Cell. Mol. Biol. (ISSN: 1165-158X)

Journal Homepage: www.cellmolbiol.org

Cellular and Molecular Biology

Original Article

Impacts of Chlorine 1-3 ıon channels on localized bladder neoplasms

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Article Info

Article history:

Received: November 20, 2023
Accepted: February 29, 2024
Published: May 31, 2024

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Abstract

Bladder tumors occur more frequently in men than in women and are the fourth most common malignancy after prostate, lung, and colon cancers. In this study, we examined the expression of chlorine ion channel 1 and chlorine ion channel 3 in localized bladder tumors according to their stage. We conducted a retrospective analysis of a prospective cohort study spanning from May 2018 to January 2020. This study involved a group of 55 patients who had been diagnosed with primary bladder cancer and underwent transurethral resection of bladder tumor under either general or spinal anesthesia. In addition, 30 patients who underwent cystoscopy due to etiology of hematuria and biopsies were taken from suspicious areas and whose results were normal were included as the control group. The collected samples were evaluated using real-time polymerase chain reaction in a medical genetics laboratory. In our study, it was observed that chlorine ion channel 3 gene expression increased significantly ($P<0.001$) in all cancer tissues compared to the control group, whereas no significant increase was found in chlorine ion channel 1 gene expression compared to the control group. The data obtained, especially for chlorine ion channel 3, are promising in terms of their use in the treatment of bladder tumors in humans.

Keywords: Bladder, Tumor, Chlorine, Ion channel, ClC-3

1. Introduction

It is worth noting that bladder tumors are more prevalent in men than in women and are the fourth most frequently diagnosed malignancy, following prostate, lung, and colon cancers. Over the past two decades, bladder cancer has seen a 0.8% increase in incidence, with regional and national variations [1]. The development of bladder cancer can be attributed to a variety of factors, including genetics, societal, environmental, occupational, and nutritional elements. Most cases of bladder cancer are diagnosed without any infiltration of smooth muscle, which makes up the majority of cases (75-85%). The standard course of treatment for such patients is transurethral resection of bladder tumor (TURBT) surgery. The primary treatment for non-muscle-invasive bladder cancer is complete transurethral resection of visible lesions. However, even with aggressive resection, bladder tumors recur in up to 70% of cases and progress in 10-30% of cases [2]. For cases of muscle-invasive bladder cancer, which are generally more aggressive and prone to spreading to other parts of the body. The 5-year overall survival rate was 37.0% with adjuvant chemotherapy and 29.1% with observation after radical cystectomy. According to reports, undergoing neoadjuvant therapy leads to a 10% reduction in the risk of death, which is equal to a 5% better chance of survival [3, 4].

Due to its electronegative properties, chlorine has the ability to alter the membrane and potential of both neurons and muscles, thereby facilitating their contraction. In mammals, there exist nine proteins that act as chlorine ion channels (CLIC) out of which four are located in the plasma membrane and function as carriers, while the other proteins in the plasma membrane facilitate Cl/H exchange [5].

The protein CLIC1 has a vital role in regulating intracellular pH and volume balance, as well as maintaining extracellular membrane integrity. Additionally, it plays a significant biological function in intracellular transport and signaling. While its precise working mechanism remains unclear, it is believed to contribute to pH-dependent cell cycle regulation and the creation of chloride channels within the cell membrane [6]. CLIC1 has been observed in both benign and malignant tumors, with its presence being demonstrated in about three-quarters of all gastric malignancies. As a result, it has been suggested that it could potentially serve as a diagnostic marker for the diagnosis of gastric malignancies [7]. In a similar vein, Zhang et al. [8] discovered that CLIC1 was excessively expressed in

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Doi: http://dx.doi.org/10.14715/cmb/2024.70.5.11

81.2% of hepatocarcinoma patients, with these cases being linked to distant metastasis and reduced survival rates. CLIC-3 is a voltage-gated Cl channel found in various tissues, including the liver, kidney, prostate cancer, pheochromocytoma, and glioma cells [9]. Previous research has shown that CLIC1 and CLIC3 are closely associated with tumor cell proliferation, apoptosis, and migration, and play a crucial role in tumor formation and development. Limited studies have shown that CLIC1 and CLIC3 levels increase significantly in advanced stages of bladder tumors, at both serum and gene levels [10-12].

