UNIVERSITY OF MEDICINE AND PHARMACY OF CRAIOVA

DOCTORAL SCHOOL

PhD THESIS

Clinical, histopathological and immunohistochemical study of bladder tumors

- ABSTRACT -

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Introduction

Bladder cancer is quite a frequent disease and it is associated with high morbidity and mortality. Clinical data in the last years showed that this form of cancer, worldwidely, is the 7th most frequent cancer in and the 17th most frequent cancer in wo (Klaile Y, Schlack K, Boegemann M, et al, 2016). The most frequent form of neoplasia of the bladder is urothelial carcinoma, previously called transition cell carcinoma, a tumor that develops within the urothelium.

The incidence of bladder carcinoma in the EU countries is approx. 100,000 new cases per year, while the mortality caused by this disease may reach over 30,000 patients every year (Siegel RL, Miller KD, Jemal A, 2015). Approximately 70%-80% of urothelial carcinomas of the bladder are represented by non-muscular invasive carcinomas (NMIBC), comprising stages of primary Ta, T1 and Tis (Babjuk M, Burger M, Zigeuner R, et al, 2013). The rest of 20% of bladder cancers are invasive carcinomas in the muscular carcinoma and are extremely metastasizing (Li Y, Lin K, Yang Z, et al, 2017).

Despite its high incidence, the molecular mechanism involved in the onset of bladder carcino,a and its progression has not been clarified so far. Histopathological and genetic data show that the tumors come from a single cell, but, once the tumors grow, they become quite heterogenous, due to genetic and phenotype changes that occur during tumorigenesis.

CHAPTER I. Anatomy and histophysiology of the urinary tract

The urinary bladder is a muscle-membrane reservoir where the urine (continuously secreted in the kidney and brought through the ureters) is accumulated in the interval between mictiuritions, from here being excreted through the urethra to the outside. In the adult, the bladder is a pelvic organ; when it is full, it exceeds the pubic symphysis through its upper part and comes into contact with the abdominal wall. The space occupied by the bladder in the pelvic excavation bears the name of “bladder bed”, limited by the pubic symphysis (front), prostate-peritoenal aponevrosis (back), peritoneum (cranial), prostate (caudal) and by the anal lifting muscles (lateral).

The average capacity of the bladder (to which the micturition is triggered) is 200-250 ml; the maximal physiological capacity is 300-350 ml, and in pathological states it may reach 5 liters.

The means of bladder fixation are multiple ones, but the most important is the perineum. In , the bladder bottom closely adheres to the prostate and by its means it strongly supports on the pelvic diaphragm; in wo, the bladder bottom comes into direct contact with the urogenital diaphragm.

The structure of the bladder presents three layers: the fibroserous external layer, the middle layer and the mucous internal layer. The thickness of the wall is about 4-7 mm in an empty bladder and 2 mm in the full bladder. The external layer (serous tunica) – is formed of the peritoneum. This covers only the posterior side and part of the lateral sides of the bladder, thus reflecting on the rectum or the uterus. The rest of the bladder

wall is limited by the adventix, a conjunctive structure that connects the bladder to the pelvic organs and the skeleton of the small basin. The middle tunica, represented by the muscular stratum, is made of smooth muscular fibers, to which there adds a rich network of elastic fibers (with a role id the distension of the bladder walls). Also at this level, we find the detrusor muscle (made of three muscular layers: external - with longitudinal fibers, middle -- with circular fibers and internal -- with fibers with a plexiform arranget). The contraction of this muscle helps emptying the bladder. The internal or mucous layer covers the entire internal surface of the bladder. It continues with the ureters and urethra mucosa. When the bladder is empty, the mucosa presents numerous uneven folds arranged irregularly that disappear when the bladder is full.

