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PhD THESIS

IDENTIFICATION OF NEW MOLECULAR TARGETS FOR THE THERAPY OF PATIENTS WITH INFILTRATIVE BLADDER TUMORS

A B S T R A C T

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CONTENT

INTRODUCTION

Oncological pathology is currently a challenge: although in recent decades progress has been made in early diagnosis and the identification of markers of high diagnostic specificity the incidence of new cases continues to increase. In recent years in economically advanced countries, the rate of cancer deaths has outpaced cardiovascular disease and the long-term survival of patients with various forms of cancer continues to be reduced despite modern acquisitions of adjuvant therapy. In the last two decades, oncological research insists on identifying new therapeutic targets. A number of antitumor substances with demonstrated in vitro and in vivo effects have already been introduced in practice, for the time being in combination with conventional therapy. The results over time are difficult to appreciate at this time, especially since some have been shown to be effective in the laboratory but have only questionable effects in the clinic. If for a long time oncology research focused almost exclusively on tumor cells and too little on the microenvironment in which they proliferate, in the current period we also see a significant change in conception in oncogenesis. Currently, research is also considering the tumor microenvironment, whose reaction stimulates or inhibits the growth of malignant cells. Numerous growth factors have been discovered and characterized, which govern not only the proliferation of normal cells, but also tumor cells. That is why we consider that it is necessary first of all the careful characterization of the molecular targets that can have a therapeutic impact. For these reasons, we focused our study on the two components - tumor cells and stroma, trying to characterize and validate known molecular targets, but also to identify new epitopes that could significantly influence current therapeutic strategies. This research focused on the identification and validation by retrospective study of potential therapeutic targets identified in tumor cells and / or tumor stroma, such as blood and lymph vessels.

In **the general part** of the paper we reviewed the normal histological structure of the bladder, then that of malignant bladder tumors, followed by a brief tour of their epidemiology, listing the risk factors known so far. We insisted on the heterogeneity of malignant tumors of the bladder; the histological types of urothelial carcinoma (formerly called "transitional cell") are reviewed in terms of their natural history and histological classification. TNM staging of bladder tumors is presented in the context of diagnostic methods and clinical evaluation. I wrote a small subchapter on the molecular profile of infiltrative bladder tumors, with special attention to the relative rarity of molecular studies on muscle infiltrative bladder tumors, most of which are modeled on malignant tumors with other locations of neoplastic disease (e.g. breast cancer). The molecular types currently defined are incompletely characterized, and in urothelial carcinoma the performance of molecular biology still does not compete with the results obtained in breast cancer. Although seemingly complicated and expensive, molecular classification has given birth to new therapeutic strategies currently in the testing stage. This paper is limited to this favorable and encouraging start in which the identification of new strategies and therapeutic targets appears as a necessity for new research and new interpretations of older observations.

The original part presents, at the beginning, the motivation and objectives of the study, followed by the presentation of the material and methods. We aimed to establish a homogeneous group of patients with infiltrative bladder tumors and to study possible new molecular targets for adjuvant therapy. For some of these biological markers - with potential impact on the therapeutic strategy - we did not find references in the literature, so we appreciate that the paper has a novelty. The objectives of the study were the following:

- Investigating the morphological features of the cases included in the study and establishing conventional clinicopathological parameters.
- Assess HER2 expression and determine the true proportion of cases that may respond to trastuzumab therapy.
- Investigate the impact of EGFR (Epidermal Growth Factor Receptor) on disease progression and therapy, as a potential target for specific inhibitors

.- Study of the tumor microenvironment by extensive evaluation of angiogenesis, given the microvascular density, endothelial cell proliferation, VEGF (Vascular endothelial growth factor) expression and specific receptors, VEGFR1 and VEGFR2.

- Analysis of the phenomenon of lymphangiogenesis, considering the frequency of lymph node metastases in these cases. Although currently there are no clearly defined targets for this aspect and no accepted medication for human cancer therapy, we believe that this approach can bring additional data to complete the profile of infiltrative bladder tumors.