In this study, we aim to evaluate the tissue levels of both CLIC1 and CLIC3 activity simultaneously in localized bladder tumors.

2. Materials and methods

We conducted a retrospective analysis of a prospective cohort study spanning from May 2018 to January 2020. This study involved a group of 55 patients who had been diagnosed with primary bladder cancer and underwent TUR-BT under either general or spinal anesthesia. In addition, 30 patients who underwent cystoscopy due to etiology of hematuria and biopsies were taken from suspicious areas and whose results were normal were included as the control group. Ethical approval was obtained from the ethics Committee and we ensured that all procedures utilizing human tissue samples for research purposes were conducted according to rigorous ethical standards. We obtained signed informed consent from every patient involved in the study. The assessment conducted a thorough evaluation of each patient, taking into account factors such as age, body mass index, use of oral anticoagulants or antiplatelet agents, and their American Association of Anesthesiologists status. Patients who presented with hematuria underwent computed tomographic urography or magnetic resonance urography for diagnosis and imaging. These procedures helped identify any local or distant invasion. Patients who were suspected to have local or distant metastasis were excluded from the study. The study divided patients into four groups. The control group (n = 30) consisted of patients with normal bladder tissue, while Group 2 (n = 21) comprised patients with pTa bladder tumors. Patients with pT1 bladder tumors were included in Group 3 (n = 25), and Group 4 (n = 9) consisted of patients with pT2 bladder tumors.

Our research was concentrated on patients who had not undergone any prior treatment for bladder tumors. However, we excluded patients with metabolic or malignant disease in other organs, those with local or distant metastases identified on imaging, those whose pathology was not confined to the bladder, and those who had previously undergone chemotherapy or radiotherapy.

2.1. Methods used to detect channels

2.1.1. RNA Isolation from Paraffin Block Sections

Sections of 0.2 mm thickness were taken from the provided paraffin blocks, stacked in epon molds, and stored at -80 ˚C for isolation. RNA isolation from the paraffin block was performed using Trizol.

2.1.2. Spectrophotometric RNA Measurement

To quantify RNA, we used the Qubit® RNA Assay Kit, which is designed for use with the Qubit® 2.0 Fluorometer from Invitrogen / Molecular Probes. We conducted a preliminary assessment using sterile water without DNAase-RNAase to ensure accuracy. We measured RNA content in ng/µl by placing 1 µl of RNA in the measurement zone of the Shimadzu BioSpec-nano device. This was done for each sample, and the lowest RNA value was taken as the standard to equalize RNA amounts for cDNA synthesis.

2.1.3. Complementary DNA (cDNA) Synthesis

To create cDNA, we utilized 10 µl of RNA samples and conducted the synthesis within a total volume of 20 µl. During the process, we combined 10µl RNA sample, 2 µl 10XRT buffer, 2 µl 10XRT random primer, 0.8 µl 25XdNTP mix, 4.2 µl nuclease-free water, and ultimately incorporated 1µl MultiScribe ™ Reverse Transcriptase enzyme. The samples were then placed in the thermal cycler and maintained at 25 °C for 10 minutes, 37 °C for 120 minutes, 85 °C for 5 minutes, and finally, at 4°C. Subsequently, the produced cDNA samples were stored at -80 °C.

2.1.4. cDNA Amplification with Real Time-Polymerase Chain Reaction

The process started by obtaining cDNAs through reverse transcription. These cDNAs were then amplified through Real Time-Polymerase Chain Reaction (RT-PCR) in the presence of sequence-specific primers. The expressions of three genes, namely glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Qiagen (Cat. No: OT00079247), CLIC-1 and CLIC-3, were determined using the Realtimeprimers Gene Panel. The $2^{-\Delta\Delta CT}$ method was utilized to calculate the differences between gene expressions.

