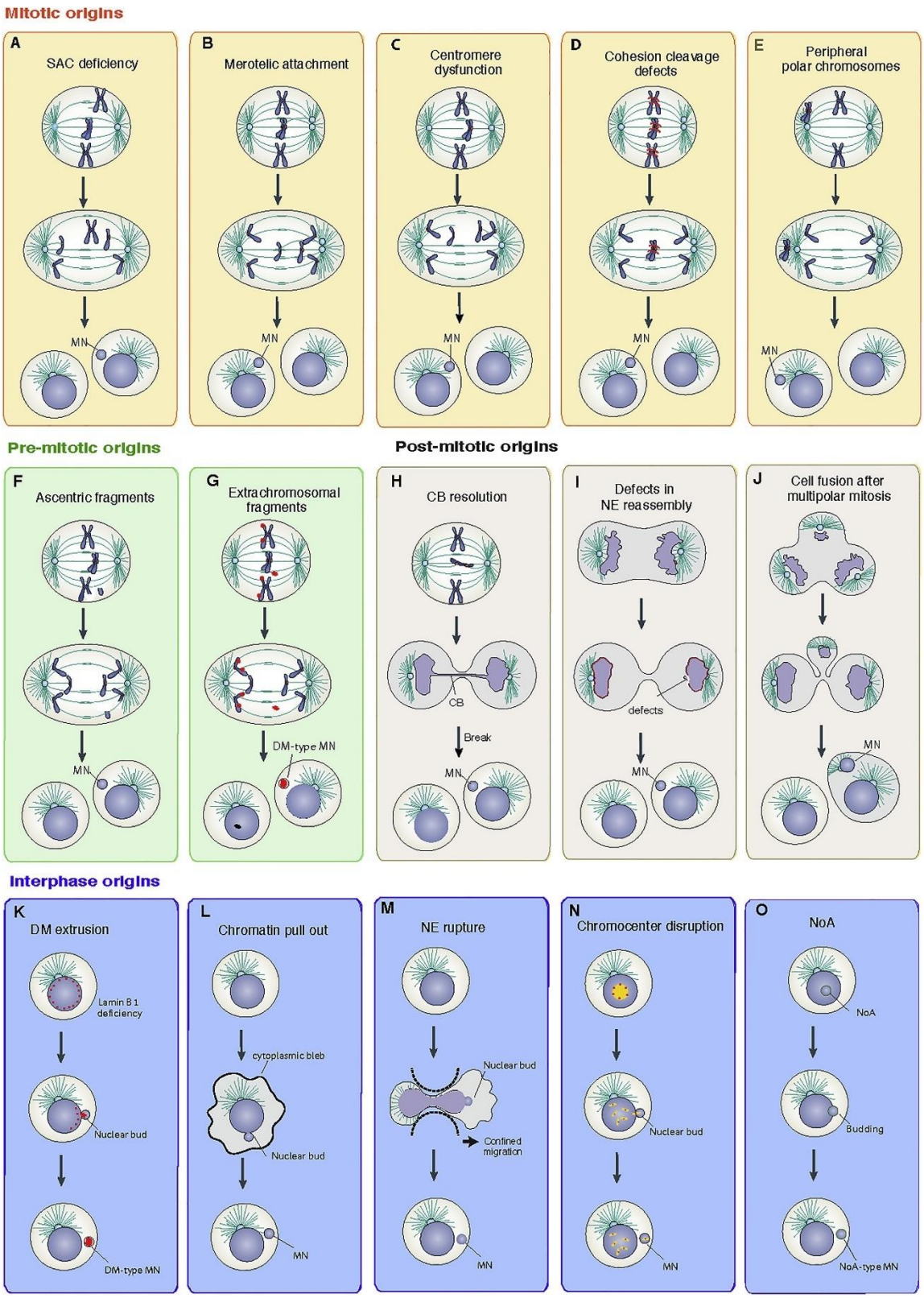

Figure 2. Different routes leading to MN formation. The underlying pathways that drive MN are complex and heterogeneous. Typically, the pathways underlying MN formation can be divided into

four groups: mitotic (A–E), pre-mitotic (F and G), post-mitotic (H–J) and interphase (K–O) origins (See further description in text). Abbreviations: SAC, spindle assembly checkpoint; DM. Double minutes; CB, chromatin bridge; NE, nuclear envelope; NoA, nucleolar aggresomes. Adopted from Guo X et al (54).

1.4.1.2. The origin of nucleoplasmic bridges

NPB originate during anaphase when the centromeres of dicentric chromosomes are pulled to opposite poles of the cell during mitosis. In the absence of breakage of the anaphase bridge, the nuclear membrane eventually surrounds the daughter nuclei and the anaphase bridge, and in this manner, an NPB is formed (Figure 3). NPB are usually broken during cytokinesis but they can be accumulated in cytokinesis-blocked cells using the cytokinesis inhibitor cytochalasin-B. Dicentric chromosomes originate either from misrepair of chromosome breaks or telomere to telomere end fusions (71, 55)

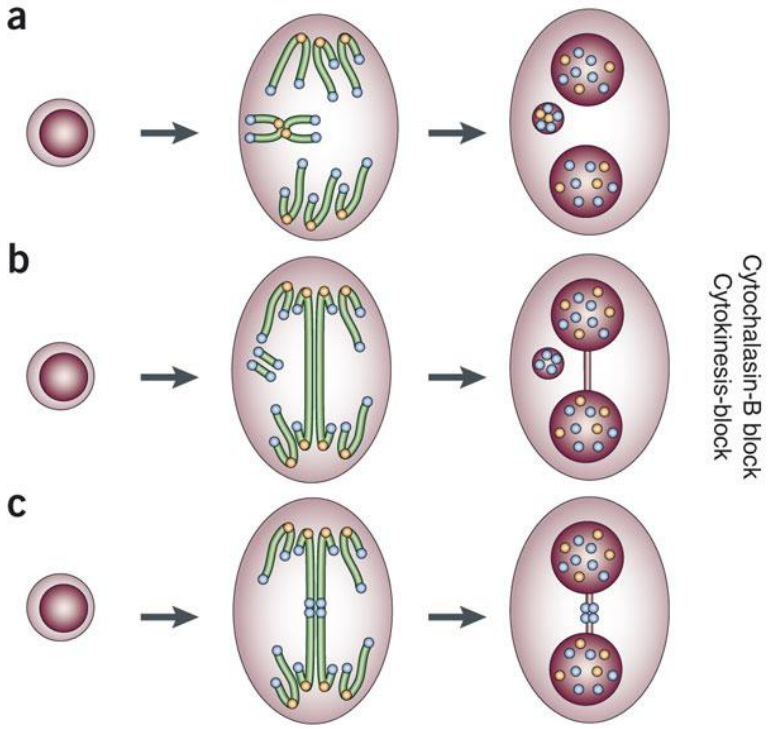

Figure 3. Pancentromeric and telomeric probes can be used to distinguish (I) between MNi originating from whole chromosome loss (**a**) and MNi originating from acentric chromosome fragments (**b**) and (II) NPBs from dicentric chromosomes resulting from misrepair of DNA strand breaks (**b**) and dicentric chromosomes caused by telomere end fusions (**c**). The yellow dots represent probes that hybridize to the centromeric region of chromosomes. The light blue dots represent probes that hybridize with the telomeric sequences in chromosomes. Adopted from Fenech M et al (95).

Telomeres are DNA–protein complexes that contain short repeat sequences added on to the ends of chromosomes by the enzyme telomerase. Telomeres serve multiple functions, including protecting the ends of chromosomes and preventing chromosome fusion. Loss of telomere function leading to chromosome fusion can occur through a variety of mechanisms, involving either endogenous events or exogenous DNA damage. Chromosome fusion can result from the loss of capping function when telomeric repeat sequences are still present, or through the loss of sufficient telomeric repeat sequences to maintain a functional telomere (96). The latter are caused by inappropriate assembly of the telosome protein structure that encapsulates and protects the telomere either because of excessive telomere shortening or deletion and/or base damage in the telomere sequence (87, 96).

Failure to cap the 3' single-stranded G-rich overhang at the end of the telomere may result in the chromosome ends being perceived as broken DNA molecules in which case they may be repaired by the DNA end-joining mechanism and cause telomere end fusions (97, 98); the latter may also be increased due to defects in recombinational repair proteins (98). These mechanisms of nucleoplasmic bridge formation can be distinguished using telomere probes, in cytokinesis-blocked binucleated cells. NPB originating from telomere end fusions are expected to be telomere positive if they retain telomere sequences and/or if the fusions are caused by telomere dysfunction due to loss of telomere-binding proteins without telomere attrition; however, if the fusion was caused due to complete erosion of telomere sequence, the NPB originating from such a mechanism can only be recognized with a specific probe that hybridizes in the subtelomeric region adjacent to the telomeric repetitive sequence track (95, 98, 99). In contrast, an NPB caused by misrepair of DNA

breaks has a low probability of occurring within the telomeric sequences and is therefore likely to be telomere negative (71, 95). Furthermore, NPB arising from misrepair of DNA breaks are also likely to be associated with a MN originating from the acentric fragment generated during misrepair (71, 100). However, a MN originating from an acentric fragment may not necessarily accompany a telomere end fusion event because the latter can occur in the absence of DNA strand breakage and misrepair (54).

1.4.1.3. The origin of nuclear buds

Nuclear buds, micronucleus-like bodies attached to the nucleus by a thin nucleoplasmic connection, have been proposed to be generated similarly to micronuclei during nuclear division or in S-phase as a stage in the extrusion of extra DNA (amplified DNA), possibly giving rise to micronuclei (101). Shimizu *et al.* (102) used *in vitro* experiments with mammalian cells to show that amplified DNA is selectively localized to specific sites at the periphery of the nucleus and is eliminated via nuclear budding during S phase of the cell cycle. The NBUD are characterized by having the same morphology as an MN with the exception that they are connected to the nucleus by a narrow or wide stalk of nucleoplasmic material depending on the stage of the budding process. It is also reported that MN may also be formed by a budding process following exposure to γ-irradiation (103). NBUD have also been shown to be formed when an NPB between two nuclei breaks and the remnants shrink back towards the nuclei (46).

Using centromere and telomere probes, Lindbergh et al. (101) investigated the mechanisms of MN and nuclear bud formation in folic acid-deficient cells. Their results suggest that NBUD and MN have a partly different mechanistic origin. Interstitial DNA without centromere or telomere labels was clearly more prevalent in NBUD (43%) than in MN (13%). Telomeric DNA only or both centromeric and telomeric DNA was more frequent in MN (62 and 22%, respectively) than in NBUD (44 and 10%, respectively). Folate deprivation increased the frequency of NBUD and MN harbouring telomeric DNA, NBUD harbouring interstitial DNA and also NBUD and MN with both centromeric and telomeric DNA. According to this model, MN in binucleate lymphocytes

primarily derive from lagging chromosomes and terminal acentric fragments during mitosis; however, most NBUD originate from interstitial or terminal acentric fragments. Such NBUD may possibly represent nuclear membrane entrapment of DNA that has been left in the cytoplasm after nuclear division or from excess DNA that is being extruded from the nucleus (101).

1.4.2. Micronuclei fate profiles

Persistence. The most general outcome of MN is persistence, where micronucleated (MNed) chromatins/chromosomes keep their MN state during one or more following cell cycles. After the complete division of MNed cancer cells, most of the MN (62%) can persist into the second generation cells (104). Most strikingly, MNed cells showed a very consistent outcome over subsequent divisions; ∼70% of the divisions led to the maintenance of MN at the end of the first division and in the remaining ∼30%, micronuclei were reincorporated; ∼75% of the daughter MNed cells maintain their MN after the second division, remaining ∼25% of them reincorporated the micronucleus (105).

The high tendency for MN to persistence suggests it is a regulated process, rather than a result of the stochastic distribution. Yet, the underlying molecular mechanism remains elusive. One explanation is the centromere of MN-enclosed chromosomes become damaged and lack proper kinetochores and are therefore unable to be aligned at the metaphase plate prior to anaphase onset [74]. Upon their formation, micronuclei already display decreased levels of important kinetochore assembly factors. Importantly, these defects favour the exclusion of the micronucleus over the reintegration into the primary nucleus over several divisions (105).