- Study of the expression and significance of CLIC1 protein (chloride channel protein) in tumor and non-tumor cells of the bladder; we did not find data on this subject in the literature. This molecule can become an attractive therapeutic target, hitherto untested for infiltrative bladder tumors.

MATERIAL AND METHODS

Patients. The retrospective study investigated 52 consecutive cases of infiltrative bladder tumors (T2-T4), treated in the Arad Municipal Hospital between 2010 and 2015. The diagnosis was established based on standardized procedures. All patients underwent radical cystectomy surgery, followed by a replacement reservoir. The elements of inclusion in the study were represented by the documented existence of the infiltrative tumor, complete data on the extension balance and staging of TNM. The samples for the present study were taken only from the radical cystectomy specimen, selecting a representative fragment from the tumor and a fragment from the tumor-macroscopic bladder wall interface without significant changes.

The primary processing was performed in a standardized automatic system with the Thermo Shandon workstation. From each block to paraffin were made multiple serial sections, adapted to morphological and immunohistochemical staining.

The morphological evaluation was performed on the colored sections with the hematoxylin-eosin method, standard technique, with Leica automatic system, establishing the histopathological type of carcinoma, the degree of differentiation and the level of invasion; In addition, lymphovascular invasion has been reported.

The immunohistochemical method was performed in a fully automated and standardized procedure for all cases, with the Leica Bond-Max immunohistochemistry machine. The sections were treated with the primary antibody and for visualization we used the Bond Polymer Refine Detection System. The final reaction product was visualized with 3, 3 diamino-benzidine dihydrochloride (10 minutes) and the nuclei were stained with modified Lillie hematoxylin. The colored sections were permanently mounted with Canadian conditioner.

The combined immunohistochemical methods (double immunostaining) that we performed in the present research were the following:

CD34 / Ki67 for the identification of the proliferative character of blood vessel endothelial cells, CD34 with cytoplasmic expression highlighted by Fast Red (red) and Ki67 (highlighted with diaminobenzidine, DAB (brown)).

D2-40 / Ki67 for the identification of proliferative endothelial cells of lymphatic vessels, D2-40 with cytoplasmic expression highlighted with Fast Red (red) and Ki67 highlighted with diaminobenzidine, DAB (brown). presented the co-expression of the two markers.

Microscopic evaluation and image analysis were performed with Zeiss Axiocam 506 and Nikon AY260 microscopes, both equipped with a real-time image capture system and digital microscopic image analysis software. The calculation of microvascular density was performed according to the standard method (Weidner, 1993; Gasparini et al, 1993).

The same method was applied to the sections stained with CD34 and D2-40. Ki67 was evaluated by the original semi-automatic method (Suciu et al., 2014), on the sections with double immunostaining. HER2 (Herceptest) and EGFR were estimated based on internationally accepted scores. Epitopes with cytoplasmic expression (growth factors) were expressed by scoring, applying the score from 0 to 3 (0, negative, 1 under 25% positive cells, 2 25-50% positive cells, 3 over 50% positive cells).

Statistical methods. The statistical analysis of the cases aimed preferentially at evaluating the correlation between the investigated markers and conventional clinical-pathological parameters. In this sense, the Student and chi square tests were applied, $p < 0.5$ being considered as statistically significant. We did not analyze the survival data due to the relatively small group for such studies.

The results we obtained were structured in eight chapters (3.3.1 - 3.3.8) in accordance with the objectives of the study. They are based on microscopic observations on colored sections with morphological and immunohistochemical methods, correlated with clinical and pathological parameters and we confirm them as original. The study had only a retrospective component. The initial selection of cases took into account the presence of a representative specimen from the primary tumor, compatible with the determination of T and G elements; from a technical point of view, five cases were excluded. Under these conditions, the results presented below are based on the analysis of 47 cases.

3.3.1. Morphological features of infiltrative bladder tumors

In this chapter we presented the results obtained on the colored sections with the usual method. From the analysis of the cases included in the study we found that most were urothelial carcinomas, and only a few squamous cell carcinomas and adenocarcinomas. The fact that most of the tumors included in the study were carcinomas with more or less obvious urothelial differentiation led to the realization of a homogeneous group of cases, on which we consider that representative conclusions can be drawn. We had a small number of patients with squamous cell carcinoma and adenocarcinoma, so we considered the results on them as indicative, requiring further studies.