Real-Time PCR was performed in 3 replicates. While preparing the RT-PCR Plate, 0.5 µl of cDNA samples were added to each well. For each sample, 1 µl qPCR mix, 0.5 µl primer, and 3 µl DNAase and RNAase-free sterile water, calculated according to the number of samples, were placed in Eppendorf and vortexed. 4.5 µl of the prepared mixture was dropped on the cDNA samples on the plate, and the plate was covered with an optical adhesive film. Plate samples were centrifuged for 1 minute in a mini plate spin device to completely collapse to the bottom and to eliminate bubbles.

The Applied Biosystems 7500 Real-Time PCR system was used to measure the expression levels. In the study, GAPDH was used as a housekeeping gene. The temperature conditions were set at 95°C for 15 minutes once, followed by 40 cycles of 95°C for 15 seconds, 60°C for 30 seconds, and 72°C for 30 seconds.

2.2. Statistical analysis

The statistical analysis was conducted using the IBM SPSS 22.0 software package (Armonk, NY: IBM Corp). To detect a significance difference using this test, the minimum sample size required for each group should be at least 17 (68 in total). This calculation takes into consideration a type I error (alfa) of 0.05, power (1-beta) of 0.8, effect size of 1.02, and a two-sided alternative hypothesis (H1). To determine if the expression level followed a normal distribution, we conducted the Shapiro-Wilk test. As the data met the assumption of normal distribution, we used the one-way ANOVA test to determine if there were any differences in expression levels between the groups. Mean and standard deviation values were provided as des-

criptive statistics. A p-value of < 0.05 was considered statistically significant.

3. Results

Out of the total 55 patients who approached our clinic for treatment of bladder tumor and underwent TUR-T operation, 49 were male (89%) and 6 were female (11%). The average age of female patients was 81.25 years, while the average age of male patients was 69.4 years. Out of the control groups in the study, 25 (83.3%) were males and 5 (16.7%) were females. The average age of male patients was 65.4 years, while that of female patients was 79.25 years. The pathological stages of the patients in the study are shown in Table 1. Based on the imaging results obtained from the abdominal and magnetic resonance imaging of the patients involved in this study, there was no evidence of any local or advanced-stage metastasis detected in any of the patients.

In our study, which used the real-time polymerase chain reaction technique, showed that there was a significant increase in the expression of the CLIC-3 gene in all cancer tissues compared to the control group. However, there was no significant increase in the expression of the CLIC-1 gene compared to the control group. *(*Figure 1, 2, Table 2). There was no significant difference between the groups in terms of CLIC-1 gene expression (F =0.739 and p= 0.236). In terms of CLIC-3, it was observed that gene expression decreased significantly as the pathological stage of the cancer increased. It was shown that CLIC-3 gene expression increased 5.9-fold in the PTA group compared to the control group (p< 0.001). Again, when compared with the control, it was determined that CLIC-3 expression increased by 3.99 in the pT1 group (p < 0.001) and it increased by 2.15 in the pT2 group (p = 0.046).