The lack of proper DNA replication could also explain the high frequency of MN persistence. Whether the persisted MN undergo DNA replication is controversial. It has been revealed that DNA synthesis takes place in a portion of MN that contain nuclear Lamin B1in the S phase before the next mitosis, whereas MN lacking Lamin B1 are never replicated (106), meaning that for DNA replication, the maintenance of Laminin B1 levels is required. DNA replication in MN is asynchronous with PN, with replication in MN persisting in otherwise G2 cells, due to improper

nuclear pore complexes (NPCs) assembly that prevents proper loading of replication proteins (104). In contrast, some MN may contain as many as ten copies of centromeric signals for X chromosome (107), indicating MN can be retained and replicated in the cytoplasm for more than one cell cycle. This was considered a speculative possibility, while there is evidence that multiple centromeric signals might simply be the consequence of centromere repeats expansion or neocentromere formation (108). Unreplicated chromatids can only form monopolar attachments that fail to align properly during cell division. There seems to be a slight bias for replication-deficient MN to be maintained as MN (105). It can be hypothesized that MN that suffer more from replication defects most likely also suffer more from other defects that could interfere with chromosome segregation (54).

Reincorporation. The mitosis of some MNed cells leads to the production of two apparently normal daughter cells, indicating that MN can be reincorporated into PN during the next mitosis (48, 104). The reincorporation frequency during the next mitosis is estimated to be 0–38%, depending on the cell type and the mode of MN induction (105, 104). Two different scenarios of reincorporation have been observed in human cells, depending on the centromere function inside MN: (1) Chromosomes in MN with an intact centromere are able to assemble kinetochore, congregate onto metaphase plate, separate and segregate into one daughter nuclei randomly (48). In some cases, chromosomes in an MN that has been replicated can separate equally during mitosis and rejoin in the PN of a daughter cell after division (109). (2) MN that do not condense nor associate with the other chromosomes during the next mitosis but can be enclosed into the daughter nucleus as nuclear envelope (NE) reassembly at the end of mitosis due to their close distance (104) being located in the periphery of daughter nuclei (105). Interestingly, the defects observed in micronuclei are likely overcomed once micronuclei are reincorporated into the primary nuclei, as they further propagate normally. Using long-term live cell imaging, a study shows that cells with the reincorporated MN do not exhibit new MN formation in the subsequent division, suggesting that kinetochore impairments in MN are likely overcomed when they are reincorporated into the nucleoplasm of PN (105).

Disruption. The NE is a physical membrane barrier that separates the nucleus from the cytoplasm. It fulfils at least two essential functions in eukaryotic cells: first it regulates the movement of molecules between the nucleus and the cytoplasm by active, signal-dependent transport via

aqueous channels that are formed by the NPCs, and second it creates a permeability barrier that prevents the passive diffusion of molecules larger than ~40 kDa across the NE. An intact nuclear permeability barrier is generally considered to be a prerequisite for nuclear transport and to be critical for proper cell compartmentalization (110). MN is thought to have the same structure of their nuclear envelope as PN. Early observations using electron microscopy revealed that some MN display an incomplete NE, with gaps in the double membrane and areas without lamina or condensed chromatin (111). This finding challenged the traditional view that MN are structurally similar to PN. Recent evidence has shown that over 60% of MN in cancer cells undergo an irreversible loss of compartmentalization due to NE collapse. MN disruption occurs throughout interphase and independently of MN origin (whole-chromosome origin and chromatin fragment origin) (112).

The molecular mechanisms implicated in NE fragility of MN are only partially discovered. It has shown that disrupted MN are frequently smaller and more condensed than intact MN. Defective NPC reassembly and disorganization/lack of Laminin B1, are shown to be indicators of the disrupted nuclear envelope in MN (112).

NE is a determinant factor that influences the functional integrity of MN, such as repair of the DNA lesions (113). Compared with intact MN, DNA transcription and replication are completely abolished in disrupted MN, and repair of existing and new DNA damage is also significantly compromised (112). The dysfunction of MN may be primarily due to the loss of Lamin B1 and NPC integrity. Therefore, NE collapse in MN may partially explain why MN have reduced functions compared to PN in the same cell.

Another factor that is thought to promote NE rupture is high nuclear curvature (114). Since the nuclear curvature of NE in MN is more extreme than that of PN, it can be a root cause of the high NE fragility of MN. It has been found that mitochondria mislocalize into the PN following NE rupture (110). It will be interesting to determine whether mitochondria are also present in disrupted MN. If dysfunctional mitochondria are present also in disrupted MN, it probably could further contribute to DNA damage by producing excessive reactive oxygen species, and it is speculated that this process could contribute to the shattering of MNed chromosomes after MN disruption (115). However, a recent study has shown that the chromatin from disrupted MN can persist into the next cell cycle and participate in a phenomenon called chromothripsis, which is defined by

small-scale DNA copy number changes and extensive intrachromosomal rearrangements that are restricted to a single chromosome or chromosome arm (116).

Degradation. MN can be subject of autophagic degradation. Autophagy is a cellular catabolic process by which cytoplasmic waste is engulfed in a double-membrane vesicle and delivered to the lysosomes for breakdown and eventual recycling. Although some studies demonstrate that MN do not colocalize with lysosome markers (104), cells with an increase in MN manifest an increase in autophagic marker LC3 and the inclusion of MN inside autophagosomes, demonstrating MN can be subjected to autophagic degradation (117, 118). The DNA in MN is found to be transported to lysosome by autophagosomes and be degraded thereby ubiquitously expressed lysosomal DNA endonucleases, including DNase2 (119, 120). Inhibition of autophagy or these DNases is found to associate with a significant increase of MN frequency (120, 121).

It is surprising that the proportion of MN undergoing autophagic processing is small (~2-5%) and autophagic MN are observed alongside clearly non-autophagic MN in the same cell (117). One of the reasons that may explain why MN are only rarely sequestered in autophagosomes is their size. However, the finding that MN-like structures sized up to10μm are subjected to autophagy (122) may preclude this possibility. Although the mechanisms underlying these phenomena require further discussion, a promising model linking MN autophagy to the failure of DNA repair in MN has been proposed (123).

Some studies have suggested that DNA in MN may also be degraded by apoptosis. Although there is no evidence of a premature or localized activation of the apoptosis pathway, it's interesting to see whether MN apoptosis represents the very early step of the apoptosis of whole MNed cell. It should be emphasized that apoptotic-like MN degradation is only observed in fixed cells but never observed in live-cell experiments (104, 112).

Extrusion. MN extrusion was first observed during the in vivo maturation of erythrocytes, and MN harbouring whole chromosomes are preferentially extruded (124, 125). In cancer cells, the extrusion of MN mediates the selective elimination of DM, and the extracellular MN have decondensed chromatin and intact lamin protein, and their DNA did not suffer any extensive degradation (102). In most cases, the extruded MN are surrounded by the cytoplasmic membranes, forming a cell-like structure (minicell). Whether MN extrusion exactly occurs remains controversial since it's never observed in live imaging studies (104).

1.5. Micronucleus assay and micronucleus index

The micronucleus was recognized at the end of the 19th century when Howell and Jolly found small inclusions in the blood taken from cats and rats. The small inclusions, called Howell-Jolly body, are also observed in the erythrocytes of peripheral blood from severe anaemia patients. These are the first description of the micronucleus itself (126). In 1959, Evans et al. did the first report attempting to evaluate chromosomal aberration by the frequency of cells harbouring micronucleus (127). In 1970, Boller and Schmid (128) developed a test method to evaluate the frequency of MNed erythrocytes among normal erythrocytes, which lack their own nuclei during haematopoiesis, using bone marrow and peripheral blood cells of Chinese Hamster treated with a strong alkylating agent, trenimon. In the paper, they named this method "Mikrokern-Test". They built the basics of nowadays “micronucleus test” (126), which was adopted to be performed in the different type of cells, in dozens of scientific investigations. In 1976, Countryman and Heddle (129) reported a method using human cultured lymphocytes. Modifications have been introduced by Fenech and Moley (130) using cytochalasin B. Later called as „cytokinesis-block micronucleus assay “(CBMN), this method was widely used for human biomonitoring of DNA damage.

The CBMN assay has evolved into a comprehensive “cytokinesis-block micronucleus cytome assay” that is used to measure not only chromosome breakage as a consequence of mutagen exposure but also chromosome loss, non-disjunction, DNA misrepair, necrosis, apoptosis and cytostasis in PBL (131).

The MN assay in PBL is a well-established method, that measures the frequency of MNi, usually reported as the number of cells containing MNi per total cells counted. The DNA damage events in PBL are specifically scored in once-divided binucleated (BN) cells that have completed the nuclear division but have been blocked at the binucleated stage prior to cytokinesis. The events scored include the following: (a) micronuclei, a biomarker of chromosome breakage and/or whole chromosome loss; (b) nucleoplasmic bridges, a biomarker of DNA misrepair and/or telomere end-fusions, and (c) nuclear buds, a biomarker of elimination of amplified DNA and/or DNA repair complexes (132).

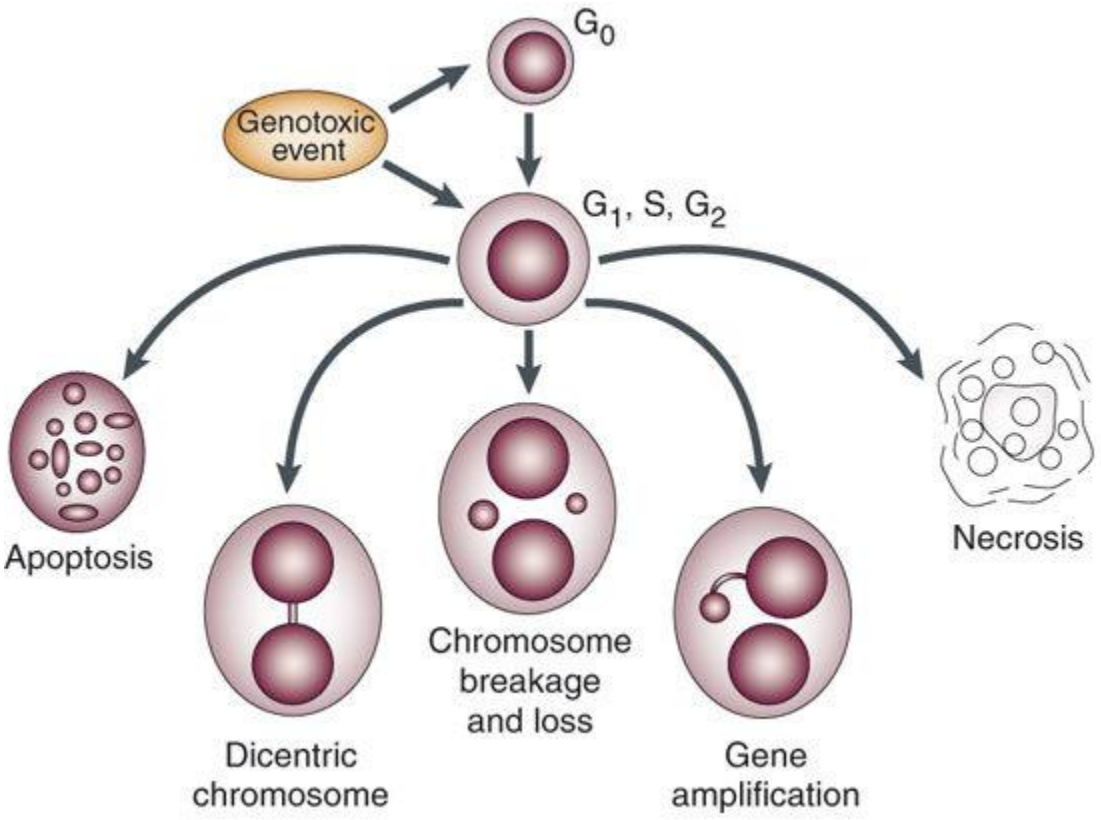

Figure 4. The various possible fates of cultured cytokinesis-blocked cells following exposure to cytotoxic/genotoxic agents. Using these biomarkers within the CBMN assay, it is possible to measure the frequency of chromosome breakage (MN), chromosome loss (MN), chromosome rearrangement, for example, dicentric chromosomes (NPB), gene amplification (NBUDs), necrosis and apoptosis. In addition, cytostatic effects are readily estimated from the ratio of mono-, bi- and multinucleated cells. Adopted from Fenech M et al (95).