CHAPTER II. Carcinogenesis of bladder tumors

The tumors derived from the epithelium of the urinary ways are described under various names: ”urothelial”, ”excretourinary”, ”paramalpighian” tumors, ”transitional cell tumors”. The term of “transitional cell tumor” is adopted and used in the Anglo-Saxon countries; the one of “urothelial tumor” was recomded by the Consensual Conference on Urothelial Neoplasms organized by WHO and ISUP (*International Society of Urologic Pathology*), in 1998 (Reuter VE*,* 2004) and accepted by WHO in 2004.

Over the past 20 years, the urothelial tumors of the bladder are divided into ”superficial tumors” and “invasive tumors”. These two terms were intriduced by the urologists for distinguishing between tumors limited to mucosa, which may be treated endoscopicallyt, and tumors invading the self-musculature (Rosai J, 2004).

From the histopathological point of view, superficial tumors include three distinct tumor categories, though its morphology, progression potential amd tumorigenesis:

- Non-invasive papillary urothelial tumors (tumors **pTa**);
- Urothelial carcinoma *in situ* (tumors **pTis**);
- Tumors that invade the mucous chorion, still with respecting the self-musculature (tumors **pT1**).

These three tumoral varieties rise a particular interest both for the pathologist and for the urologist, as from the anatomopathological point of view, it is difficult to appreciate with certainty the stage pT; tumors pTa continue to raise problems of classification, the frontier between benign tumors and the malignant ones being unclear yet; tumors pT1 are tumors with risk of progression, whose evolution is quite difficlut to predict.

At this mot, researchers and clinicians recognize two distinct molecular ways involved in the tumorigenesis and progression of bladder urothelial carcinomas: one of them leads to the genesis of superficial urothelial tumors that have the tendency to frequent local recurrences, still they present a low risk of tumor progression, and the other way generates more aggressive, invasive tumors.

Genetically established urothelial tumors, represented by the non-invasive papillary urothelial tumors, have a low level of cytoarchitectural atypias, frequent recurrences and low risk of tumoral progression (tumors G1/G2, pTa). They present only a few genetic changes, deletions of chromosome 9 (often affecting the whole chromosome) and mutations of gene FGFR3; in these tumors, mutations and amplifications of gene TP53

are rare, while tumor aneuploidy appears in less than 50% of the cases (Hartmann A, 1999).

Invasive urothelial carcinomas (pT1-4) originate either in a non-invasive papillary urothelial carcinoma with a high degree of malignity (G3, pTa), or in an ”in situ” urothelial carcinoma (CIS/pTis). Genetically, these three morphological entities are quite different from the non-invasive papillary urothelial tumors with a low degree of malignity. According to the available genetic information, there should be considered a new subdivision of bladder urothelial carcinomas, being recomded to avoid, as much as possible, the inclusion of urothelial tumors pTa and pT1 in the group of “superficial urothelial tumors”.

The molecular changes that appear in bladder urothelial carcinomas may be classified in three main categories: chromosomal changes with a role in carcinogenesis; loss of cellular cycle regulation, responsible for cellular proliferation; metastasizing, induced by events such as tumor angiogenesis (Williams SG, 2004).

CHAPTER III. Treatt of bladder tumors

The election method in the treatt of superficial bladder tumor is transurethral endoscopic resection (TURV). Endoscopic resection represents the primary treatt of superficial bladder tumors, its characteristic of radicality thus allowing, besides the removal of the entire tumor that is visible macroscopically, the obtaining of samples for the histopathological examination, control of resection by harvesting a biopsy sample from the resection bed, harvesting randomized biopsy samples for establishing the state of the urothelium. Harvesting biopsies of bladder urothelial biopsies, apparently normal, is quite a disputed attitude nowadays between the supporters of the idea that in this way there could be possible the discovery of Tis simultaneity, much more aggressive than the exophytic primary tumor, and those claiming the risk for an iatrogenic implantation of tumoral cells, exfoliated during cystoscopy and resection, in the areas of mucosa cropped out by these biopsies.