The incidence of cases with grade 3 differentiation was higher than the data in the literature, although they came from consecutive patients. We attributed this aspect to the advanced stage of natural evolution of the disease and late diagnosis by late presentation to the doctor. Associated lesions, such as dysplasia and carcinoma in situ, have been observed in a relatively small number of cases compared to data from the literature. One explanation for this may be the removal of a small number of fragments from the area adjacent to the tumor, even if all cases were treated surgically by radical cystectomy. The aim of our work, however, was to identify therapeutic targets in the primary tumor itself.

Particular forms of urothelial carcinoma, such as clear cell carcinoma and sarcomatoid carcinoma, have distinct morphological features, but in terms of current therapeutic strategies are treated by the same procedures as classical urothelial carcinomas, although both are characterized by marked aggression and molecular profile. different.

In conclusion, the morphological study of the case study selected for the present study reveals the predominance of urothelial carcinoma. Most cases showed deep muscle invasion and were poorly differentiated. Particular forms of urothelial carcinoma have developed with clear cells and sarcomatoids. Squamous cell carcinomas and adenocarcinomas are a minority but are characterized by extremely aggressive clinical and pathological parameters.

3.3.2. HER2 expression

HER2 is the receptor for epidermal growth factor 2, known in oncology by its overexpression in the homonymous molecular subtype of breast cancer. Overexpression of HER2 induces cell proliferation, growth and survival - particularly of tumor cells, being poorly expressed in most normal tissues.

The introduction into practice of trastuzumab (humanized monoclonal antibody), one of the first medications to address a specific molecular target, has been a real breakthrough in cancer therapy.

HER2 overexpression in urothelial carcinomas was reported over 15 years ago; however, there is currently no accepted strategy for trastuzumab therapy.

Considering the potential therapeutic impact, in this chapter we investigated the overexpression of HER2 on a homogeneous batch of infiltrative bladder tumors only by the immunohistochemical method; because trastuzumab acts only on tumor cells that overexpress HER2 at the membrane level, we did not perform in situ hybridization.

Results On the examined sections the morphologically normal urothelium - but not the other tissue structures! - did not express HER2. The only positive elements were the tumor cells which signals the high specificity of this reaction.

Of the total of 45 cases included in this part of the study, 12 were positive, ie 26.66%, correlating statistically significant with the degree of differentiation (all were G3). Sarcomatoid urothelial carcinomas, squamous cell carcinomas and clear cell carcinomas were negative. Our observations also confirm the heterogeneous nature of HER2 overexpression, large areas of positive tumor ranges alternating with negative ones.

Overexpression of HER2 in urothelial carcinoma of the bladder correlates with tumor aggressiveness and has a predictive role on recurrence.

Conclusions Our observations on protein HER2 overexpression signal a positive immunoreaction (+2 and +3) in 26.66% of cases and draw attention to a subgroup of patients with infiltrative bladder tumors who may benefit from trastuzumab therapy in combination with conventional chemotherapy. We consider that our results, although obtained on a relatively small number of cases, bring major arguments for the introduction of trastuzumab therapy or similar drugs to certain cases of invasive bladder tumors in our own muscle, but only after prior immunohistochemical testing.

3.3.3. EGFR as a therapeutic target

EGFR (the receptor for epidermal growth factor 1, also known as HER1), also belongs to the family of tyrosine kinase receptors. EGFR activation stimulates cell proliferation and survival and inhibits or limits. Aberrant expression of EGFR in various cancers (including bladder tumors) compared to normal tissues correlates with unfavorable prognosis. Although there are several studies on EGFR expression in bladder tumors, its clinical significance is still uncertain.

There are active EGFR inhibitors, already introduced in medical practice (iressa or gefitinib), but it seems that their association with conventional therapy does not significantly improve the evolution. The lack of clear evidence currently excludes EGFR inhibitors from therapy. The controversial data presented led us to approach the research of EGFR expression in the present study.