This assay was first used in exfoliated cells in 1984 by Stich et al. (133), and continue being one of the most preferred micronucleus tests due to easier accessibility and non-invasiveness. More than 90% of cancers arise in epithelial tissues (134). Exfoliated epithelial cells have traditionally been used for cancer screening by cytopathologists, and these cells can also be used for biomonitoring of the genotoxic effects in humans (135). The epithelial cells are in immediate contact with inhaled and ingested genotoxic agents, and bladder cells are also in contact with metabolites of the chemicals (134), thus, the chromosomal damage caused by carcinogens to dividing basal cells of the epithelium results in the production of micronuclei in the daughter cells,

which migrate up through the epithelium and are exfoliated. Exfoliated cells can be readily obtained from several tissues, including the oral buccal mucosa (scrapings of oral cells), bronchi (sputum), urinary bladder and ureter (centrifugation of urine), cervix (smears) and oesophagus (imprints from biopsies) (133).

The MN index appears to increase in carcinogen-exposed tissues long before any clinical symptoms are evident (136).

Measurement of micronucleus (MN) index in peripheral blood lymphocytes (PBL) is extensively used in molecular epidemiology and cytogenetics to evaluate the presence and the extent of chromosomal damage in human populations exposed to genotoxic agents or bearing a susceptible genetic profile (63). In the last years, the CBMN assay has become a thoroughly validated and standardized technique to evaluate in vivo radiation exposure of occupational, medical and accidentally exposed individuals. There is a number of studies (137, 138) that used this assay as a biological dosimeter for detecting DNA anomalies in cancer patients treated with different doses of radiation. They revealed that after the completion of radiotherapy, the persistence of elevated MN yield in PBLs was a reflection of the surviving population of radiation-induced genetically aberrant cells (137). There are studies indicating the effect of chemotherapeutics in MN formation due to DNA damage. The anticancer mechanism of antineoplastic drugs is the generation of reactive oxygen species (ROS) which are responsible for various side effects in patients undergoing chemotherapy and the healthcare personnel occupationally exposed to them (139-141).

Some meta-analyses and nested studies, show that chronic ingestion of arsenic in drinking water (142, 143), lifestyle habits (tobacco smoking and alcohol consumption, micronutrient deficiencies) (77, 144-148), occupational exposure to formaldehyde, polycyclic aromatic hydrocarbons, petroleum, pesticides, organic solvents, etc. (61, 149-154) are linked to the enhanced incidence of micronuclei in all three types of cells, slightly higher level of micronuclei being observed in lymphocytes compared with oral mucosa and urothelial cells.

It was found that a deficiency of any of the micronutrients: folic acid, Vitamin B12, Vitamin B6, niacin, Vitamin C, Vitamin E, iron, or zinc, mimics radiation in damaging DNA by causing single- and double-strand breaks, oxidative lesions, or both (155). A number of recent investigations show that these damages are reflected as an increased micronucleus index in PBL, especially in the folate

and vitamin B12 deficiencies (156), which causes extensive incorporation of uracil into human DNA, leading to DNA breaks (157).

The presence of an association between MN induction and cancer development is supported as well, by the high index of this biomarker shown in untreated cancer patients and in subjects affected by cancer-prone congenital diseases, e.g. Bloom syndrome or ataxia telangiectasia (158).

The micronucleus test in exfoliated cells is a cost-effective, non-invasive method, through which the formation of MN in exfoliated cells from different organs, such as oral and nasal cavity, bladder, cervix, and oesophagus, is used as an endpoint to detect endogenous, lifestyle, occupational and environmental exposures to genotoxins as well as chemoprotection of various compounds in intervention studies (159). The non-invasive nature of this technique, where cultured cells are not needed for performing the MN assay, makes it an attractive candidate for biomonitoring of human populations or individuals (160).

The analysis of micronuclei has gained increasing popularity as an *in vitro* genotoxicity test and a biomarker assay for human genotoxic exposure and effect. The main reasons for this development are obvious. In comparison with chromosomal aberrations (CA), the scoring of MN is simpler, requires shorter training and is less time-consuming. In principle, the MN assay can be expected to be more sensitive than the CA assay, because of the increased statistical power brought by the fact that the number of cells analysed can easily be increased to thousands when only a hundred or a few hundred cells are usually scored for CA (64).

Recently some investigators have explored the benefits of performing this test in exfoliated urothelial cells in urine samples, as a biological marker of the genotoxic effects of bladder mutagenic/carcinogenic agents (161). The MN in exfoliated bladder cells results from genotoxic damage in basal cells of the urothelium. These cells have a high turnover rate; within 1-3 weeks they exfoliate along with any chromosomal damage to basal cells in their place of origin (162, 163). Because epithelial cells are derived from basal cells, recent genetic damage to the basal layer can be reflected in the presence of micronucleated (MNed) exfoliated cells (164). The mechanisms involved in the induction of MN in urothelial cells can be multiple. Exposure to genotoxic agents can explain part of the observed effects, but in certain cases, the effects occur by indirect mechanisms as occurs with arsenic exposure, which doesn't have a direct effect on the DNA damage (165). Arsenic cytotoxicity action involves the metabolic generation of trivalent arsenicals

and their reaction with sulfhydryl groups within critical proteins in various cell types which leads to the biological response. In epithelial cells, the response is cell death with consequent regenerative proliferation and, ultimately, to an indirect induction of MN formation and cancer (166). Increased MN indices were detected in patients with chronic bladder inflammation as well (167). Additionally, MN formation was detected in the bladder cells of rats exposed to orto-phenylphenol, a fungicide and antibacterial agent well known as a bladder tumour inducer in rats (168).

It has been postulated that exfoliated mucosa cells have a high predictive value for the detection of carcinogenesis since the majority of human tumours are of epithelial origin (169). Cultured cells are not needed to perform an MN assay in exfoliated cells, and its non-invasive quality makes the assay an attractive candidate for biomonitoring human populations or individuals (160).

In the 1980s, several exciting developments with regards to MN assays in humans emerged, which included the development of improved methods for scoring MNi in lymphocytes, buccal cells and erythrocytes. The cytokinesis-block micronucleus (CBMN) technique (as mentioned above), significantly improved the reliability of MN measurements in lymphocytes and led to its exponential adoption by numerous laboratories worldwide and has since been the most widely used method for studying micronuclei in human populations (170). In the cytokinesis-block method, enumeration of MNi is restricted to cells that are blocked from undergoing cytokinesis and which are consequently easily recognizable as large binucleate cells. The biological effects of Cytochalasin B (Cyt-B) were first described for lymphocytes by Carter (1967, 1972) who showed that Cyt-B at low concentrations (1 /~g/ml) inhibited cytoplasmic cleavage, apparently without blocking mitosis (130). But, does the cyt- B itself induces the micronucleus formation? Fenech and Morley (1985) have well investigated the use of Cyt-B as a method for accumulation of binucleated lymphocytes, and they claimed that under the concentration of 3.0 /~g/ml there was no evidence that Cyt-B itself causes an increase in micronucleus frequency (130).

As the time went by, it became increasingly evident that the possibility of measuring DNA damage in human populations worldwide was feasible and that there was, therefore, a need for an internationally coordinated effort to achieve this goal reliably and sustainably. So far, in 1997 The International Human Micronucleus (HUMN) Project was founded, with a mission to coordinate worldwide research efforts aimed at using MN assays to study DNA damage in human populations.

Their goals where to determine the main variables affecting micronucleus frequencies, establishment of the scoring criteria for this assay, set up a unified database with data collected from a big number of laboratories, all around the world, and possibly launch worldwide projects with the focus on different types of cells (like is the HUMNxl Project focused on MN in exfoliated cells) (170). Among the laboratories engaged in these projects was as well the Laboratory for Cell Biology of the Department of Biology, Faculty of Natural Sciences, University of Pristina (171), which has made a number of investigations using MN assay in different types of human and animal cells, contributing to the pooled analyses of the International Collaborative Project on Micronucleus Frequency in Human Populations (HUMN).

Furthermore, micronucleus assay has gained application in biomonitoring of consequences from aquatic pollutants, performed in bivalves and fish (172). Field plants were used as well to be tested for cytogenetic changes for biomonitoring of radiation-induced genotoxicity of contaminants in natural environments (173).

2. HYPOTHESIS

The index of MN in the epithelial exfoliated cells of urothelium in patients with bladder urothelial cell carcinoma increases with the gradus of the cell atypia. The increase of MN index of urothelial cells in patients with bladder urothelial cell carcinoma correlates with MN index in epithelial cells of buccal mucosa and peripheral blood lymphocytes of the same patients.

3. GENERAL AND SPECIFIC AIMS

General aim: The aim of this study was to establish the relationship of the MN indices between the urothelial exfoliated cells, buccal mucosa exfoliated cells and peripheral blood lymphocytes in patients with bladder urothelial cell carcinoma.

Specific aims:

1. The assessment of the MN indices in epithelial exfoliated cells of urothelium and buccal mucosa, as well as in peripheral blood lymphocytes of healthy individuals, patients with low-grade urothelial cell carcinoma and patients with high-grade urothelial cell carcinoma.
2. To analyse whether the MN indices in exfoliated urothelial and buccal cells, as well as in peripheral blood lymphocytes increase with the extent of the cell atypia in bladder cancer.
3. To establish the coexistence of genomic instability in different cell types, located in distance from the primary lesion.

4. PATIENTS AND METHODS

4.1. Patients

The study sample consisted of 60 male participants. Subjects were divided into two groups as follows:

1. Patient group – 40 patients diagnosed with bladder UCC

2. Control group – 20 healthy men

This study was conducted at the Urology Clinic and Institute of Pathology of the University Clinical Centre of Kosovo (UCCK) and Department of Biology, Faculty of Natural Sciences, University of Pristina "Hasan Pristina". The investigated patients were collected prospectively from 2014 to 2017. The age range of the patient group was 37 to 78, while the controls were between 31 and 72 years of age.

To be chosen for this study, each prospective subject was required to conform to the following criteria: non-smoker, non-alcohol consumer, and no history of chemo- or radiotherapy, as confounding factors (138, 145, 141, 174). Patients were first verbally briefed regarding the purposes of the study and were introduced to the sample collection procedure. Afterwards, patients were asked to provide information regarding their relevant personal information (name, age, occupation), family data (diseases with familiar predispositions) and medical data (present diseases, past diseases, habits). Informed written consent for sample collection was also obtained from each participant. The following three tissue samples were taken from all participants prior to the operation and diagnosis: urine, buccal mucosa exfoliated cells and blood.

This research was approved by the Scientific Ethical Committee of UCCK (Nr. 566, date: 7 February 2012). All methods were performed in accordance with the relevant guidelines and regulations.

4.2. Methods

4.2.1. Micronucleus assay in urothelial exfoliated cells

Midstream urine of the second or third void of the day was requested from each participant. The urine was collected in sterile urine containers and was processed within two hours. The volume of the voided urine ranged from 150–200 ml.

Urine samples were transferred to centrifuge tubes and centrifuged at 2000 rpm for 15 min. The supernatant was then discarded without disturbing the pellets of urothelial cells. The pellets were washed twice with 0.9% NaCl and centrifuged. Cell suspensions were spread onto clean, preheated (40 °C) glass slides and allowed to air dry for 5–10 min. The slides were fixed in Carnoy I fixative (methanol: glacial acetic acid, 3:1) at 0 °C for 20 min and air dried. The slides were stained with May-Grunwald's stain (0.25%) for 5 min, counterstained with Giemsa (4%) for 10 min and mounted in DPX. Four slides were prepared for each individual. Following the method of Reali et al. (175) and Fortin et al. (176), at least 1,000 urothelial cells per individual were analysed under a light microscope and confirmed by a second observer (Fig. 5: A, B, C).