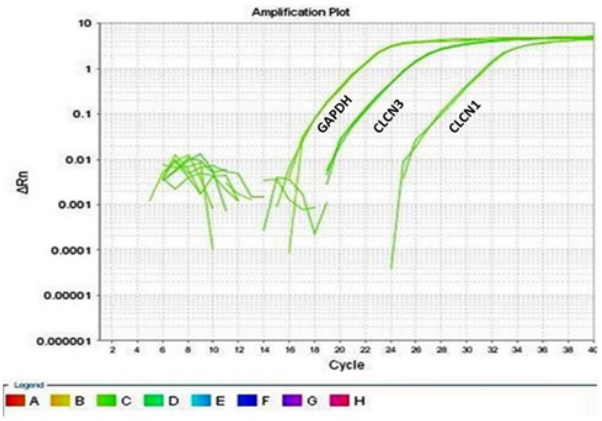

Fig. 2. Real-time PCR amplification plot for control samples is displayed on a graph, with cycle number on the x-axis and change in florescent intensity (ΔRn) on the y-axis. CLCN3 has a Ct number of 22 and CLCN1 has a Ct number of 28, with CLCN3>CLCN1 for ΔR n 0.1. **GAPDH:** Glyceraldehyde 3-phosphate dehydrogenase**. CLCN3:** Chloride Voltage-Gated Channel 3 (CLIC-3)(4q33). **CLCN1:** Chloride Voltage-Gated Channel 1 (CLIC-1) (7q35).

Table 1. Pathology results of patients who underwent TURBT operation.

Stage	Average	N (patients)
pTa	%38	21
pT1	%46	25
pT2	%16	9

TURBT: Transurethral resection for bladder tumor.

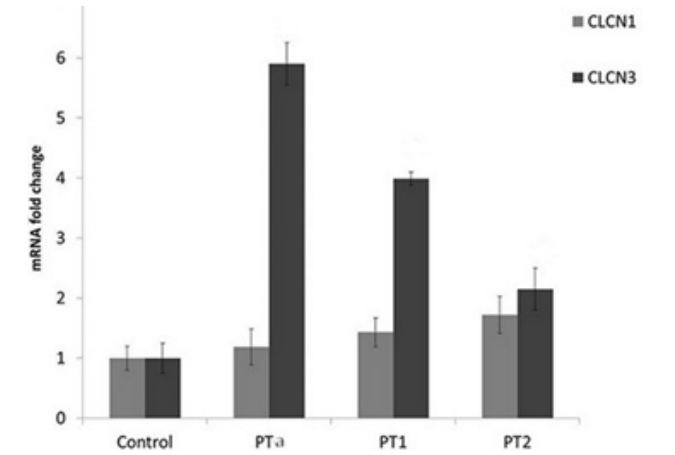

Fig. 1. Increasing of mRNA according to groups pTa: noninvasive papillary urothelial carcinoma; **pT1:** Invasion of lamina propia, **pT2:** Muscle invasive **CLCN 1:** Chloride Voltage-Gated Channel 1, **CLCN 3:** Chloride Voltage-Gated Channel 3.

4. Discussion

In this study, we observed a significant increase in CLIC3 activity in patients with localized bladder tumors compared to the control group. However, no statistically significant difference in CLIC1 activity was detected. Bladder cancer is the second most common cancer of the genitourinary system and the third leading cause of death worldwide. Shockingly, about 20% of cases result in death. A significant proportion of patients diagnosed at non-invasive stages. In fact, an astonishing 75%-85% of patients are diagnosed before smooth muscle invasion occurs. Such patients typically undergo Transurethral Resection of Bladder Tumor (TURBT) surgery as the primary treatment modality. In order to reduce the chances of cancer recurrence, additional intravesical chemotherapy or immuno-

Table 2. mRNA increase rates by groups.

Groups/stages	CLIC-1	CLIC-3
Control	1±0.08	1±0.05
pTa	1.19±0.11	5.9±0.15
pT1	1.43±0.17	3.99±0.1
pT2	1.72±0.13	2.15±0.08
F and P-value	F=1.739 and p=0.236	F=88.072 and p<0.001

The values are presented as the average with the standard deviation, and this range is within a 95% confidence interval. **pTa:** Noninvasive papillary urothelial carcinoma; **pT1:** Invasion of lamina propia, **pT2:**Muscle invasive; **CLIC:** Chlorine ion channel.

therapy treatments are used along with surgical interventions [1, 3]. Researchers have recently been focusing on ion channels due to their involvement in neurological and cardiovascular diseases [13]. Recent studies have shown that ion channels and transporters not only regulate cell proliferation, differentiation, and apoptosis but also have abnormal ion activity that promotes high proliferation of tumor cells. It is believed that ion channels play a role in the development and progression of cancer, as well as in the resistance to treatment. While their exact function in cancer is not fully understood, it is thought that they contribute to cancer by altering the cell cycle and volume [14-16].