Results We evaluated the immunohistochemical expression of EGFR by its own way of scorification, which we present in the following table:

Score	Percentage of positive cells	Intensity of reaction
0	0	-
+1	Under 10%	Weak
+2	10-30%	Moderate
+3	Over 30%	Intense

Normal urothelium in the vicinity of the tumor was positive for EGFR throughout its height.

Invasive urothelial carcinomas showed a positive reaction distributed according to three models:

- intense cytoplasmic (considered by some authors unspecific; we consider that membrane intensification is simply masked by the high intensity of the final reaction product);
- cytoplasmic with membrane intensification;
- membrane only:

We obtained a higher percentage of positive cases (82.22%.) Than in other authors. We also observed a significant correlation between EGFR expression and the degree of tumor differentiation, the reaction being intensely positive in all poorly differentiated areas. Interesting is also the relationship of EGFR with HER2 overexpression, in our study appearing three groups of patients, respectively, who express only EGFR (n = 24), co-express EGFR and HER2 (n = 13), and the third, who express only HER2 (n = = 1).

In conclusion, we identified EGFR expression in tumor cells in 82.22% of invasive bladder tumors included in the study. We imagined and implemented in the present study a new immunoreaction scoring system for EGFR. Most urothelial carcinomas (including particular microscopic forms), and squamous cell carcinomas were positive, and well-differentiated adenocarcinomas and urothelial tumors were negative. Based on the comparison with HER2 overexpression, we identified three different subgroups of patients (EGFR + / HER2-, EGFR + / HER2 + and EGFR- / HER2 +), who can benefit from specific and differentiated targeted therapy.

3.3.4. Vascular endothelial growth factor (VEGF) and correlation with vascular microdensity

In this chapter we approached the study of microvascular density (MVD) in correlation with the expression of vascular endothelial growth factor (VEGF) and specific receptors, from which we selected VEGFR2, considered by many authors as a potential therapeutic target. MVD provides information on local progression and development of hematogenous metastases. In most studies published to date, MVD with high values is considered an unfavorable prognostic element, correlating with the advanced stage of the tumor, high degree of malignancy, rapid local progression and metastasis. On the other hand, MVD values do not inform about the potential response of the tumor to anti-vascular therapy, and the relationship with growth factors and especially with VEGF is still uncertain (Raica et al, 2010).

VEGF is a growth factor, being the strongest angiogenic substance known in the present. VEGF is secreted by a wide variety of normal cells, but is overexpressed in tumor cells, particularly under conditions of hypoxia caused by rapid proliferation. VEGF stimulates in vitro and in vivo endothelial cell proliferation, differentiation, survival and migration, both under normal and tumor conditions. However, VEGF may not be the only growth factor involved in the formation of new blood vessels. VEGF becomes active after binding to specific receptors expressed by endothelial cells, the most effective of which is VEGFR2. Although VEGFR2 is intensely expressed by blood vessels associated with urothelial carcinoma, no significant antitumor effect has been demonstrated to date with treatment with specific inhibitors (Li et al, 2015).

We consider that based on existing data, the hypothesis of the present study relates to the effect of multiple growth factors that cooperate in stimulating tumor initiation and progression, in cascade, which would justify the lack of correlation between VEGF expression, VEGFR2 and MVD values. The aim of this study was to identify vascular and tumor targets for anti-vascular and antiangiogenic therapy, respectively. Our results suggest the introduction of a standardized protocol for the molecular profile of invasive urothelial carcinoma. This methodology could be useful in refining personalized therapy and increasing the effectiveness of biologic therapies.

Based on the results obtained, we consider that VEGF is not a therapeutic target in infiltrative bladder tumors. VEGF expression does not correlate with clinicopathological parameters of the tumor and does not represent an element of individual or predictive prognosis for the response to therapy.

Invasion of blood vessels by tumor cells is optimally detected on stained preparations for CD34. Given the clinical importance of this parameter, the application of the method in infiltrative urothelial carcinomas becomes mandatory.