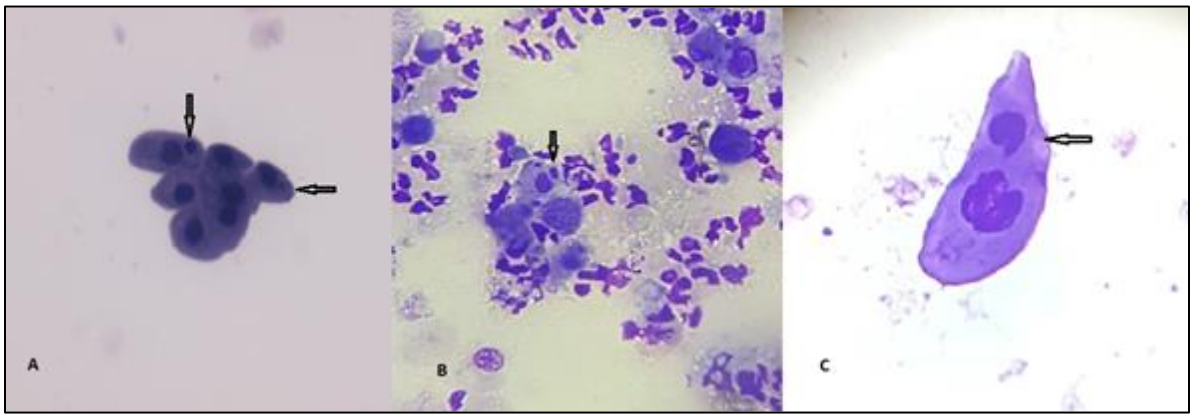

Figure 5. (A, B, C) Micronucleus in urothelial exfoliated cells (arrows). May Grunwald and Giemsa stain (magnification x400).

4.2.2. Micronucleus assay in buccal exfoliated cells

Buccal exfoliated cells were prepared and analysed after the method of Thomas et al. (177) and Tolbert et al. First, subjects removed unwanted debris from their oral cavities using a distilled water rinse. BEC were then obtained by rolling a cytobrush against the buccal mucosa ten times in a circular motion. Next, the heads of the brushes were individually placed into separate 30 ml yellow-top containers, each of which contained a mixture of the following components: buccal cell buffer (0.01 M Tris-HCl; Sigma T-3253), 0.1 M EDTA tetrasodium salt (Sigma E5391), and 0.02 sodium chloride (Sigma S5886) at pH 7.0. The containers were then agitated to disperse cells. Cells were transferred into separate TV-10 centrifuge tubes and centrifuged for 10 min at 1500 rpm (MSE Mistral 2000). Slides containing two spots of cells were air dried for 10 min and then fixed in ethanol: acetic acid (3:1) for 10 min. Slides were then air dried for 10 min prior to staining with Giemsa (5%). At least four slides were prepared per individual, and 2,000 cells were analysed per case. The results are expressed as the number of micronucleated cells per 1,000 counted cells (‰). All the scoring criteria for observing these nuclear anomalies (Fig. 6: A, B, C) were taken from the corrected version of the Protocol of Thomas and Fenech (177).

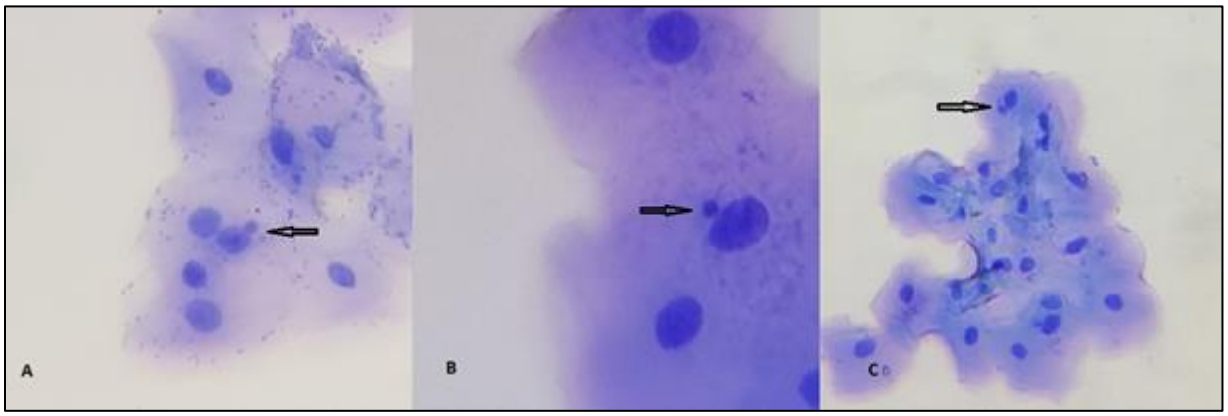

Figure 6. (A, B, C) Micronuclei in buccal exfoliated cells (arrows). Giemsa stain (magnification x400).

4.2.3. Cytokinesis-block-micronucleus cytome assay in peripheral blood lymphocytes

Blood samples (5 ml) were collected via venipuncture into vacutainer blood tubes containing a lithium heparin anticoagulant (BD Plymouth. Pl6 7BP. UK). Whole heparinized blood (0.5 ml) was added to 5 ml of complete PB-Max Karyotyping medium for cell cultivation (Invitrogen, California, USA). All cultures were duplicated and incubated at 37 °C for up to 72 hours. Cytochalasin B (Sigma, St Louis, MO, USA) at a final concentration of 4 μg/mL was added 44 hours after the incubation was initiated. The cells were centrifuged (Sigma, Germany) at 800 rpm for 10 min and treated with a hypotonic solution (0.075 M KCl). The cell suspension was then fixed in Carnoy I fixative (methanol: acetic acid, 3:1) three times and centrifuged (300 g/8 min) after each fixation. Finally, the centrifuged cells were resuspended in a small volume of fixative and spread onto specially prepared, cold, and lamp-dried slides. The slides were stained with Giemsa solution (5%). At least six slides per individual were prepared. Finally, using a light microscope, the MNi (Figure 7: A, B, C), NPBs (Figure 8: A, B) and NBUDs in PBL (Figure 9: A, B, C) were scored independently by two scorers for 1000 binucleated cells (500 per culture) according to the method of Fenech (95).

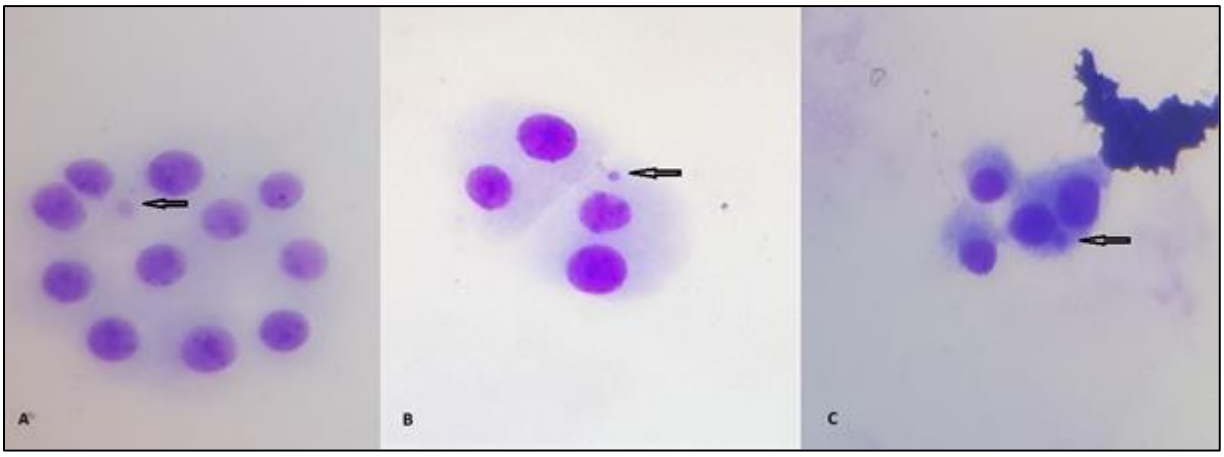

Figure 7. (A, B, C) Micronucleus in binucleated peripheral blood lymphocytes (arrows). Giemsa stain (magnification x400).

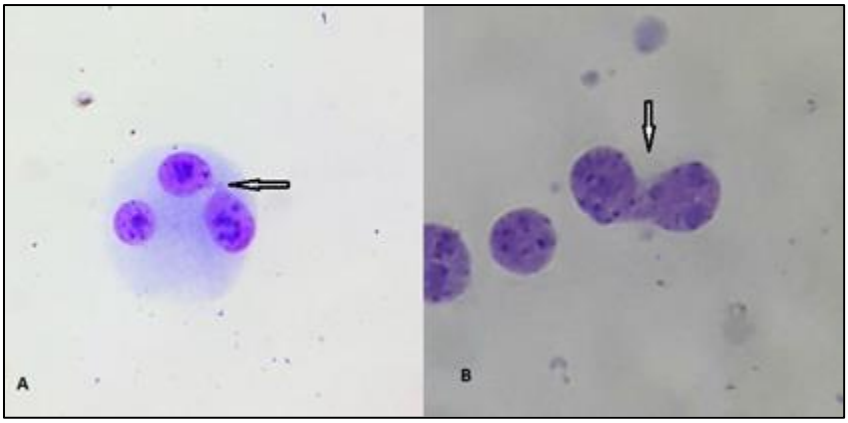

Figure 8. (A, B) Nucleoplasmic bridges in binucleated peripheral blood lymphocytes (arrows). Giemsa stain (magnification x400).

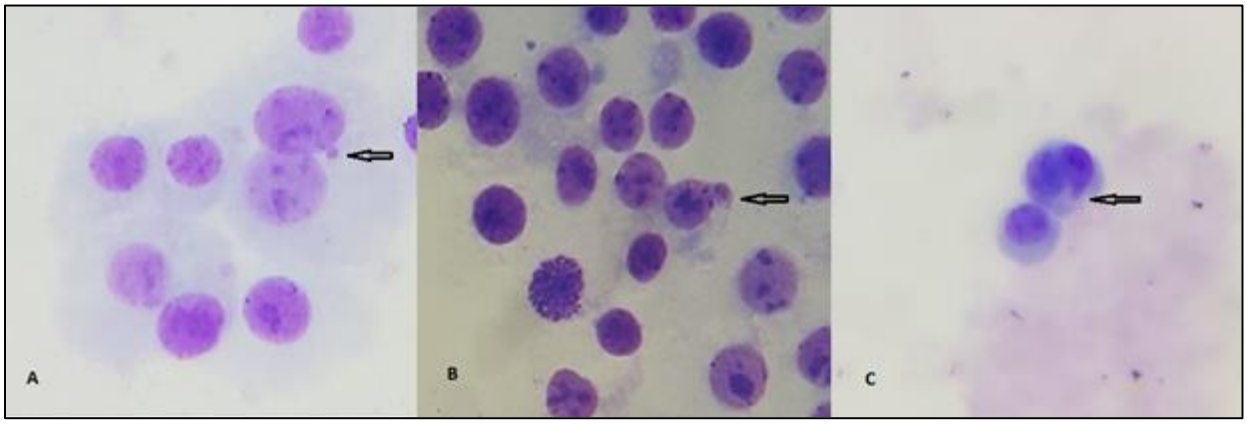

Figure 9. (A, B, C) Nuclear buds in binucleated peripheral lymphocytes (arrows). Giemsa stain (magnification x400).

4.2.4. Scoring criteria for micronuclei, nucleoplasmic bridges and nuclear buds

All the scoring criteria for observing these nuclear anomalies were taken from the corrected version of the Protocol of Michael Fenech, first published in 2007 (132).

Criteria for selecting BN cells suitable for scoring MN, NPBs and NBUDs. The cytokinesis-blocked BN cells that may be scored for MN, NPB and NBUD frequency should have the following characteristics:

- The cells should be binucleated.
- The two nuclei in a binucleated cell should have intact nuclear membranes and be situated within the same cytoplasmic boundary.
- The two nuclei in a binucleated cell should be approximately equal in size, staining pattern and staining intensity.
- The two nuclei within a BN cell may be attached by a nucleoplasmic bridge, which is no wider than 1/4th of the nuclear diameter.
- The two main nuclei in a BN cell may touch but ideally should not overlap each other. A cell with two overlapping nuclei can be scored only if the nuclear boundaries of each nucleus are distinguishable.

The cytoplasmic boundary or membrane of a binucleated cell should be intact and clearly distinguishable from the cytoplasmic boundary of adjacent cells. The cell types that should not be scored for the frequency of MNi, NPBs and NBUDs frequency include mono- and multinucleated (with three or more nuclei) cells and cells that are necrotic or apoptotic.

