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Chloride Intracellular Channel Protein 1 (CLIC1) Is Over-expressed in Muscle Invasive Urinary Bladder Cancer

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Abstract. *Background/Aim: Invasive bladder cancer mortality remains high despite progresses made in early diagnosis and surgical procedures. Thus, there is a need to define new markers for bladder cancer. CLIC1 has not been previously studied in bladder cancer and thus, we aimed to assess its immunohistochemical expression in relation to different stages of bladder cancer development. Materials and Methods: Immunohistochemistry for CLIC1 was applied in 50 cases of muscle invasive bladder cancer. Results: CLIC1 was not expressed in the normal urothelium, but a strong reaction was observed in dysplastic urothelium, carcinoma in situ and in 94% of the cases with invasive urothelial carcinoma; however, it was not expressed in squamous cell carcinoma cases. No correlation was found between the immunohistochemical expression of CLIC1 and the stage and grade of the tumour. Conclusion: CLIC1 was overexpressed in urinary bladder dysplastic epithelium, carcinoma in situ and invasive carcinoma. CLIC1 constitutes a new potential marker of invasive bladder cancer.*

Cancer of the urinary bladder is a frequent neoplasia in human, and more than 550,000 new cases are worldwide reported each year. Patients affected by bladder cancer are usually stratified into two groups: non-muscle invasive, which accounts for 70-75% of all cases, and muscle invasive carcinoma. This classification reflects significant differences concerning clinical presentation, natural evolution, prognosis, therapeutic possibilities and overall survival (1, 2). Muscle invasive bladder cancers are aggressive tumours, frequently associated with lymph nodes and distant metastases. Unfortunately, neoadjuvant and adjuvant therapy were not as efficient as expected and provided only a modest impact on overall survival (3). Nowadays, clinical trials with checkpoint inhibitors are in progress, and preliminary reports are promising (4). The lack of response of urothelial carcinoma to adjuvant therapy may be due to the limited knowledge of the molecular profile of these tumours.

Human malignant tumours are extremely heterogeneous and are only arbitrary classified based on some common morphologic features. Almost two decades ago, coincident with the publication of the human genome, the first molecular classification of malignant tumours was introduced in the clinical practice (5). Unfortunately, only few tumour types fall into this category, like breast cancer or colorectal carcinoma. In an attempt to propose a molecular profile of bladder cancer, patients were stratified into five subgroups, very similar with the molecular classification already in use in breast cancer. Although this attempt showed some impact in the prognosis and prediction of response to systemic therapy (6), the molecular landscape of urothelial carcinoma is still unclear. The molecular markers involved in diagnosis were largely investigated but with limited benefits in the clinical practice. Therefore, there is the need for new subcellular markers, which may represent potential targets for therapy. To fulfil this condition, the marker should be expressed in a significant percentage of patients affected by a specific cancer, with a strong expression in the majority of tumour cells. For these reasons, we focused on chloride intracellular channel protein 1 (CLIC1), previously shown to be expressed in a variety of human tumours.

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CLIC1 is a member of the human CLIC family of proteins. Under physiological conditions, it is involved in a large variety of biological processes by regulating chloride transmembrane transport. *CLIC1* gene is encoded as GC06M032030, and is located on chromosome 6p21.33. The gene encodes a nuclear protein that is also expressed in the plasma membrane, and on occasion, in the cytoplasm. The cytoplasmic protein is soluble and the membrane component most probably consists of a single transmembrane domain. Mutations of the *CLIC1* gene cause loss of dimerization and supress the active transport of ions (7). CLIC family includes a variety of proteins that have multiple roles, such as the stabilization of the plasma membrane, transepithelial transport, regulation of the cell volume and intracellular pH. CLIC1 protein is involved in a variety of biological processes, like chloride transport, platelet aggregation, and regulation of transmembrane transport or signal transduction. The mechanism of action of CLIC1 is not completely understood. The protein is inserted into the membrane to form channels for chloride ions. The activity of these channels is pH-dependent and involves the regulation of the cell cycle (8-10). Because of its role in cell proliferation and the cell cycle, CLIC1 became of interest in a broad spectrum of human diseases, including neoplastic diseases.

Until now, a lot of normal human tissues have been tested for the immunohistochemical expression of CLIC1 protein. In most of the cases, the pattern was granular nuclear and/or continuous at the plasma cell membrane. CLIC1 expression was not found in the normal central nervous system, and only a weak and variable staining was found in the endocrine glands, pancreas, testis and liver. A strong expression has been reported in clear cell renal cell carcinoma (11), gallbladder, colon, placenta and the tubular system of the nephron (12).