Vascular micro-density is a useful prognostic parameter, it correlates with vascular invasion and the degree of differentiation, but we do not recommend its constant application in all cases. Immature and intermediate vessels are an attractive target for anti-vascular therapy and may be indicative for assessing the response to specific therapy.

3.3.5. Endothelial cell proliferation compared to tumor cell proliferation and tumor-associated vessel type

The blood vessels in the stroma of malignant tumors are morphologically different from the normal ones in terms of size, trajectory, wall thickness and absence of the lumen. Since 2003, Gee et al have classified tumor-associated blood vessels as immature, intermediate, and mature. Although applied in several studies with promising results, this classification is rarely discussed in routine pathological diagnosis (probably because not all vessels respond to this therapeutic model).

We investigated this aspect on the cases included in the study, in the hope of finding the optimal target for anti-vascular medication. This is a novelty of the thesis, as, so far, however, there is no publication on infiltrative bladder tumors from this point of view.

Sleeping endothelial cells have a long lifespan, with an extremely low rate of division. In normal tissues, the doubling time of the endothelial cell population is over 1000 days, but it becomes only a few days if the cells are activated - a process signaled by the early expression of endoglin (Ceausu et al., 2011; Ferician et al., 2017).

The working hypothesis has two purposes: it highlights the prognostic value of endothelial proliferation and demonstrates the value of a potential indicator of the response to antiangiogenic and anti-vascular therapy.

Results The types of vessels associated with infiltrative bladder tumor were evaluated by the double immunohistochemical staining method, based on a cytoplasmic endothelial marker (CD31, final reaction product visualized in brown) and a specific marker for contractile filaments found in perivascular cells (muscle actin smooth, red reaction final product). For the immature and intermediate vessels, only the endothelial marker was obvious.

In all the infiltrative bladder tumors studied, we identified all three types of vessels associated with the tumor. We noticed that immature vessels are more numerous in the tumor area in the central area, where they represent 62.25% of all positive structures for the endothelial marker, while intermediate vessels represent 35.1%, and mature vessels only 6.65%.The ratio between the types of vessels changes in the tumor area, the peripheral area, where the intermediate vessels increase numerically, up to 54% and the mature ones, up to 18.6%. In the peritumoral area I practically did not notice immature vessels.

Quantification of endothelial proliferation on the double immunoreaction for CD31 and Ki67 is problematic, because in infiltrative bladder tumors a large number of malignant cells are also positive at the nuclear level. We considered only cells that express Ki67 at the nuclear level and co-express in red cytoplasmic smooth muscle actin. We report the presence of colored endothelial cell nuclei for Ki67 only for immature and intermediate vessels, and never for mature ones.

Conclusions In this chapter we demonstrated for the first time the existence of immature and intermediate vessels in infiltrative bladder tumors. Immature vessels predominate intratumorally, and intermediate ones intratumorally peripheral and peritumorally. Only these two types of vessels can be a viable therapeutic target for antivascular medication. The microvascular density calculated only for immature and intermediate vessels has high prognostic value. The endothelial cell proliferation rate accurately reflects the activation and progression of tumor angiogenesis and may be a major candidate for evaluating the efficacy of antivacular and antiangiogenic therapy.

3.3.6. Expression and significance of podoplanin D2-40 in bladder cancer

The formation of new lymphatic vessels is called lymphangiogenesis and is often associated with the natural evolution of carcinomas. For a long time the lymphatic vessels and their formation in normal and pathological conditions was neglected, especially due to the lack of specific markers of the lymphatic endothelium. The identification of podoplanin as a high-specific marker for lymphatic endothelial cells (LEC) allowed the study of lymphatic vessel density (LVs) independently of blood vessels. Currently, most research uses the D2-40 antibody, which recognizes the formalin-insensitive epitope of podoplanin expressed by LEC, but does not stain the endothelium of blood vessels. By this method the density of lymphatic micro-vessels (LMVD) can be calculated and the lympho-vascular invasion is much easier to identify (Raica et al., 2013; Raica et al., 2015; Cobec et al., 2016).