Criteria for scoring micronuclei. MNi are morphologically identical but smaller than nuclei. They also have the following characteristics:

- The diameter of MNi in human lymphocytes usually varies between 1/16th and 1/3rd of the mean diameter of the main nuclei, which corresponds to 1/256th and 1/9th of the area of one of the main nuclei in a BN cell, respectively.
- MNi are non-refractile and they can, therefore, be readily distinguished from artefact such as staining particles.
- MNi are not linked or connected to the main nuclei.
- MNi may touch but not overlap the main nuclei and the micronuclear boundary should be distinguishable from the nuclear boundary.
- MNi usually have the same staining intensity as the main nuclei but occasionally staining may be more intense.

In Table 1. are listed the mimickers of MN that can be misinterpreted during MN scoring procedure(178).

Table 1**. Differential Diagnosis of Micronucleus**	
Source of doubt	*Differentiating points*
Nuclear debris	• Background material • Smooth • Usually multiple
Bacteria / bacterial colony	• Single or just a clump of multiple round structures • Different texture than that of the nucleus • Present in the background
Candida	• Spores along with hyphae may be present • Located extra-cellular also • Multiple, same size, and monotonous staining
Kerato-hyaline granule	• Numerous • All over the cytoplasm • Polymorphic in size and texture, solid and monotonous
Stain deposit	• Polymorphic granules in the smear, generally over the cells – nucleus or cytoplasm or outside the cell. • Darker colour
Platelet	• Platelets have nebulous margin and woolly texture. • Lighter stain

Criteria for scoring nucleoplasmic bridges. An NPB is a continuous DNA-containing structure linking the nuclei in a binucleated cell. NPBs originate from dicentric chromosomes (resulting from misrepaired DNA breaks or telomere end fusions) in which the centromeres are pulled to opposite poles during anaphase. They have the following characteristics:

- The width of an NPB may vary considerably but usually does not exceed 1/4th of the diameter of the nuclei within the cell.
- NPBs should also have the same staining characteristics as the main nuclei.
- On rare occasions, more than one NPB may be observed within one binucleated cell.

- A binucleated cell with an NPB may contain one or more MNi.
- BN cells with one or more NPBs and no MNi may also be observed.

Criteria for scoring nuclear buds. An NBUD represents the mechanism by which a nucleus eliminates amplified DNA and DNA repair complexes. NBUDs have the following characteristics:

- NBUDs are similar to MNi in appearance with the exception that they are connected with the nucleus via a bridge that can be slightly narrower than the diameter of the bud or by a much thinner bridge depending on the stage of the extrusion process.
- NBUDs, usually, have the same staining intensity as MNi.
- Occasionally, NBUDs may appear to be located within a vacuole adjacent to the nucleus.

A small protrusion of nuclear material from the nucleus without an obvious constriction between the nucleus and the protruding nuclear material should not be classified as a nuclear bud.

4.2.4. Statistical analysis

Since the study variables (the indices of MN in UEC, BEC and PBL as well as NBUD and NPB in PBL) did not follow the normal distribution (tested by Kolmogorov-Smirnov test), non-parametric methods were used. Mann-Whitney U test was applied for comparison of two independent groups. Correlation analysis between study variables was calculated as Spearman's rank correlation coefficients. All the analyses were performed by Statistica (data analysis software system), version 13 (TIBCO Software Inc. (2018). The results were expressed as the mean ± standard deviation (SD). A value of $p < 0.05$ was considered statistically significant.

5. RESULTS

The characteristics of the study population are shown in table 2. The MN indices in UEC, BEC and PBL in the patient group (n=40) were about 17 times higher, 3 times higher, and 4 times higher, respectively, than the MN indices in UEC, BEC, and PBL in the control group (n=20) (Figure 10: A, B, C). Furthermore, the NPB and NBUD indices in PBL in the patient group were 10 times higher and 12 times higher, respectively, than in the control group (Figure 10: D, E).

Table 2. Baseline characteristics of bladder urothelial carcinoma patients and controls

COVARIATES	**CASES (%)**	**CONTROLS**
Number	40	20
NMIUC	16 (40%)	-
MIUC	24 (60%)	-
Age range	37-78yrs	31-72yrs
Smoking status	Non-smokers	Non-smokers
T stage		-
Ta	15 (37%)	-
T1	14 (35%)	-
T2	8 (20%)	-
T3	1 (2.5%)	-
T4	2 (5%)	-
Tis	-	-
Tx	-	-
Grading (WHO 2004) *		-
Low grade	20 (50%)	-
High grade	20 (50%)	-

Recurrence		-
Recurrent	12 (30%)	-
Non-recurrent	28 (70%)	-

Abbreviations: NMIUC – Non-muscle invasive urothelial carcinoma; MIUC- Muscle invasive urothelial carcinoma.

***For reference see: Eble JN et al. Pathology and Genetics of Tumors of the urinary system and male genital organs, (2004).**

Since study variables did not follow a normal distribution (tested by Kolmogorov-Smirnov test), non-parametric tests were employed to verify differences among groups: Man-Whitney U test was applied to see the differences between two independent groups, and Spearman's rank correlation was applied to show the relationship between study variables.

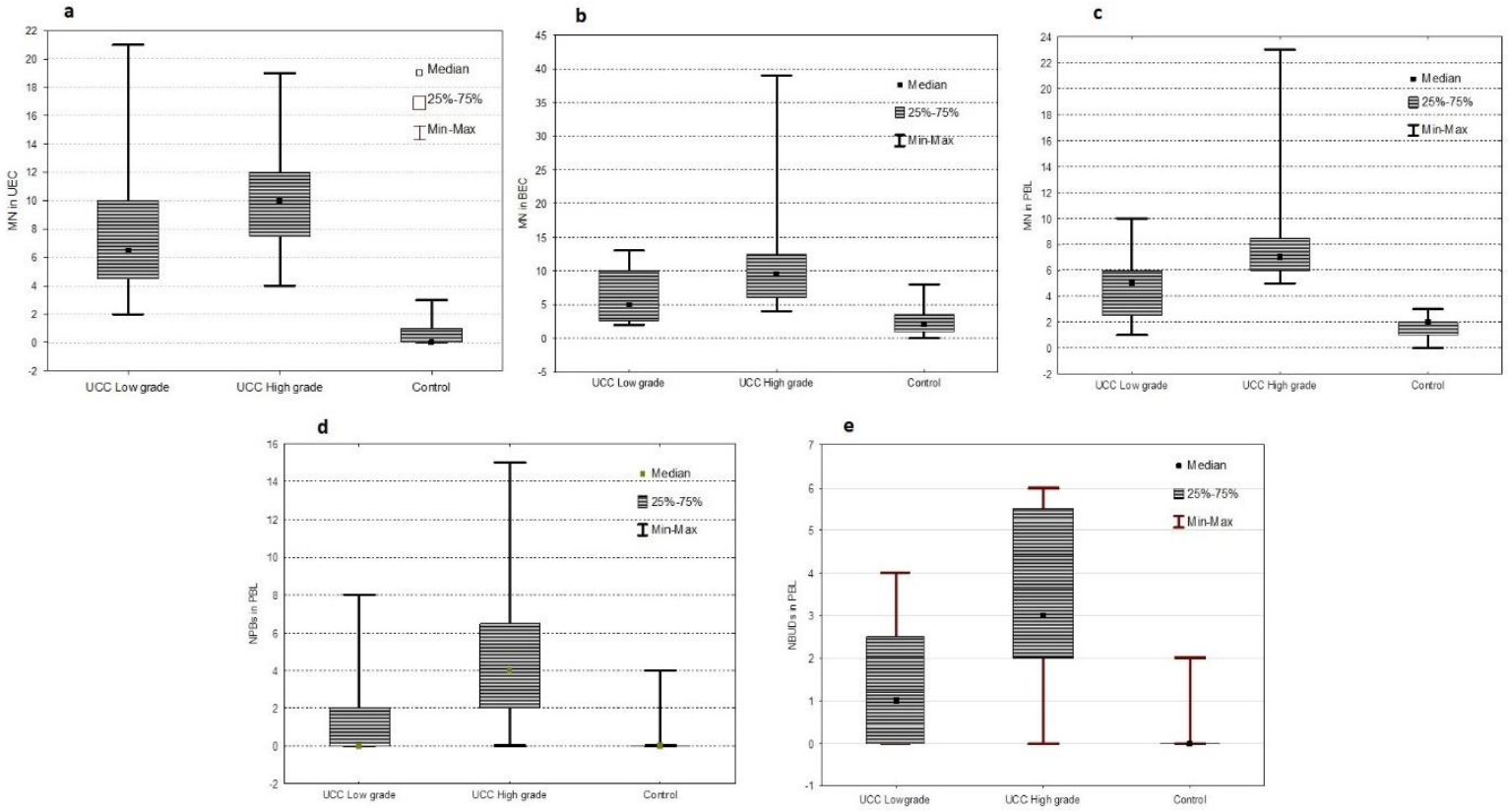

Figure 10. Box plots showing MN indices in: (a) urothelial exfoliated cells (UEC), (b) buccal exfoliated cells (BEC) and (c) peripheral blood lymphocytes (PBL), as well as (d) nucleoplasmic bridges (NPBs) and (e) nuclear buds (NBUDs) indices in PBL in the control group, low grade and high grade patient groups.

According to Man Whitney U test, statistically significant differences were found in all study variables when the patient group (n=40) was compared to the control group (n=20) ($p< 0.001$; Table 3).

Table 3. Mann-Whitney U test (with continuity corrections) between UCC patients (n=40) and controls (n=20)

	Mean rank UCC	**Mean rank control**	**U**	**z**	**p value**	**z adjusted**	**p value**	**Two-sided exact p value**
MN in UEC	40.33	10.85	7.0	6.155	<0.001	6.202	<0.001	<0.001***
MN in BEC	38.04	15.43	98.5	4.720	<0.001	4.739	<0.001	<0.001***
MN in PBL	39.41	12.68	43.5	5.583	<0.001	5.638	<0.001	<0.001***
NPBs in PBL	36.66	18.18	153.5	3.858	<0.001	4.105	<0.001	<0.001***
NBUDs in PBL	37.50	16.50	120.0	4.383	<0.001	4.594	<0.001	<0.001***

***** p<0.001**

UCC- urothelial cell carcinoma; MN-micronucleus; UEC-urothelial exfoliated cells; BEC- buccal exfoliated cells; PBL- peripheral blood lymphocytes; NPBs- nucleoplasmic bridges; NBUDs- nuclear buds.

Spearman's rank correlation showed no significant correlation between study variables in the control group (Table 4). However, in the patient's group (Table 4), Spearman's correlation showed: moderate positive correlation between the MN indices in UEC and BEC (rho=0.427, p=0.006, Figure 11); between the MN indices in BEC and PBL (rho=0.382, p=0.015, Figure 12), and between the MN index in PBL and the NPB index in PBL (rho=0.327, p=0.040, Figure 13). Furthermore, a strong positive correlation was found between the indices of NPBs and NBUDs in PBL (rho=0.701 and p<0.001, Figure 14). There was a positive correlation between the MN and

NBUD indices in PBL, but it did not reach the level of statistical significance (rho=0.271, p=0.091, Figure 15) (Table 4).

As it can be seen in the patient group statistically significant positive correlations were found pairwise between all variables except between MN in PBL with NBUD (rho=0.271, p=0.091, Figure 15) and MN in UEC and MN in PBL (rho=0.197, p=0.224, Figure 16), which might also be statistically significant on a larger sample of patients.

Table 4. Spearman's correlation of the MN indices in UEC, BEC and PBL and the NPB and NBUD indices in PBL in the control group, all cases group, the NMIUC and MIUC subgroups and the low grade and high-grade subgroups

		MN in UEC: MN in BEC	**MN in UEC: MN in PBL**	**MN in BEC: MN in PBL**	**MN in PBL: NPB**	**MN in PBL: NBUD**	**NPB: NBUD**
Control group (n=20)	rho	-0.091	0.082	0.264	0.049	0.049	-0175
	p	0.702	0.732	0.261	0.831	0.837	0.459
Patients (n=40)	rho	0.427**	0.197	0.382*	0.327*	0.271	0.701***
	p	0.006	0.224	0.015	0.040	0.091	<0.001
NMIUC (n=16)	rho	0.421	0.018	0.245	-0.093	-0.194	0.861***
	p	0.104	0.947	0.361	0.731	0.472	<0.001
MIUC (n=24)	rho	0.343	0.141	0.478*	0.552**	0.450*	0.622**
	p	0.100	0.511	0.018	0.005	0.027	0.001
Low grade UCC (n=20)	rho	0.468*	0.277	0.328	0.039	0.055	0.579**
	p	0.038	0.236	0.158	0.871	0.818	0.007
High grade UCC (n=20)	rho	0.213	-0.163	0.339	0.185	-0.180	0.710***
	p	0.367	0.492	0.144	0.435	0.447	<0.001

***statistically significant positive correlation p<0.05**

**** statistically significant positive correlation p<0.01**

***** statistically significant positive correlation p<0.001**

UCC- urothelial cell carcinoma; NMIUC-non-muscle invasive carcinoma; MIUC- muscle invasive carcinoma; MN-micronucleus; UEC-urothelial exfoliated cells; BEC- buccal exfoliated cells; PBL- peripheral blood lymphocytes; NPBs- nucleoplasmic bridges; NBUDs- nuclear buds.