Total cystectomy is rarely required for the patients with superficial tumors; exception is made only by non-resectable papillary tumors and/ or ”in situ” carcinomas *that do not respond to* intravesical treatt. For these selected cases, the rate of survival is good.

The indications for cystectomy in patients with bladder tumors Ta, T1, Tis (Soloway MS, 1996) are: young age patient, with multi focal tumor, grading 3; simultaneous Tis, located in a difficult to resect position (anterior, posterior bladder wall) with T1 at least at re-TURV; recurrence under BCG treatt.

Some authors state that the patients in stages Ta and T1 treated by cystectomy had a survival rate comparable to the one of same aged persons in the healthy population.

In the patients with high degree bladder tumors, with a low response to conservatory treatt and in whom there was performed an immediate cystectomy, the survival rate after 5 years was almost 80%, unlike other patients, in whom cystectomy was delayed and at the mot of taking the decision for cystectomy, they already presented muscular infiltration or even more, thus a reserved prognosis (Freeman şi colab., 1995).

The adjuvant treatt of superficial bladder tumors is varied and includes: instilational chemotherapy, instilational immunotherapy, chemo-immunotherapy.

CHAPTER IV. Clinical and statistical study

The main objectives of the study were to determine the number of patients diagnosed with bladder tumors, admitted between 2013 and 2015 in the Clinic of Urology of the County Emergency Hospital of Craiova, to find out the number and type of procedures they were subjected during hospitalization, as well as the stage of the condition when the diagnosis was made, reasons for admission and the used paraclinical investigations in diagnosing the disease. In the study, there were included 1073 patients with bladder tumors. The data regarding age, sex and living environt of the patients, admission reasons, pathological medical history, present risk factors, objective examination, paraclinical examinations, stage of condition, type of intervention, were obtained from the clinical observation sheets.

During the three years of study, the number of patients admitted with bladder tumors varied quite a little, from 323 (in 2015) to 369 (in 2014).

The distribution of patients in our group depending on sex, during the studied years, was the following:

- in 2013, out of a total number of 369 patients, 234 were males and 135 females, the male/ female ratio being 1.73/1.
- in 2014, out of 381 cases, 269 were males and 112 females, the male/ female ratio being 2.4/1;
- in 2015, out of 323 patients, 238 were males and 85 females, male/ female ratio being 2.8/1;

Out of a total number of 1073 patients with bladder tumors, 741 (69.06%) were and 332 (30.94%) were women, male/ female ration being 2.23/1.

Regarding the distribution of patients according to social environment, out of 1073 cases, 709 (66%) came from the rural area and 364 (34%) from the urban area.

The evaluation of the patient distribution of our group according to age allowed us to observe that bladder tumors were diagnosed in adult patients aged between 40 to 99 years old; most patients with bladder tumors were recorded in the age decades between 60-69 years old (362 patients) (33.74%) and between 70-79 years old (372 patients) (34.67%), respectively.

According to the macroscopic aspect of the tumor and the morphological and progressive features, in our group we identified:

- 528 cases of vegetation tumors, of which: 163 were primary tumors, 101 relapse tumors, 141 unique vegetation tumors and 123 multiple vegetation tumors;
- 846 superficial tumors, of which: 246 were primary tumors, 171 relapse tumors, 204 cases of superficial unique tumors, and 219 cases of multiple superficial tumors;
- 116 sessile-pediculated tumors, of which: 35 were primary tumors, 23 relapse tumors, 39 unique tumors and 19 multiple tumors;
- 88 sessile tumos, of which: 26 cases of primary tumors, 18 relapse tumors, 32 unique tumors and only 12 multiple sessile tumors;

- 216 were pediculated tumors, of which: 46 were primary tumors, 62 were relapse tumors, 63 were unique pediculated tumors and 45 multiple tumors;

- 352 were infltrating bladder tumors, of which: 92 were primary tumors, 84 relapse tumors, 99 infiltrating unique tumors and 77 multiple tumors.