The first purpose of this part of the study was to demonstrate the correlation between LMVD and clinical-pathological prognostic parameters. Unlike angiogenesis, in lymphangiogenesis there are currently no inhibitors accepted for application in human cancer therapy. There are currently only experimental studies that have applied anti-podoplanin (NZ-1) and anti-VEGF-C monoclonal antibodies in the animal model, but the results are unconvincing. For these reasons, we consider it mandatory to accurately characterize therapeutic targets, as we have further addressed for bladder tumors.

The first aim of the present study was to validate the method and test the sensitivity and specificity of the D2-40 antibody for the endothelium of the lymphatic vessels. In terms of blood vessel endothelial specificity, the D2-40 immunoreaction was positive only in the lymphatic endothelium. In all lumen structures and morphological features of blood vessels the reaction was negative.

The reaction to podoplanin was positive in the endothelium of the lymphatic vessels in all cases included in the study. On the one hand, the method has a high sensitivity for the lymphatic endothelium, because it does not constantly stain the endothelial cells of the blood vessels.

Lymphatic vessels were present in both the intratumorally area and the peritumoral area, and the number of lymphatic vessels is higher in the peritumoral area than in the intratumorally area, except for two cases of invasive urothelial carcinoma.

Of course, the results are extrapolable for urothelial carcinomas, with a representative number of cases, but we cannot make definite statements about the other histopathological forms from this point of view.

In most of the studied tumors, respectively 43 of the 50 cases, in the tumor stroma were present in large numbers cells intensely stained with anti-podoplanin, arranged in the immediate vicinity of tumor cell plaques, with morphology characteristic of myofibroblasts.

Podoplanin expression in tumor cells is another aspect that has been observed in 8 of the 47 invasive urothelial carcinomas, including clear cell forms, in both cases of squamous cell carcinoma, and has been negative in sarcomatoid form and adenocarcinoma. We found a statistically significant correlation, directly proportional, between the expression of podoplanin in tumor cells and the degree of differentiation. It should be noted that the expression of podoplanin in proliferative cells is an early acquisition during carcinogenesis, as we found positive cells in the squamous metaplasia of the urothelium and in the dysplastic urothelium.

Lympho-vascular invasion is a particularly important element for pathological reporting, because it correlates statistically significantly with the risk of lymph node metastases in a very large number of cases. Lympho-vascular invasion is relatively easy to detect on sections stained with D2-40, a method we routinely recommend for evaluating this parameter.

Our observations reveal the identification of lymphatic vessels in all cases included in the study. We noticed higher density lympho-vascular invasion in the peritumoral vessels than in the intratumoral ones. We consider that the immunoreaction for podoplanin is particularly effective for the diagnosis of lympho-vascular invasion. LMVD correlates with tumor stage and degree of differentiation for urothelial carcinomas. Podoplanin is expressed by tumor cells in 17.02% of cases, thus being an attractive therapeutic target. Our results support the testing of podoplanin expression in infiltrative bladder tumors, given that there

are three potential therapeutic targets: myofibroblasts, the endothelium of lymphatic vessels and selectively, tumor cells.

3.3.7. Lymph endothelial cell proliferation and Prox-1 expression

In this chapter we aimed to characterize lymphatic vessels in the tumor and peritumoral area, elucidate the prognostic value of lymphatic microvascular density (LMVD), study the incidence of lymphovascular invasion and characterize the expression of LECs markers in tumor cells.

Current data on lymphangiogenesis show that the formation of new LVs is based on the transdifferentiation of cells in the postcapillary venules, which subsequently lose the connection with the venous system. This process is initiated under the action of Prox-1, it seems the best indicator of cells programmed to become LEC. No exogenous inhibitors of Prox-1 are currently known, but it could be an attractive therapeutic target even in the early stages of lymphangiogenesis.

We studied the proliferation of LECs in the tumor and peritumor area by the method of double immunohistochemical staining with D2-40 and Ki67. Under these conditions, the working hypothesis considered as a therapeutic target not only tumor cells, but also LECs.For all cases included in the study, the double immunoreaction was positive for Ki67 seen in brown and for podoplanin, seen in red. Under these conditions, lymphatic vessels were identified in all cases included in the study. Only endothelial cells expressing podoplanin relative to coexpression with the proliferation marker were considered for quantification. In areas with inflammatory infiltrate, the lymphatic vessels did not have a proliferative character, having an irregular contour and dilatations that suggest that they are pre-existing.