Although the impact of chloride, an essential ion linked to the development of pathological conditions, on cell proliferation was discovered almost 100 years ago, there is no report of targeted therapy for cancer patients that focuses on chloride channels. The lack of information about the identity of these channels seems to be the primary reason why targeted therapies to trigger apoptosis in cancer cells have not been developed yet [13].

Chloride channels are widely expressed on cell membranes and organelles. They are responsible for balancing ions and pH, regulating electrical excitability, transporting fluids across epithelial cells, and maintaining cellular volume. Ion channels play a critical role in the migration and infiltration of cancer cells, influencing the regulation of the cell cycle, and potentially serving as key factors in the progression from the G1 to S phases. In addition to cancer, dysfunction of ion channels has also been linked to various endocrine and neurological disorders, such as arrhythmias, skeletal muscle disorders, epilepsy, migration, cystic fibrosis, and diabetes [17].

CLIC-1 is a newly discovered member of the seven-member p64 chloride channel protein family. It is identified on chromosome 6p21.3 and consists of 241 amino acids. CLIC-1 plays a vital role in regulating various physiological cellular processes, including intracellular regulation, proliferation, membrane potential regulation, and differentiation [18, 19]. During the G2/M phase of cell division, CLIC-1 is detected. However, the exact function of CLIC-1 remains unclear, although it has been suggested that high expression of CLIC-1 may have an impact on anti-apoptotic signaling and cell division [18]. CLIC-1 plays an important role in several critical functions. However, it has also been identified as an oncogene in prostate and gastrointestinal tract cancers. Various clinicopathological analyses have shown that high expression of CLIC-1 can lead to a poor prognosis in some cancer types, thereby serving as an independent prognostic marker [20-22]. In addition, CLIC-1 is a promising marker for ovarian cancer, and it can accurately predict patient survival [23]. It has been shown that high levels of CLIC-1 in patients with gallbladder carcinoma can indicate a poor prognosis [24-25]. While the activity of CLIC-1 has been studied in various types of cancer, there is not enough information available about its role in bladder tumors. One study by Adelmann et al [10] found that CLIC-1 activity was increased in patients with muscle-invasive, stage 3-4, and carcinoma in situ bladder cancer. This suggests that CLIC-1 may play a role in the distant spread of cancer cells, as well as in the proliferation and migration of tumor cells. The study conducted by Wang et al [12] clearly establishes a positive correlation between the stage of bladder tumors and the increased level of CLIC1. Our research aimed to investigate the activity of CLIC-1 in the tissues of patients diagnosed with bladder tumors who underwent TURBT surgery at our clinic. However, our study did not find any significant difference in the activity of CLIC-1 between the tumor group and the control group. The discrepancy between our findings and those reported by in previous studies could be attributed to the predominance of localized and low-grade tumors in our study cohort. This suggests that while the activity of CLIC-1 significantly increases in cases of high-grade and aggressive tumors, it does not experience a noteworthy increase in localized and low-grade tumors.

CLIC-3 is a chloride channel located in the plasma membrane that regulates cell volume and plays an essential role in cell proliferation. Overexpression of CLIC-3 can cause apoptosis by inhibiting the PI3K/Akt/mTOR signaling pathway [26]. CLIC-3 is a vital component of the natural volume-regulated anion channel (VRAC) found in various cancer cells such as epithelial nasopharyngeal carcinoma, prostate cancer, and malignant glioma. The presence of CLIC-3 in cancer cells promotes abnormal cell proliferation, particularly in malignant cells.