CLIC1 is expressed in various benign and malignant human tumours. Chen *et al*. (13) detected CLIC1 expression in 67.9% of gastric cancer cases, and the expression in tumour tissues was 1.95 times stronger than that in the normal mucosa. The increased expression of CLIC1 in gastric cancer significantly correlated with lymph node metastases, lympho-vascular invasion, perineural invasion, and advanced stage. In addition, CLIC1 expression seems to correlate with prognosis, as survival after 5 years follow-up was lower in cases with low expression, and significantly higher in patients with over-expression. Therefore, preliminary results indicate that CLIC1 over-expression is a potential prognostic marker in gastric cancer.

Although CLIC1 is involved in normal conditions in the regulation of the cell cycle and cell proliferation, its significance in malignant tumours is unclear. In patients with hepatocarcinoma, it was found that both the protein levels of CLIC1 and the mRNA expression were significantly higher than those in the normal liver. Zhang *et al*. (14) have shown that over-expression is noticed in 81.2% of the cases, and correlates with distant metastasis, pTNM stage, and low survival. Wei *et al*. (15) have found that strong expression of CLIC1 in gastric cancer correlates with vascular invasion and poor prognosis. In the ductal carcinoma of the pancreas, the expression of CLIC1 has been reported but its clinical significance remains elusive. Jia *et al*. (16) investigated 79 specimens by immunohistochemistry and found strong expression in 67.1% of tumour cells, and in 25.7% of the non-tumour tissues. Over-expression of CLIC1 showed a positive correlation with grading and size of the tumour, but not with other parameters. Although a multivariate analysis showed a decrease in overall survival in CLIC1 positive patients, further studies on larger series are needed to clearly demonstrate its relationship with lymph node and distant metastases.

The expression of CLIC1 at the protein level has not yet been reported in muscle-invasive urothelial carcinoma of the urinary bladder. In the present study, we examined the immunohistochemical expression of CLIC1 in a subset of urothelial carcinoma and associated lesions, and its correlation with prognosis. To the best of our knowledge, this is the first report on CLIC1 expression in invasive urothelial carcinoma of the urinary bladder.

Materials and Methods

Patients. Fifty consecutive patients with muscle-invasive tumours of the urinary bladder (pT3-T4) were included in the study; age ranged between 56 and 72 years. Diagnosis was based on clinical data, imaging, endoscopy, and pathological criteria, according to standard procedures. All patients were treated by radical cystectomy with lymphadenectomy followed by configuration of the orthotopic low-pressure reservoir or urinary diversion, depending on the location and local extension of the disease. Biopsies were taken from the primary tumour and from the border between the tumour and the apparently normal tissue. Informed consent was obtained from all patients before surgery, and all procedures respected the ethical principles regarding the use of human tissue specimens for research purposes, according to the WMA Declaration of Helsinki.

Primary processing. Biopsies were processed according to standard histological techniques. Briefly, they were washed in buffer saline and fixed in buffer formalin pH7.2 for 72 h. After dehydration and clarification, they were embedded in paraffin and 3 μm thick sections were cut from each block. The full procedure was fully automated by using ThermoShandon carousel (ThermoScientific Fischer, Cambridge, UK). We used haematoxylin-eosin for microscopic diagnosis and estimation of the grade. Additional sections were prepared for immunohistochemical evaluation.

Immunohistochemistry. Following an initial evaluation, additional paraffin-embedded slides from each case were stained using the monoclonal mouse anti-human CLIC1 antibody (Clone 356.1, dilution 1:2,000) (Santa Cruz Biotechnology, Heidelberg, Germany), incubated at room temperature for 30 min. Incubation with the primary antibody was followed by the use of Bond Polymer Refine

Detection System (Leica Biosystems, Newcastle Upon Tyne, UK) specific for BOND MAX autostainer according to a well-standardized protocol. The automated process included dewaxing for 30 min, incubation with primary antibodies as described above, and incubation with polymer for 30 min, followed by incubation with the diamino-benzidine chromogen for 10 min. Nuclear staining was the final step, performed with Lillie's modified haematoxylin. Prostate tissue expressing CLIC1 was used as external positive control. A dark brown colour detected by microscopy on stained slides, showing cytoplasmic and/or membrane pattern was considered as positive for CLIC1. All immunohistochemical steps were fully automated and controlled by the Bond Max autostainer (Leica Biosystems).

CLIC1 score. We stratified cases according to the relative number of CLIC1 positive tumour cells as follows: less than 10% of cells, noted with 0, low expressing tumours (10-30% of tumour cells) scored as 1, mild expression (30-50% of tumour cells), scored as 2, and high expression (>50% tumour cells positive for CLIC1), scored as 3. When positive, the reaction was strong in all positive tumour cells, and therefore, the intensity of the staining was not considered as a useful parameter. Endothelial cells and inflammatory cells that were occasionally positive, where not taken into account during scoring.