The localization of the tumors was quite varied: 51 patients presented bladder tumors in the trigon; 152 in the posterior wall, 156 cases presented tumors in the anterior wall, 308 tumors were found in the right lateral wall and 406 on the left lateral wall.

Regarding the pathology associated with bladder tumors, in our study we observed that the highest number of patients (657) presented associated urinary infection, 609 patients presented haematuria, 273 patients presented prostate adenoma, 199 ureterohydronephrosis, 209 cases HBP, while 476 patients presented ischemic cardiopathy, diabetes mellitus, vertebrobasilar failure.

CHAPTER V. Histopathological aspects of bladder tumors

The biological material subject to a histopathological study was represented by 32 tumor fragments of the bladder, harvested during a transurethral endoscopic resection of tumors present in the bladder, coming from a number of 32 patients diagnosed clinically, paraclinically and cytoscopically with bladder tumors.

The histopathological study of urothelial carcinomas highlighted a multitude of microscopic aspects, showing that bladder tumors present a marked structural polymorphism. Tumoral heterogeneity may be explained through a monoclonal theory, namely through the possibility of the occurrence of multiple tumors from one single cell transformed malignantly, which has the capacity to generate different clones of tumoral cells or, by the malignant field theory showing that the exposure of the urothelium to risk factors for bladder cancer, such as smoking, work carcinogens, etc, may cause different cellular changes in the cells of the covering epithelium, in various places, leading to a simultaneous occurrence of more neoplastic cells.

The microscopic aspects of the urothelial papillary carcinoma were different from one patient to another, thus showing that the etiopathogenic factors involved in urothelial carcinogenesis may be different, or that the degree of their involvement in the carcinogenesis process may be different.

The urothelial carcinoma may present two clinical and histopathological forms that have different onset physiopathological mechanisms: the papillary or non-invasive form and the muscular-invasive form. In our study, most types of urothelial carcinomas were papillary, characterized by papillary growths around some conjunctive-vascular stromal axes. The tumoral stroma, made of the extracellular matrix, conjunctive cells, blood and lymph vessels, plays an essential part in the growth of tumoral cells. In our study, there were identified more types of stromal cells in the tumoral micro environment: fibroblasts, endothelial, pericyte cells, cells of the immune system, tumor associated macrophages, etc. We consider that all these cells from the stromal micro environment cooperate with each other for the proliferation and metastasizing of tumors, due to the fact that we observed that the number and morphology of stromal cells differ according to the degrees of differentiation and tumor invasiveness.

In our study, we observed that some invasive urothelial carcinomas, found in the lamina propria or even in the muscular tunica of the urinary bladder, were associated with necrosis focci, more or less extended that affected all the structural elements of the conjunctive stroma.

The invasive urothelial carcinoma had a cellular stroma with numerous fibroblasts and a variable collagenization or a hypocellular stroma with a myxoid background. Quite rarely, the tumor induces an exuberant proliferation of the fibroblasts, which present an alarming cellular atypia. This characteristic, although an useful clue for invasion, should not be confused with the cellular component of the sarcomyoid urothelial cancer. In our study, in about 25% of all the examined cases of invasive urothelial carcinomas in the muscular tunica, there were highlighted lymphovascular invasions, which shows a possibility of metastases in other organs.

CHPATER VI. Immunohistochemical study of bladder tumors

The biological material selected for the immunohistochemical study was the same used in the histopathological study, namely the 32 fragments of bladder tumor harvested during transurethral endoscopic resections, practiced in the case of some tumors present in the bladder.

Starting from the observations that the immunohistochemical and molecular markers used as adjuvants of the classical histopathological method for reaching a diagnosis, more precisely of bladder lesions, and that these provide more data for the immunohistochemical characterization of urothelial carcinomas, we used the antibodies anti-cytokeratin 20 (anti-CK20), anti-protein p53 (anti-p53) and anti-Ki67, anti-CD34 and anti-VEGF.