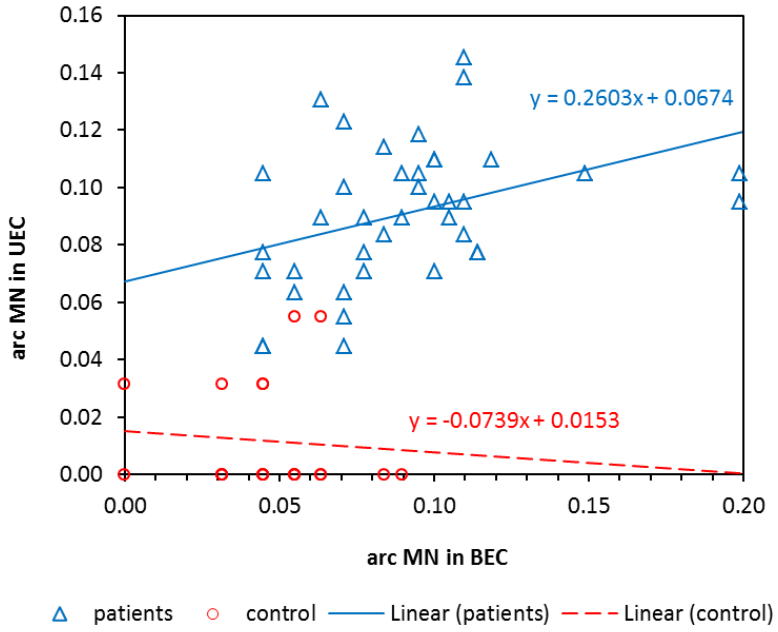

Figure 11. Spearman's rank correlation between MN indices in UEC and BEC in patient group (n=40) and control group (n=20) (arcsin transformed data).

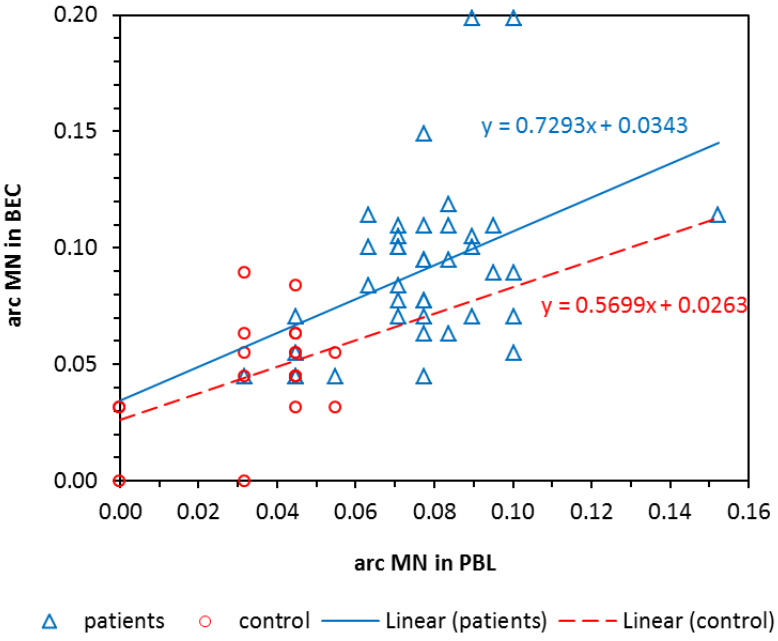

Figure 12. Spearman's rank correlation between MN indices in UEC and BEC in patient group (n=40) and control group (n=20) (arcsin transformed data).

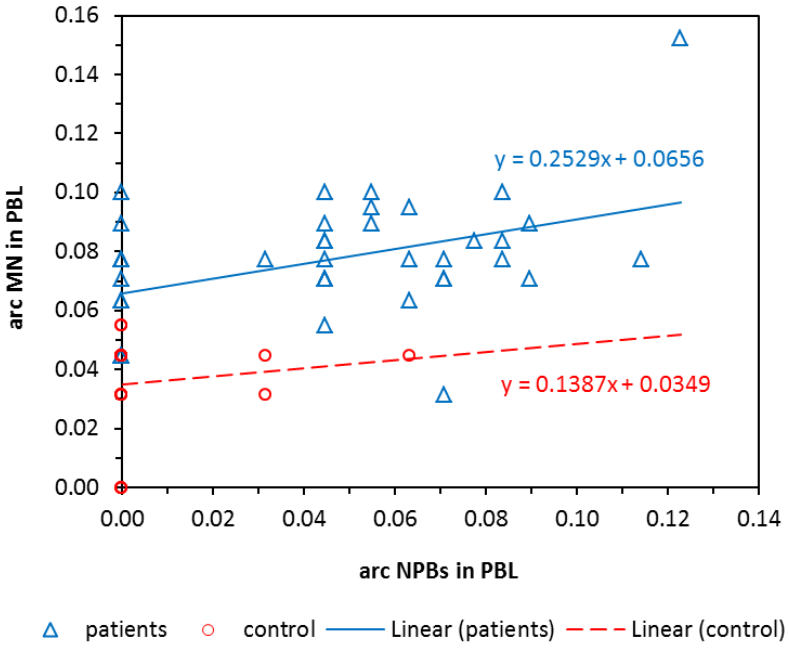

Figure 13. Spearman's rank correlation between MN and NPBs indices in PBL in patient group (n=40) and control group (n=20) (arcsin transformed data).

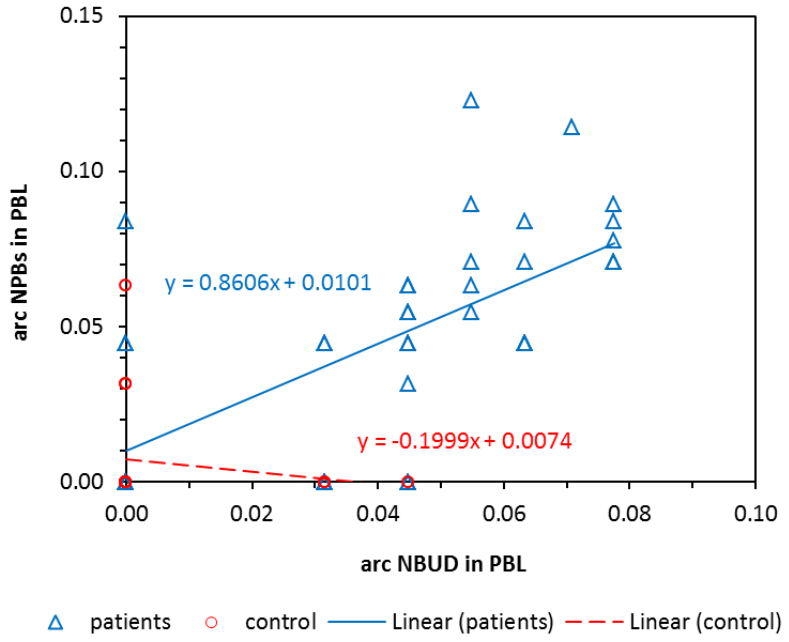

Figure 14. Spearman's rank correlation between NPBs and NBUDs indices in PBL in patient group (n=40) and control group (n=20) (arcsin transformed data).

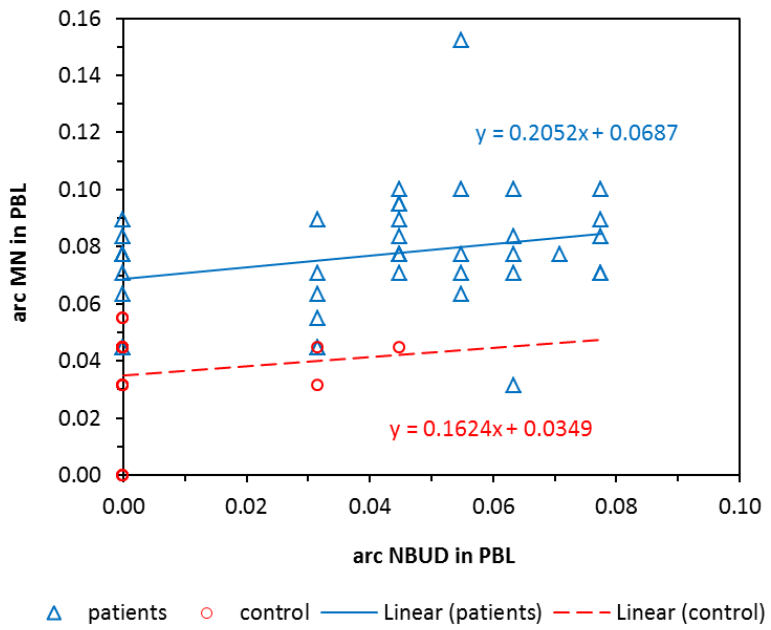

Figure 15. Spearman's rank correlation between MN and NBUDs indices in PBL in patient group (n=40) and control group (n=20) (arcsin transformed data).

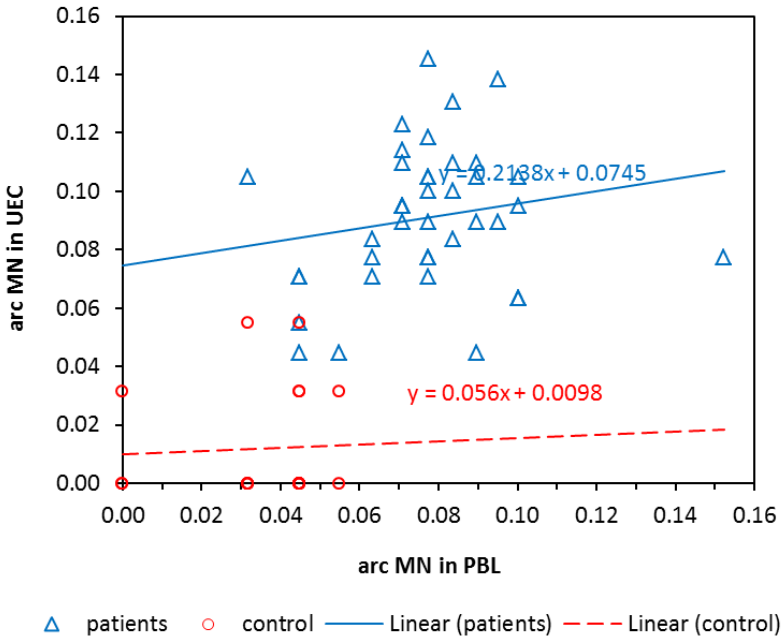

Figure 16. Spearman's rank correlation between MN indices in UEC and PBL in patient group (n=40) and control group (n=20) (arcsin transformed data).

Statistically significant differences were found in all study variables when patients, stratified by tumour grade into the low grade (n=20) and high grade (n=20) subgroups, were compared between one another ($p<0.05$; Table 5).

Table 5. Mann-Whitney U test (with continuity corrections) between UCC low grade (n=20) and UCC high grade (n=20) patients

	Mean rank Low grade	**Mean rank High grade**	**U**	**z**	**p value**	**z adjusted**	**p value**	**Two-sided exact p value**
MN in UEC	16.88	24.13	127.5	-1.948	0.051	-1.954	0.051	0.049*
MN in BEC	15.65	25.35	103.0	-2.610	0.009	-2.620	0.009	0.008**
MN in PBL	13.98	27.03	69.5	-3.517	0.000	-3.554	0.000	<0.001***
NPBs in PBL	14.58	26.43	81.5	-3.192	0.001	-3.258	0.001	0.001**
NBUDs in PBL	15.00	26.00	90.0	-2.962	0.003	-3.010	0.003	0.002**

*** p<0.05; ** p<0.01; *** p<0.001**

UCC- urothelial cell carcinoma; MN-micronucleus; UEC-urothelial exfoliated cells; BEC- buccal exfoliated cells; PBL- peripheral blood lymphocytes; NPBs- nucleoplasmic bridges; NBUDs- nuclear buds.

