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IMPACT OF CHLORIDE ION CHANNEL *CLC4* ON BLADDER CANCER PROGRESSION

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Abstract

Oncogene and tumour suppressor gene mutations cause heterogeneous cancer. About 10 million people die due to cancer annually. Bladder cancer is one of the most common cancers worldwide, predominantly in men. It is reported that ion channels are involved in cell proliferation, differentiation, apoptosis, tumour cell motility, invasion, and metastasis; hence, cancers are channelopathies. This study examined the expression of chloride ion channel *CLC4* in bladder cancer. We collected 20 pairs of tumours and normal tissue samples from bladder cancer patients for the expression analysis. We observed an increase in *CLC4* mRNA expression. Mutations in *CLC4* gene cause chloride ion channel changes that promote cancer. Understanding chloride channel molecular mechanisms require more research.

KEYWORDS: Bladder Cancer**,** Chloride Ion Channels, CLC4 Expression**,** Cancer Channelopathies**,** Tumour Suppressor Genes

Introduction

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Bladder cancer or urinary bladder cancer is one of numerous kinds of cancer on the globe. Several inherent and environmental factors contribute in bladder cancer, leading to transmutations in crucial genes that control maintenance, cell progress and tumour microenvironment (Chiu, Wu, Chia, Hsu, & Tzeng, 2016). It is common in males as compared to female counterparts. Generally, women's incidence of bladder cancer is about three to four times lower than its occurrence in men (Rafique, 2005). Bladder cancer is the 12th most frequent cancer type globally (Cao, Chen, Yu, Li, & Chen, 2021), the 9th leading cancer-related deaths (Bray et al., 2018)**,** and ranked seventh in men and seventeenth in women. It is responsible for two-thirds of all urinary tract malignancies, with an estimated diagnosis in 2.7 million people (Mithani et al., 2018). The prevalence of bladder cancer is increasing globally, especially in industrialized countries (Bray et al., 2018).

Several factors are involved in cancer development, including environmental and genetic factors. Earlier studies have shown that ion pumps or channels are also involved in cell proliferation, differentiation, apoptosis and help the tumour cell in migration, invasion and metastasis. Recently, cancer has been considered channelopathies. Ion pumps and channels are crucial for maintaining homeostasis, excitable cell signaling and controlling pH and membrane potential. However, these channels have also been linked to other cellular processes e.g., growth, migration, differentiation, and cell death (Litan & Langhans, 2015). Consistently, ion channels and pumps play important part in cell signaling, forming macromolecular complexes with growth factors implicated in cellular adhesion. Due to their unconventional function, it is hypothesized that they also play a part in cancer development and progression (Huang & Jan, 2014; Litan & Langhans, 2015). When ion channels are overstimulated, tumour cells multiply rapidly. Recent research focus on potassium channels, however, chloride channels have recently emerged as important targets for understanding the causes of cancers (Cuddapah & Sontheimer, 2011; Pardo & Stühmer, 2014; Peretti et al., 2015). There are five major classes of chloride channels, all of which are categorized by the type of regulation they undergo: calcium-activated chloride channels (*CaCCs*), voltage-gated chloride channels (*ClCs*), ligand-gated chloride channels, cystic fibrosis transmembrane conductance regulator (*CFTR*), and volume-regulated chloride channels (Peretti et al., 2015).

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The anoctamin protein family is expressed in a wide variety of cell types that function as CaCCs e.g., *ANO1* (*TMEM16A*) and *ANO2* (*TMEM16B*). *ANO1* has been reported to play a role in cancer metastasis. Cell swelling is responsible for activating *ANO1* resulting in increased intracellular Ca^{2+} to reduces cell volume (Almaça et al., 2009; Ruiz et al., 2012). Little is known about anoctamins other than *ANO1* and *ANO2*; our comprehension of them is only beginning to scratch the surface (Wanitchakool et al., 2014). *ANO6* (*TMEM16F*) has recently discovered a link between cell shrinkage and phospholipid scrambling, which indicates apoptosis (Kmit et al., 2013; Kunzelmann et al., 2014; Yang et al., 2012). Increased expression of the gene encoding *ANO7* (*TMEM16G*) has been observed in prostate cancer (Metsälä, Wahlström, Taimen, Kellokumpu-Lehtinen, & Schleutker, 2023). Glioma cells invade and migrate with help from CLC, namely *ClC3*. Neutrophils and nasopharyngeal cancer cells' movement with the help of *ClC3* has been observed (Mao et al., 2007; Peretti et al., 2015; Volk et al., 2008). Chloride channels have been studied extensively, but questions remain about their molecular identity and composition in volume-regulated channels (Liu, Lu, Liu, Huang, & Wang, 2012). Furthermore, the precise function of CLC during carcinogenesis and metastasis is unknown. Here, we looked into the expression of chloride channel *ClC4* in both healthy and diseased tissues. It opened a new door for us to investigate the cellular process of tumours and aid in creating cutting-edge cancer treatments by zeroing in on the specific chloride channels involved in cancer cell proliferation, invasion, and dissemination.