Image acquisition and data analysis. All slides were scanned using the Pannoramic Desk slide scanner (3D Histech, Budapest, Hungary). Digital slides were stored in Case Center and assessed by using the Pannoramic Viewer Platform (3D Histech, Budapest, Hungary). By using these methods, we evaluated the whole section of each specimen for CLIC1 expression pattern, percentage of positive cells and CLIC1 signal intensity. Statistical analysis, using Pearson, Spearman and Kendall tests, was performed with SPSS version 17.0. A p-value less than 0.05 was considered statistically significant.

Results

The microscopic analysis showed urothelial cell carcinoma in 48 cases and squamous cellular carcinoma in 2 cases. The degree of differentiation was as follows: well differentiated (G1) in 8 cases, moderately differentiated (G2) in 27 cases, and poorly differentiated (G3) in 13 cases. Both squamous cell carcinomas were graded as G2.

CLIC1 immunohistochemical expression was evaluated in all 50 cases included in the current study. Beside the invasive carcinoma, we noticed the presence of normal urothelium in 9 cases, urothelial dysplasia in 14 cases, and carcinoma *in situ* in 4 cases. Normal urothelium was negative for CLIC1 expression in 7 of the 9 cases. In two cases, the reaction was slightly positive, with a cytoplasmic pattern, without membrane enhancement. Particular structures of the normal bladder wall, like von Brunn nests, were negative.

Urothelial dysplasia was identified in 14 cases, in close proximity to urothelial carcinoma. Dysplasia showed a strong reaction in 11 cases, moderate in 2, and weak in 1 case. The pattern of reaction was cytoplasmic diffuse, heterogeneous in four cases, and homogeneous in ten. Carcinoma *in situ* (n=4) showed a strong, diffuse and homogeneous positive reaction in all cases (Figure 1a). The expression pattern in the carcinoma *in situ* was granular, cytoplasmic and strong, usually without nuclear positive staining.

CLIC1 immunohistochemical reaction was positive in 47 of the 50 cases (94%). A strong positive reaction was found in 32 cases that showed a final score >3 (Figure 1b). Two models of distribution of expression were detected: heterogeneous in 6 cases, and homogeneous in 41 cases (Figure 1c), both with strong intensity, particularly close to the proliferation zone. Particularly, in one sarcomatoid urothelial carcinoma, a strong reaction in tumour spindle cells and their cytoplasmic processes was observed (Figure 1d). In addition to tumour cells, small blood vessels close to the invasion front showed positive reaction in the endothelium. We found no statistically significant correlation between the immunohistochemical expression of CLIC1 and the degree of differentiation or the stage of the tumour.

Discussion

In the last four decades, ion channels were characterized in both normal and pathological conditions. More recently, ion channels became targets for therapy in some diseases, such as neurological and cardiovascular disorders. Chloride channels are the main class of anion channels associated with different pathological conditions (17). The role of chloride in cell proliferation was recognized almost 100 years ago (18), but a specific targeted therapy for cancer patients has not yet been found. The lack of data regarding the identity of chloride channels seems to be the main reason for the failure to develop a targeted therapy to restore apoptosis in cancer cells (17). The CLIC family includes six members, encoded by six different genes. CLIC family proteins have been extensively investigated in cancer cells, in both primary tumours and metastases (19).

CLIC1 expression has been demonstrated in some human malignant tumours, such as in the carcinoma of the lung (20), stomach (21), pancreas (22), head and neck squamous cell carcinoma (23, 24), or ovarian cancer (25). Recently, we reported for the first time the expression of CLIC1 in clear cell renal cell carcinoma; using immunohistochemistry, we showed a differential expression of the protein, and a correlation with the grading of the tumour and the presence of distant metastases (11). The majority of these observations were recently confirmed on a relative short series of patients. In addition, although the normal central nervous system does not express CLIC1, its expression has been reported in glioblastoma (26, 27).

Its mechanism of action in tumor cells is not completely understood, nevertheless CLIC1 is a promising marker of ovarian cancer cells and predicts patient survival (28). In addition, overexpression of CLIC1 reflects a poor prognosis in patients with gallbladder carcinoma (29, 30). The