When chloride current is activated in human prostate cancer epithelial cells, inhibiting CLIC-3 results in reduced growth. The same is true for nasopharyngeal carcinoma and malignant glioma cells, which also show decreased growth upon CLIC-3 inhibition [27]. It is important to note that CLIC-3, like CLIC-4 and CLIC-5, acts as a Cl/H transporter in intracellular membranes, playing a crucial role in the acidification of endosomes and lysosomes. This heightened acidification of intracellular compartments can affect the effectiveness of essential anticancer drugs, potentially decreasing their concentrations and leading to drug resistance [5].

Multiple studies have shown that CLIC-3 plays a significant role in the development of resistance against chemotherapeutic drugs such as etoposide and cisplatin in cancer cells, through various mechanisms [18]. Therefore, inhibiting CLIC-3 could be a potential strategy to increase sensitivity to chemotherapy. In cancers, CLIC-3 functions as a VRAC, which leads to an increase in cell proliferation and migration. However, if CLIC-3 is upregulated for an extended period, it can also cause apoptosis. This result may seem paradoxical, but it's unclear how cancer cells evade apoptosis induced by the upregulation of CLIC-3. One possible mechanism for cancer cell growth is their ability to increase the production of antiapoptotic proteins such as BCL2, which inhibits the proapoptotic effect of CLIC-3 [28]. The use of CLIC-3 inhibitors can block the proliferation of cancer cells and increase their sensitivity to chemotherapy. On the other hand, CLIC-3 activators can promote cancer cell apoptosis, making them a potential strategy for cancer treatment. Therefore, the development of new CLIC-3 inhibitors and activators could be a promising approach for cancer treatment [6].

There have been numerous studies on the activity of CLIC-3 in various types of cancer. A study conducted by Knowles et al [29] has shown the strong binding of the antiangiogenic peptide CLT1 to clinical bladder cancer tissues, exhibiting a specific pattern of expression of integrin α5β1 and CLIC-3. In another study, Chen and colleagues [11] reported that CLIC3 mRNA expression was significantly higher in bladder cancer tissues than in normal tissues. In our study, similar to the literature, CLIC-3 activity

was found to be increased in the tissues of patients who applied to our clinic with a diagnosis of bladder tumor and underwent TURBT surgery, compared to the control group. It is important to note that even localized tumors show an increase in CLIC3 levels.

It is important to note certain limitations of this study. Firstly, the sample size is small and the distribution among groups is uneven. Additionally, due to the limited number of women included in the study, we were unable to make accurate comparisons between genders, age. Secondly, we excluded patients in stage 3 and 4 from this study. Due to economic constraints, we were unable to validate our findings at the tissue level through immunohistochemistry. Despite these limitations, our study represents an initial effort to comprehend the expression and activity of CLIC-1 and 3 in localized bladder cancer.

5. Conclusion

Bladder tumors are a common pathology in urology practice. Detecting and treating them early is critical, as they can spread quickly to other parts of the body. Researchers are working to understand the complex molecular factors that cause bladder tumors. Our study suggests that CLIC-3 could serve as a valuable diagnostic tool for localized bladder tumors and as a biomarker for the prognosis of bladder cancer patients. However, further research is needed to fully understand the potential of this approach. Studies with a larger number of participants at all stages of bladder tumors are necessary to develop new and innovative medical treatments.

Conflict of Interests

The author has no conflicts with any step of the article preparation.

Consent for publications

The author read and approved the final manuscript for publication.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Firat University, Institutional Ethical Board, Number: 2016/09/02.

Informed Consent

Informed consent was obtained from all individual participants included in the study.

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request

Author's contribution

AK, MO conceptualized and conceived the research design, analyzed the data, and drafted the manuscript. MO, EO, AT, AK, and TO, carried out the Study. IO, AK EO contributed to the finalizing of the manuscript. All authors read and approved the final manuscript.

Funding

This study was funded by the Scientific Research Fund of Firat University (Project No: TF 17.07).

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