In most cases in which we observed tumor cell emboli, the corresponding lymphatic endothelial cells are not positive for Ki67. It should be noted that in tumor emboli most tumor cells express Ki67 (over 90%), regardless of the rate of proliferation of the original tumor.We did not find a statistically significant correlation between lymphatic microvascular density and Ki67 expression in tumor cells. The rate of lymphatic endothelial cell positivity was variable, but constantly in locally advanced tumors we identified proliferating vessels, especially lymphatic capillaries in the intratumoral area.

In conclusion, lymphatic vessels were present in all cases

- Lymphatic capillaries with Ki67-positive endothelium were identified in 23 of the 50 cases
- Ki67 positivity of lymphatic endothelial cells does not correlate with the degree of tumor cell positivity, but correlates with local tumor extension
- In terms of our data, the proliferation of endothelial cells in the intra- and peritumoral lymph capillaries is an unfavorable prognostic element.
- It is the first report in the literature of the proliferative nature of lymph endothelial cells associated with invasive bladder tumors.

3.3.8. CLIC1 (intracellular channel 1 chloride) is overexpressed in infiltrative bladder tumors

Intracellular channel protein for chloride 1 (CLIC1) is a member of the human CLIC family, a group of substances that regulate transmembrane transport of chloride and consecutively, are involved in a wide variety of biological processes, such as chloride transport, platelet aggregation, regulation of transmembrane transport or signal transduction. The mechanism of action of CLIC1 protein is not fully elucidated. The protein is inserted into the cell membrane to form channels for chloride ions. The activity of these channels is pH dependent, and is involved in the regulation of the cell cycleCLIC1 is expressed in a wide variety of human tumors, both malignant and benign.

Regarding the normal and tumor urothelium we did not find available data. Protein CLIC1 expression has not yet been reported in invasive urothelial bladder carcinomas. In fact, the PubMed search with the data: CLIC1 / bladder cancer / urothelial carcinoma / transitional cell carcinoma does not report any article published in the world literature.

In the present study we report CLIC1 expression, demonstrated immunohistochemically, in tumor cells of invasive bladder carcinoma and in synchronous associated urothelial lesions.

Results

Normal urothelium was negative for CLIC1 in 12 of the 14 cases in which it was present in sections, and in two it was weakly positive. Incidentally, observed structures in the mucosa, such as von Brunn nests, were also negative.

Dysplastic lesions, identified in 14 cases in the immediate vicinity of the tumor, were intense positive in 11 cases, moderate in 2 and weak in one case. The reaction was evident even in moderately and weakly positive cases, compared to normal urothelium.

All four carcinoma lesions identified in situ were intensely positive for CLIC1, across the entire epithelium.

The immunohistochemical reaction for CLIC1 was positive in 47 of the 50 cases (94%), and the intensely positive tumors showed several distribution patterns.

The small blood vessels at the front of proliferation and invasion were constantly and intensely positive in the endothelium. The appearance was constant in the lamina propria and the tumor stroma, and only occasionally observed in the vessels of the submucosa and / or the invaded muscle. The poorly differentiated urothelial carcinoma with sarcomatoid appearance had the highest intensity of expression, which points to a possible therapeutic target.

Both two squamous cell carcinomas, both developed in the bladder trigone, showed the same aspects, i.e. poor, diffuse, and heterogeneous reaction in tumor cells. Unlike poorly differentiated urothelial carcinomas, in squamous cell carcinomas the appearance was diffuse but with moderate or low intensity. However, most tumor cells are positive, where the heterogeneous distribution pattern of the final reaction product is not observed.