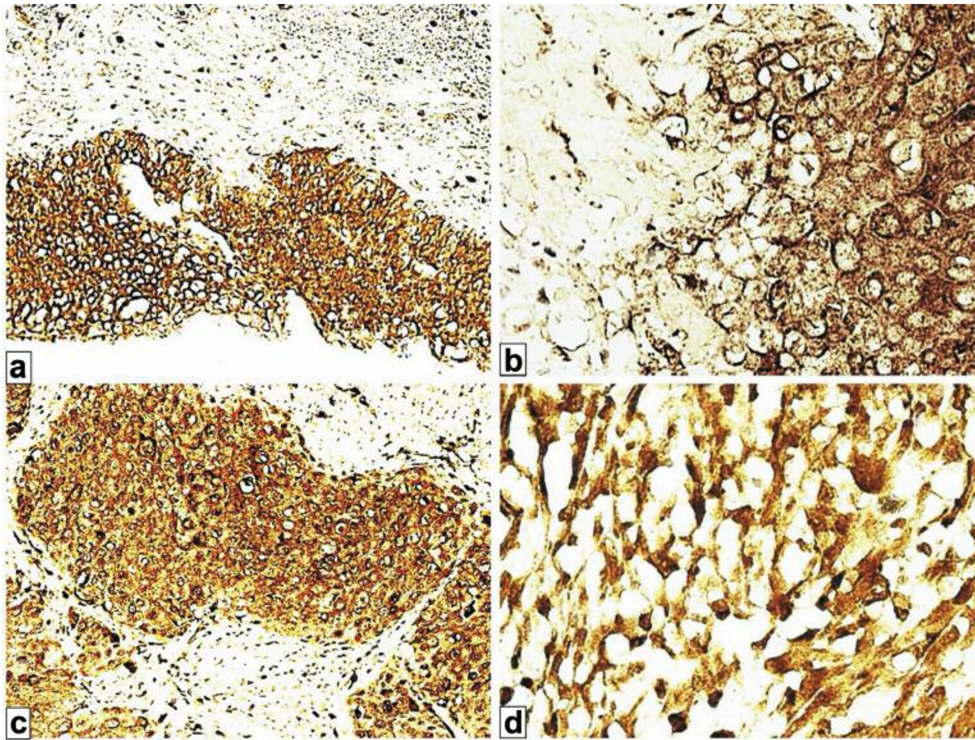

Figure 1. *Immunohistochemical expression of CLIC1 in in situ, invasive and sarcomatoid urothelial carcinoma. (a) In situ carcinoma staining showing strong positive reaction in almost all cells of the urothelium (×200). (b) Invasive urothelial carcinoma with strong CLIC1 expression (anti-CLIC1, ×400). (c) Strong, homogeneous reaction for CLIC1 in invasive carcinoma (×200). (d) Sarcomatoid urothelial carcinoma (×400).*

prognostic significance of CLIC1 over-expression in malignant tumours is still a matter of debate, as it is associated with poor prognosis in breast and liver cancers, and with better survival in gastric cancer.

CLIC1 is involved in the proliferation and migration of tumor cells, playing a role in the development of distant metastases. Therefore, the inhibition of CLIC1 activity may limit or even prevent migration and invasion of tumor cells. This aspect was previously shown in glioblastoma cells, where CLIC1 suppression reduced both proliferation and self-renewal properties (31).

The presented data suggest that CLIC1 could represent a potential therapeutic target in cancer treatment. Due to its over-expression in various malignancies, CLIC1 is an attractive potential marker. However, the expression and distribution of CLIC1 in normal tissues is still unknown. This is why currently, a humanized anti-CLIC1 antibody and an experimental model to support this hypothesis are not available.

In the current paper, we report for the first time the expression of CLIC1 in muscle invasive tumours of the urinary bladder. To the best of our knowledge, this is the first report in the literature regarding this topic. Our data revealed a strong correlation with transitional cell carcinoma; unfortunately, only very few cases of other histotypes were available in our series, therefore, no conclusions can be drawn for rare bladder cancer diseases. Surprisingly, in 94% of the cases CLIC1 positivity was found in the majority of tumour cells without a correlation with grade. The results of our study suggest the need for further investigations to define weather CLIC1 is involved in the evolution from non-muscle invasive to muscle invasive transitional bladder cancer, and its potential prognostic role in invasive urinary bladder cancer.

Conclusion

In this study, for the first time, we showed the immunohistochemical expression of CLIC1 in muscle invasive urinary bladder cancer. CLIC1 positivity was found in the tumours of 94% of the cases; it strongly correlated with urothelial cell proliferation, but not with the grade and stage of the tumour. Further investigations are needed to better define the precise role of CLIC1 in transitional cell carcinoma, and its eventual involvement in the transition from non-muscle invasive to muscle invasive disease.

Conflicts of Interest

The Authors have no conflicts of interest to declare regarding this study.

Authors' Contributions

TGA designed the study and wrote the paper. TCC and MM kindly provided the antibody, coordinated the technical part of the study and revised the manuscript. ARC performed immunohistochemistry. MR was involved in the routine histopathological evaluation of the cases and interpretation of CLIC1 immunohistochemical expression in bladder cancer, and together with AMC, statistically analysed the results. MR and AMC also revised the final draft of the manuscript.

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