When patients were stratified by tumour muscle invasion into non-muscle invasive urothelial carcinoma (NMIUC, n=16) and muscle-invasive urothelial carcinoma (MIUC, n=24), and compared statistically significant difference was found only in MN indices in UEC ($p=0.044$ or $p<0.05$), whereas MN indices in BEC, PBL, as well as NPB and NBUD indices in PBL, did not differ between NMIUC and MIUC subgroup ($p>0.05$ in all cases, Table 6).

Table 6. Mann-Whitney U test (with continuity corrections) between subgroups stratified according to tumour muscle invasion: MIUC (n=24) and NMIUC (n=16)

	Mean rank MIUC	Mean rank NMIUC	U	z	p value	z adjusted	p value	Two-sided exact p value
MN in UEC	23.52	15.97	119.5	1.988	0.047	1.994	0.046	0.044*
MN in BEC	22.15	18.03	152.5	1.077	0.282	1.081	0.280	0.279
MN in PBL	22.44	17.59	145.5	1.270	0.204	1.283	0.199	0.202
NPBs in PBL	21.42	19.13	170.0	0.594	0.553	0.606	0.545	0.557
NBUDs in PBL	22.17	18.00	152.0	1.091	0.275	1.108	0.268	0.279

*** p<0.05**

NMIUC-non-muscle invasive carcinoma; MIUC- muscle invasive carcinoma; MN-micronucleus; UEC-urothelial exfoliated cells; BEC- buccal exfoliated cells; PBL- peripheral blood lymphocytes; NPBs-nucleoplasmic bridges; NBUDs- nuclear buds.

Spearman's rank correlation coefficients were calculated for two subgroups formed according to muscle invasiveness i.e. within subgroups MIUC (n=24) and NMIUC (n=16). Within both groups a very strong positive correlations were found between NPBs and NBUDs (rho=0.622, p=0.001 and rho=0.861, p<0.001 for MIUC and NMIUC subgroup, respectively) (Table 4). In the same time not any other pair of variables showed correlation in NMIUC subgroup while statistically significant correlation coefficients were found in MIUC subgroup between MN in BEC and MN in PBL, MN in PBL and NPBs as well as MN in PBL and NBUDs (p<0.05 in all three cases) (Table 4). According to the tumour grade, in the low-grade subgroup a positive correlation was found between the MN index in UEC and the MN index in BEC (rho=0.468, p<0.038), as well as between the NPBs and NBUDs indices within the low grade and high-grade subgroups (rho=0.579, p<0.007; rho=0.710, p<0.000, respectively; Table 4).

Finally, when MN in UEC, BEC and PBL, as well as NPBs and NBUDs, were correlated with the tumour stage and tumour recurrence, we found that among all of these variables, only the MN

index in PBL showed a positive correlation with the tumour stage but it did not reach the level of statistical significance (rho=0.276, p=0.085 or 0.05<p<0.10) (Table 7).

Table 7. Correlation between the index of MN in UEC, BEC, PBL as well as NPBs and NBUDs with tumour recurrence and tumour stage

Frequency	Recurrence		Tumour stage	
	rho	p-value	Rho	p-value
MN in UEC	-0.101	0.536	0.248	0.122
MN in BEC	-0.140	0.389	0.61	0.706
MN in PBL	-0.031	0.848	0.276	0.085
NPBs	-0.265	0.098	0.234	0.147
NBUDs	-0.128	0.432	0.180	0.267

Statistically significant correlation at p<0.05
Rho-Sperman's Rank Correlation
MN-micronucleus; UEC-urothelial exfoliated cells; BEC- buccal exfoliated cells; PBL- peripheral blood lymphocytes; NPBs- nucleoplasmic bridges; NBUDs- nuclear buds

6. DISCUSSION

It is well established that cancer has a genetic basis. Cancer development is a multistep process that involves the progressive accumulation of DNA damage, leading to the activation of oncogenes and to the loss of tumour suppressor functions, which in turn, result in changes in cell function and in the transformation of cells into the malignant cell (179). The search for cytogenetic biomarkers for the identification of groups and individuals who are at high risk of developing cancer is an important initiative in public health. By identifying and validating the markers for cancer risk, the global community can improve disease outcomes and increase the frequency of early diagnoses. In this research project, we used the MN assay in UEC and BEC and the CBMN cytome assay in PBL to evaluate genomic instability in bladder UCC patients. To the best of our knowledge, this is the first study performed on all three cell types of first-diagnosed non-smoker bladder cancer patients.

Two previous studies performed MN assays in three types of cells (UEC, BEC and PBL) to evaluate those cells in individuals exposed to arsenic exposure (143) and cigarette consumption (148), but none in cancer patients. These studies reported a slight increase in the prevalence of micronuclei in lymphocytes compared to exfoliated epithelial cells. However, those results may be confounded by the discrepancy in the turnover rates of these two cell types, with lymphocytes exhibiting a longer lifespan than epithelial cells (180). In our observation, different MN means were found among the three cell types in patients with developed UCC. Specifically, exfoliated cells had a greater prevalence of MN than PBL. Recently, there has been a growing emphasis on the value of utilizing the MN assay in exfoliated cells as a method for the early detection and/or monitoring of cancer patients, as well as the evaluation of different exposures, due to the method's cost-effectiveness and non-invasiveness (170, 181).

Given that epithelial cells are highly proliferative and that they represent the origin of more than 90% of all human cancers (182), it is evident that epithelial cells represent a reasonable alternative to PBL for biomonitoring studies, thus the use of the micronucleus assay has been extended to cells other than lymphocytes, basically exfoliated epithelial cells. Approximately more than 200 studies with epithelial cells have been published in the last 30 years and apart from cells of the oral mucosa, cells from other organs, such as nasal mucosa, cervix, bladder, oesophagus and bronchi, were also used (183). MN frequency in PBL is considered a general biomarker more than a specific one of a

particular type of cancer. Furthermore, while CBMN in PBL requires cell culturing, isolation of UEC can be accomplished via urine sample centrifugation alone. In the approximately thirty years since epithelial cells from human urine were first used for MN analyses, approximately 56 studies have been published (181), most of which were performed to predict the risk of UCC based on the presence of MN-promoting genotoxic metabolites in urothelial cells.

Our results show that the MN index in UEC is significantly higher in patients with UCC than in individuals in the control group as well as between subgroups according to tumour grade and the tumour muscle invasion. Several confounding factors (lifestyle habits (smoking, alcohol, etc.), age, exposure to mutagen agents) may modulate MN formation and frequency (184), but the abovementioned association in our study was independent of these factors, suggesting that endogenous factors are likely to be the most relevant causative factors that contribute to DNA damage in UCC. Endogenous sources of DNA damage, in particular oxidative DNA damage, may contribute to genomic instability and to a mutator phenotype in some tumours (185). This result agrees with a previous study that phenotypically profiled urothelial cells in unexposed patients with a prior history of UCC (186). They claimed to have found genetic unstable cells in the normal appearing urothelium of resected patients. Similarly, the results of a separate previous study indicated that the MN index in urothelial cells from bladder washings of patients with a history of UCC was higher than that of the control group (47). Furthermore, in a retrospective study of urine samples, slides that had been identified as atypical urothelial cells and subsequently diagnosed as UCC positive were found to be positive for MN. By contrast, slides from the control group did not contain MN (187).

There is a lack of literature in performing MN assay in UEC of UCC patients. Only a few studies have been carried out with bladder cancer patients until now, and all of them used low numbers of patients. The most recent one, results to be the largest, using bladder washings from 383, from them 77 patients were negative in their first cystoscopy, and were considered as a reference group; 79 were positive and were classified as patients with tumour; and 227 with previous bladder cancer submitted to follow-up monitoring were negative and classified as BC patients without tumour (165). Their findings are in line with our results, pointing out the presence of genomic instability in patients with UCC, their positive correlation with tumour grade, but not with tumour recurrences.

In contrast, in a recent association study elevated MN frequencies were observed in patients with recurrent neoplasia, compared with patients with no-recurrent neoplasia, supporting MN as appropriated tool to detect individuals with increased risks (47).

Based on our findings and those of aforementioned studies, it is obvious that subjects who are at high risk for UCC, who have UCC, or who have a history of UCC harbour and accumulate genetically unstable cells in the bladder urothelium, which may represent early precursors of new UCC or subclones from previous UCC (47), and that these changes can be readily detected via an MN assay performed on urine.

Use of the MN assay in buccal cells is a well-established and standardized method (177) that has been widely used in biomonitoring processes. In the present study, observation of the MN index in buccal mucosa cells revealed significantly higher levels of genomic instability in patients with UCC than in controls, similar to what was found in PBL and UEC. The MN index in BEC was found to have a statistically significant positive correlation with UEC and PBL in the patient group ($p < 0.006$, $p < 0.015$, respectively; Table 4). Our findings are in line with Feki-Tounzi et al (188), who observed a higher MN index in BEC of patients with UCC than in controls. While there is a lack of literature studying the MN index in BEC among bladder cancer patients, a number of papers have explored the MN index in BEC among patients with head and neck cancer (189), oral cancer (190), breast cancer, and uterine cancer (191), as well as among those with cervical pre-cancer and cancer (192, 193). These studies are consistent with our findings, showing that the MN index in PBL is significantly reflected by the MN index in oral exfoliated epithelial cells, as surrogated cells, even in precancerous lesions.

The CBMN assay is a genotoxicity assay that provides information on a variety of endpoints that reflect chromosomal breakage and rearrangements as well as gene amplification (194). Measurement of the MN index in PBL is extensively used in molecular epidemiology and cytogenetics to evaluate the presence and extent of chromosomal damage in human populations exposed to genotoxic agents (134). Most lymphocytes are short-lived, with an average life span of a week to a few months, but a few live for years, providing a pool of long-lived T and B cells, that recirculate through the secondary lymphoid tissues (195). PBL circulate and accumulate DNA damage over their lifespans due to their close contact with different tissue microenvironments or

tumour-derived substances. Thus, chromosomal aberrations in PBL are considered to be valuable biomarkers of genomic instability and predictors of cancer risk (196).

The association between the MN index in PBL and cancer risk is supported by a number of observations (63, 197). The HUMN International Collaborative Project assembled a large international cohort of subjects, whose lymphocytes had been screened for MN frequency in a period of time from 20 years. They gathered data from about 20 laboratories, and of about 6718 individuals. They found that subjects in medium and high tertile had a higher risk for cancer, relative to the low tertile. This study suggested that increased MN formation is associated with early events in carcinogenesis (63).

In the present study, the MN index in PBL in UCC patients was significantly higher than that in healthy subjects, confirming the results of Pardini et al., who found a higher index of MN in PBL and an increased index of NPBs and NBUDs in patients than in controls. Moreover, they found a statistically significant difference between non-muscle invasive UCC patients with controls but not between controls and muscle invasive UCC patients nor among tumour grades and recurrence (197). In contrast, the present study found statistically significant differences in the MN, NPB and NBUD indices in PBL when comparing the two subgroups (according to tumour grade) between one another, but not when these variables were compared between subgroups according to tumour muscle invasion.

Investigations of genomic instability on primary tumour and/or tumour precursors cells, recently have been a field of interest for researchers, which used MN assay to show different levels of genomic instability in different grades of preneoplastic and neoplastic lesions. Our results show a grade-dependent relationship with the levels of MN formation, which is equivalent with the data in the literature. Similar results were found, for example: in breast cancer cells provided from fine needle aspiration (FNA) the MN index was significantly correlated with tumour grade (198), (199); in cervical dysplasia was revealed a statistically significant difference between different grades of dysplasia (135), as well as between invasive squamous cell carcinoma (SCC) and high grade squamous intraepithelial lesions (HSIL) compared with low grade squamous intraepithelial lesions (LSIL) and control group (200, 201); similar results were shown also in oral squamous cell carcinoma where the level of genomic instability correlated positively with the tumour

histopathological grade (182, 202). These investigations are in line with our finding, pointing out the positive correlation of the MN indices with different tumour grades.