The aberrant expression of CK20 in the urothelial cells, plus the overexpression of p53 and Ki-67, are indicators of the dysplastic change occurring in the epithelial cells from the urothelial mucosa. In our study, all the urothelial carcinomas presented a positive immunohistochemical reaction to the marking with anti-CK20, which suggests that this immunomarker could be used for a positive and differential diagnosis od urothelial carcinomas, especially in the metastatic lesions whose starting point could not be identified. Still, we should mention that the reaction of tumoral cell to CK20 was a variable one, thus showing a great phenotype variability of tumoral cells.

Protein p53 is the product of gene TP53, called the ”genome guardian”, a gene that regulates the progression of the cellular cycle, scenescence and apoptosis. In our study, the immunohistochemical expression of protein p53 was extremely variable. Thus, we identified well-differentiated, non-invasive urothelial carcinomas, in which the expression of p53 varied from moderate to intense. A similar situation was recorded in the invasive urothelial carcinomas. In conclusion, the immunohistochemical expression of protein p53 was not correlated in our study with the tumor stage or invasive vs non-invasive forms. The variable intensity of the immunohistochemical reaction of protein p53 shows the existence of some variate stages of damaging the cellular genome, through the mutant accumulation of gene TP53 in variable quantities, which leads us to the conclusion that the pathogenic factors involved in the process of urothelial carcinogenesis are extremely varied, and the intensity with which it acts upon the urothelium is, also, a variable one. Our results concur with those of the International

Agency for Cancer Research, which showed that the gene changes are expressed by changes of the tumor; cell phenotype, inducing variable clinical aspects regarding the progression and prognosis of the disease.

For evaluating the proliferative capacity of the tumoral cells in the urothelial carcinoma, we used as an immunohistochemical marker, the antibody anti-Ki67. In our study, the reaction of urothelial carcinomas to anti-Ki67 varied according to the tumor stage, namely it was moderate or low in the low-degree papillary carcinomas and intense in the high-degree papillary carcinomas.

Angiogenesis is an essential part of many physiological processes, such as the adaptation of hypoxia tissues or tissue regeneration after traumatic, inflammatory or acute ischemic lesions. Angiogenesis is of an utmost importance for tumor growth and survival of neoplastic cells. For the evaluation of the tumoral angiogenesis process, we used the antibody anti-CD34. The specific immunomarking of endothelial cells allowed us to establish that tumoral vascularization is extremely variable from one tumor to another, even if it was the same type of carcinoma. As a consequence, we may state that the vascularization of urothelial carcinomas was not correlated with the tumor staging.

One of the key factors in tumoral angigenesis is the vascular endothelial growth factor (VEGF). In our study, the immunohistochemical expression of VEGF was identified in all tumoral cells, being more intense in the poorly differentiated carcinomas.

CHAPTER VII. General conclusions

In our study, we evaluated a number of 1073 patients diagnosed with bladder tumors, admitted between 2013-2015, in the Clinic of Urology of the Emergency County Hospital of Craiova, which shows that every day a patient with bladder tumor is admitted to hospital.

Bladder cancer mainly affects men. In our study, 741 (69.06%) were men and 332 (30.94%) women, the men/ women ratio in the entire group being 2.23/1.

Regarding the distribution of patients according to social environment, 709 (66%) came from the rural area and 364 (34%) from the urban area.

Age represents a risk factor; bladder cancer occurs in adults and mainly affects men over 60 years old. In our study, in the age decades 60-69 years old and 70-79 years old, respectively, there were recorded 362, namely 372 patients, representing 68.40% of the total of patients.

The histopathological and immunohistochemical study of urothelial carcinomas highlighted a multitude of microscopic aspects showing that bladder tumors present a pronounced structural polymorphism. Of the immunohistochemical markers, the most constant expression was for cytokeratin 20 (CK20), for the cellular proliferation marker Ki-67 and for the vascular endothelial growth factor (VEGF)