CONCLUSIONS

1. The morphologic study of the 50 selected cases of urinary bladder cancer reveals predominance of urotelial carcinoma, majority of them being low differentiate and with musculary invasion (MIBC). Particular types of urothelial carcinoma have been developed as clear cell or sarcomatoid. Scuamous cell carcinomas and adenocarcinomas were minoritar, but characterized by aggresive clinical parameters.
2. Our observations on HER2 overexpression show positive immunoreaction (+2 and +3) in 26.66% of cases. The method points out a subgroup of patients with infiltrative urinary bladder tumours that might benefit of transtuzumab therapy, associated to conventional chemotherapy. Although our results came out from a relatively reduced number of cases we consider them an important argument for introduceing transtuzumab or similar products in treatment of advanced MIBC with low differentiation but only after careful immunohistochemical assesment and in situ hibridization.
3. In 82.22% of studied cases we have identified EGFR expression in tumoral cells. We have proposed and applied a new scoring for EGFR immunoreaction. The majority of urothelial carcinomas have been positive, including particular microscopical forms as well as scoamous cells carcinomas; adenocarcinomas and highly differentiate urothelial tumours have been negative. Based on the comparisson with HER2 overexpression we have been able to identify 3 sub-groups of patients (EGFR+/HER2-, EGFR+/HER2+ și EGFR-/HER2+) that might beneficiate of highly targeted and differentiate therapy.
4. VEGF didn't confirm as therapeutic target in MIBC. Our study has show only 13.3% cases where VEGF was expressed at proteic level, with low intensity and high heterogenity. VEGF expression does't correlate with tumoral clinical and pathological

parameters noe with individual prognostic or therapy response. Vascular invasion by tumour cells was optimaly shown on CD34 coloration. The clinical importance of this parameter makes it an compulsory method applied on MIBC cases. Despite MVD correlates well with tumoral vascular invasion and with degree of cell differentiation, thus an useful prognostic parameter, we do not consider to apply in all cases. Imature and intermediary vessels might be considered an attractive target for anti-vascular therapy and might develop as orrientative landmarks to evaluate speciffic therapy response.

5. Our study demnostrates for the very first time existence of imature and intermediary vessels in MIBC. Imature vessels are predominant into the tumour while intermediate vessels are found at tumour's perifery. Only those two types of vessels are considered to be a potential target for anti-vascular therapy. We consider prognostic value only for MVD calculated for imature and intermediary vessels. The proliferation rate of endothelial cells reflects strictly the activation and progression of tumoral aangiogenesis, thus being major candidate for evaluation of anti-vascular and anti-agiogenic efficency.
6. In all cases included in this study we have been able to reveal limphatic vessels. A higher in density limpho-vascular invasion was noticed rather in peritumoral vessels than in those intratumoral. Therefore we consider immunoreaction to podoplanin to highly efficient to diagnose limpho-vascular invassion. LMVD correlates well with the stade of tumour and differentiation grade for urothelial carcinomas. Podoplanin was expressed in tumoral cells of 17.02% of the studied cases. That makes us to consider it another attractive therapeutic target. Our results encourage testing podoplanin expresion in infiltrative bladder tumours as there are three potential therapeutic targets: miofibroblasts, limphatic endothelial cells and, selectively, tumoral cells.
7. Studying limphatic endothelial cells proliferation by double immunocoloration technique with podoplanin and Ki67 allowed us to conclude that limphatic vessels have been present in all cases but limphatic capilaries with endothelia positive for Ki67 have been found in 23 out of the 50 cases. Positivation for Ki67 of limphatic endothelial cells doesn't correlate with tumoral cells positivation degree but correlates well with local tumoral extension. Out of this data, the proliferation of endothelial cells in intra- and peri-tumoral limphatic capilaries turns to be a bad prognosis indicator. To our knowledge, this is the firs report in literature that associates the limphatic endothelial cells proliferative characteristic with invasive tumours of urinary bladder.
8. In 94% of cases we've been able to present expression of CLIC1. The overexpression corelates significantly with differentiation grade and with histological form, being more evident for urothelial carcinomas and negative or very low positive for any other types (adenocarcinoma or scuamous cell carcinoma). Our results support the realization of an experimental model in which CLIC1 should be used as therapeutic target in infiltrative urinary bladder tumours. We have to emphasize that this study is the first report of CLIC1 in infiltrative bladder tumors.