An increased MN frequency may be considered to be a biomarker of chromosome loss and/or breakage, whereas other anomalies, such as NBUDs, are biomarkers of gene amplification and/or the removal of unresolved DNA repair complexes, and NPBs are biomarkers of DNA misrepair and/or telomere end-fusions (203). The increased nuclear anomalies in PBL and BEC indicate that the induction of chromosomal damage employs similar mechanisms in different tissues and that the levels of DNA damage measured in surrogate tissues may reflect those present in cancer-prone tissues (203, 204). It is estimated that each human cell is subjected to 70,000 lesions per day (205) as a result of exogenous and endogenous factors. Because the majority of DNA repair pathways are eliminated, these important pathways can be inactivated due to the action of such factors, leading to genomic instability, which in turn causes an increase in the gene mutation rate at other genomic sites and leads to cellular transformations (206). Furthermore, abnormal active tumour substances leak from cell membranes and may enter blood circulation and interact with other tissues, probably destabilizing their genome.

The increased MN index in the PBL and BEC of UCC cases compared with that of controls could be interpreted as an altered status of the DNA damage repair system or to reflect an unknown past exposure (197). Although our patients were non-smokers and non-alcohol consumers and they denied any professional exposure, we cannot rule out other unknown confounding factors that might have an impact on their genomic instability. There are contradictory endpoints after a number of investigations performed in NATO soldiers who served in the Kosovo war regarding the effect of the weapons of war, especially those with depleted uranium, on cancer risk elevation. A Norwegian investigation showed a 5-fold increase in the incidence of bladder cancer among soldiers who served in Kosovo compared with the general Norwegian population (207, 208). Other studies show an increased number of cancer and MN frequencies in the Bosnian population in regions exposed to depleted uranium during the Bosnian war (209, 210). In Eastern Croatia was reported an 3.5 times increased incidence rate for testicular tumours in Croatians from the pre-war period to the post-war period (211). In Kosovo, there is no investigation that shows the level of contamination with such agents, so this hypothesis remains at the level of speculation and should be investigated in the future.

Chromosomal instability correlates with tumour metastasis, but it remains unclear whether it is merely a bystander or a driver of metastatic progression (212). In our study, the nonsignificant differences in most study variables between subgroups (NMIUC and MIUC) reveal that the invasive feature of the tumour is likely not directly linked with the level of CIN. It is known that higher tumour grades harbour higher levels of CIN, but it is also known that low-grade and high-grade UCC can both be invasive. Therefore, there may be other mechanisms that generate invasive clones within UCC (213, 214) in a persistent established state of CIN.

Our data indicate that in UCC, a systemic genomic destabilization occurs and that it can be easily detected using the MN assay in UEC, BEC and PBL, which means that these cells reflect the CIN in the UCC state. Bladder cancer continues to be challenging to detect and treat. Early diagnosis, prior to muscle invasion, can greatly impact the clinical outcome, whether measured by disease-specific survival, morbidity, or quality-of-life variables (215). While cystoscopy and cytology are established standards, the UroVysion™ FISH test is a new but expensive and technically demanding approach that focuses on chromosomal aneuploidy using fluorescent in situ hybridization (FISH) (216) and is mainly used as a surveillance test for NMIUC patients. It is well recognized that high pre-diagnostic levels of MN are associated with an elevated risk for cancer development. It is also known that tetrasomy of chromosomes 3, 7 and 17 and loss of 9p21 can be detected in urine samples 1–3 years before diagnosis (217) . Although more extensive studies are needed, we recommend the following practical approach for cancer screening: combined testing for bladder cancer, in which the MN assay in UEC might serve as a triage-test for the identification of people who are at high risk for bladder cancer development so that they can be referred for UroVysion™ FISH testing.

It is known that cost concerns are a strong determinant of testing, so it can contribute to low testing in at risk for cancer population. Hence, identification of additional tests that would enhance the detection rate and will lower the cost, can be of high importance for the health system's cancer screening programs. The identification of biomarkers of bladder cancer susceptibility in easily accessible tissues, like UEC, may allow targeting of screening efforts in the way of improving the bladder cancer prognosis. In this regard, standardized MN assay in UEC could be considered as a promising biomarker of bladder cancer susceptibility. Its cost-effectiveness might expand access to screening for bladder cancer early detection, especially in low- and middle-income countries,

what might be probably reflected in the patient treatment, in setting of better clinical outcomes due to early detection, and lower costs in cancer treatment. This method could reduce the number of unnecessary tests with UroVysion™ FISH.

Strengths and limitations. This study used a very homogenous sample. The inclusion criteria for eligibility, especially the non-smoking status, made the sample collection phase to be a long and challenging process, that resulted in a relatively small sample size. The lack of the literature for MN test done in bladder cancer patients was another problem in the process of writing. Despite this, the study is valuable as an explorative one, significantly pointing to the presence of a systemic genomic instability due to bladder cancer disease. Although, further studies are needed to set up or to standardize MN frequencies in UEC, BEC, PBL as well as NPBs and NBUDs in PBL, to be routinely included as predictive diagnostic criteria for bladder cancer early diagnosis, and cancer surveillance.

7. CONCLUSIONS

Based on the study outcomes, regarding the micronucleus indices in urothelial exfoliated cells (UEC), buccal exfoliated cells (BEC) and peripheral blood lymphocytes (PBL), as well as nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs) in peripheral blood lymphocytes between urothelial cell carcinoma (UCC) patient groups (stratified according to tumour grade and tumour muscle invasion), it can be concluded that:

1. The increased genomic instability in patients with urothelial cell carcinoma is manifested with higher MN indices in UEC, BEC and PBL, as well as NPB and NBUD indices in PBL.
2. MN index in UEC is significantly higher in high grade UCC and muscle invasive urothelial carcinoma (MIUC) compared to opposite subgroups.
3. Our results show a grade-dependent relationship with the study variables: all of them showed statistically significant differences between low grade UCC and high grade UCC subgroups ($p<0.05$ in all cases)
4. The study variables did not show any statistically significant difference between subgroups according to tumour muscle invasion, except for MN in UEC ($p<0.04$).
5. Statistically positive correlation is found between MN indices in UEC and BEC and between BEC and PBL in patient group.
6. MN indices in UEC and PBL are significantly higher in the patient group than in the control group, but they did not show any positive correlation between them.
7. In PBL positive correlations are found between indices of MN and NPBs and between indices of NPBs and NBUDs.

In conclusion, our data show that genomic instability can be detected through the MN assay in target cells (UEC) as well as in surrogate cells (BEC and PBL). The extent of DNA damage varies among the three cell types, but their simultaneous appearance suggests that similar mechanisms can cause a systemic genomic instability in different types of cells during carcinogenesis. The positive correlation between MN indices in PBL and BEC shows that the genomic instability due to bladder cancer disease is a generalized state, and it can equally be detected in both surrogated

cells. The statistically nonsignificant correlation between MN in UEC and MN in PBL can be attributed to the relatively small sample size.

Although further studies are needed for standardization, our findings support the data in the field that indicate that the MN index in exfoliated cells (especially in UEC) may be a potential biomarker for the early detection of bladder cancer and for patient surveillance. The MN index in exfoliated cells might also be able to be used as an additional triage test prior to the UroVysion™ FISH test.

8. ABSTRACT IN ENGLISH

Microncronucleus index in epithelial exfoliated cells of urothelium and buccal mucosa, and peripheral blood lymphocytes of patients with papillary urothelial carcinoma, Arjeta Podrimaj-Bytyqi, 2019

Introduction: Bladder urothelial cell carcinoma (UCC) is an increasingly prevalent cancer worldwide, and thus, gaining a better understanding of its identifiable risk factors is a global priority.

Aim: This study addressed this public health need with the understanding that cancer-initiating events, such as chromosome breakage, loss and rearrangement, can be reasonably used as biomarkers to evaluate an individual's cancer risk.

Methods: Overall, forty bladder cancer patients and twenty controls were evaluated for genomic instability. To the best of the investigators' knowledge, this is the first study to perform micronucleus (MN) assays simultaneously in urothelial exfoliated cells (UEC), buccal exfoliated cells (BEC), and peripheral blood lymphocytes (PBL) in first-diagnosed, non-smoker bladder UCC patients. Additionally, the frequency of nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs) in PBL was evaluated.

Results: The MN indices in UEC, BEC, and PBL, as well as the indices of NPBs and NBUDs, were significantly higher in patients than in controls. Different levels of genomic instability were found also in relation to tumour grade and tumour muscle invasion.

Conclusions: MN assays, particularly in UEC, may be used to identify individuals who are at high risk of developing UCC, as single or as additional triage test to UroVysion FISH test. Our results further validate the efficacy of such biomarkers as predictors of genomic instability.

Keywords: micronucleus assay, urothelial cell carcinoma, buccal exfoliated cells, urothelial exfoliated cells, peripheral blood lymphocytes.

9. SAŽETAK (ABSTRACT IN CROATIAN)

Uvod: Karcinom urotela mokraćnog mjehura sve je učestaliji oblik karcinoma u svijetu te je stoga bolje razumijevanje njegovih prepoznatljivih čimbenika rizika globalni prioritet.

Cilj rada: Ovo istraživanje se bavi tom javnozdravstvenom potrebom da se događaji koji iniciraju rak, kao što je lom, gubitak i preraspodjela kromosoma, racionalno iskoriste kao biomarkeri za procjenu rizika od karcinoma urotela.

Ispitanici i metode: Četrdeset bolesnika s *de novo* karcinomom urotela mokraćnog mjehura i dvadeset kontrolnih ispitanika analizirano je na genomsku nestabilnost. Prema dostupnim saznanjima istraživača, ovo je prva studija takve vrste koja je provela istodobno ispitivanje mikronukleusa (MN) na izljuštenim urotelnim stanicama, izljuštenim epitelnim stanicama sluznice usne šupljine i limfocitima periferne krvi bolesnika s *de novo* dijagnosticiranim karcinomom urotela mokraćnog mjehura. Osim toga, procijenjena je i učestalost nukleoplazmatskih mostova (NPB) i nuklearnih pupoljaka (NBUD) u limfocitima periferne krvi bolesnika i kontrolnih ispitanika.

Rezultati: Učestalost MN u izljuštenim urotelnim stanicama, izljuštenim epitelnim stanicama sluznice usne šupljine i limfocitima periferne krvi, kao i učestalost NPB i NBUD u limfocitima periferne krvi bila je značajno viša u bolesnika s karcinomom urotela nego li u kontrolnih ispitanika. Dodatno je utvrđen i stupanj genomske nestabilnosti u odnosu na stupanj tumora i invaziju tumorskih mišića: MN indeks bio je značajno viši u karcinomu visokoga stupnju i u invazivnom karcinomu nego u neinvazivnim podskupinama.

Zaključak: Testiranje mikronukleusa, a posebice na izljuštenim urotelnim stanicama, može se koristiti za otkrivanje pojedinaca koji se nalaze pod visokim rizikom za razvoj karcinoma urotela, bilo kao pojedinačni test ili kao dodatni trijažni test za UroVysion FISH test. Dobiveni rezultati dodatno potvrđuju učinkovitost analiziranih biomarkera kao prediktora genomske nestabilnosti.

Ključne riječi: mikronukleusni test, karcinom urotela, izljuštene epitelne stanice sluznice usne šupljine, izljuštene urotelne stanice, limfociti periferne krvi.

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11. CURRICULUM VITAE

Arjeta Podrimaj-Bytyqi was born in 1980 in Istog, Kosovo, where she finished both Primary and High School (Gymnasium). Moved to Pristina, in 1999 to pursue her education in the Faculty of Medicine, University of Pristina, and graduated in 2006. Since 2007, she has been working as Teaching Assistant of Pathology at the Faculty of Medicine. In the academic year 2010/2011, she enrolled in the PhD Program on "Biomedicine and Health Sciences" at the School of Medicine, University of Zagreb. She finished the residency of Pathology in 2013, and since 2015 she has been working as a pathologist in the Institute of Pathology, University Clinical Centre of Kosovo.

Besides her education, she has attended and actively participated in a number of professional congresses, seminars, courses, in Kosovo, Albania, Croatia, Serbia and Austria. She is author of four scientific papers published in peer-reviewed journals and many abstracts presented in pathology congresses.

She is married, and mother of two children.