The primary purpose of this research was to examine the expression of chloride channel in a range of tumour cell types. To examine whether or not malignant and healthy cells express the same gene.

Material and Methods

Tissue Specimen and Data Collection

This research agreed with past consents from the Ethical Review Board (ERB). Informed consent was taken from the individuals, and a Performa was filled out for every patient with all the required data like age, gender, addiction, and ethnic background. Twenty paired bladder cancer tissue samples and adjacent healthy control tissues were collected. The samples were collected from the different cancer hospitals of Pakistan. The normal tissues or controls were histopathologically confirmed, and their reports were collected from the lab. The average age

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was determined. Tumour and healthy tissue samples from the immediate surrounding area (about 2 cm) were collected and frozen in RNAlater® (Invitrogen, California). United States of America) Furthermore, kept at 4 degrees Celsius in an ice bucket on the way to the lab. Patients who underwent surgery were surveyed to collect demographic data and clinicopathological features such as tumour grade, nodal involvement, stage, and age.

RNA isolation and quantification

The standard TRIzolTM reagent technique was used for RNA extraction from tissue samples (Qadir et al., 2021). Extracted RNA was measured using a nanodrop (IMPLEN GmbH, Germany). One microliter of RNA was injected into a Nanodrop for each sample. Absorbance was measured at 260 and 280 nm to examine the role of chloride channel dysregulation in the development and progression of bladder cancer. Models with a 260/280 ratio greater than 1.9 were considered high enough RNA quality for further analysis.

cDNA Synthesis

cDNA synthesis was used to analyse the *CLC4* expression in each sample. The RNA was transcribed into cDNA using a Thermo Scientific cDNA synthesis kit (Cat#K1622) per the manufacturer's instructions. So generated cDNA was verified by electrophoresis in 2% agarose gel.

Quantitative Real-Time Polymerase Chain Reaction

Real-Time PCR, also well-known as quantitative PCR (qPCR), was used to study gene expression at the RNA level. After careful and measured preparation of the reaction mixture, it was transferred into the microcentrifuge tubes. These tubes are designed to fit the PCR rack. The reaction mixture boxes were placed in an RT-PCR frame after properly closing them. The qPCR was carried out using a Step-One Plus RT-PCR system (Applied Biosystems). As an internal control GAPDH was used for *CLC4* gene data normalization (Yarotskyy et al., 2022).

Statistical analysis

2 -ΔΔ CT method was used to determine the relative mRNA expression of the gene/reference gene. Graph Pad Prism software was applied to statistically analyze the data obtained from experimentation in this study. The relative expression of gene was determined using Student t-tests. The data was considered to be statistically significant at a p-value of 0.05.

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Results

Baseline characteristics of the study population

A total of 20 individuals participated in the study. Among patients, 14 (70%) individuals were men and 6 (30%) were women. 6(30%) have low while 14(70%) have high grade tumor. (Table.1A) The data in (table.1B) indicates that different variables including age, Gender, Smoking and expression of chloride ion channels in bladder cancer patients. Smoking and chloride channels has significant role in increased expression of genes which are involved to spread cancer. But the data has shown significant decreased expression of gene and decreased standard mean values.

Table 1A: Association between clinic pathological parameters in bladder cancer tissues. (1B) Characteristics of Bladder cancer patient samples: Values are given as mean ± SD, mean values < 0.05 indicates the statistical significance. Cl= Chloride Channels.

A

Parameters	No of Cases	Percentage
Sex		
Males	14	70
Female	6	30
Age		
<59	11	55
>59	9	45
Grade		
Low	6	30
High	14	70

B

Characteristics	Cancer Patients	p values
Age	160.24 ± 34	0.67
Gender	29.79 ± 22	0.57
Smoking	156.45±28	< 0.05
Cl	195.24± 27	< 0.05

Gene expression of *CLC4*

The expression analysis of *CLC4* was done by using real time polymerase chain reaction (qPCR) among normal (control) and tumor tissues. We analyzed the 20 patients paired tissue samples. The data showed that, *CLC4* (10.97 folds) expression was significantly (P< 0.05) up regulated in tumor as compared to controls (Fig. 1A) Separate samples t test was used to compare two groups. CLC4 were considerably upregulated in patients compared to controls (Table 4.3).

TNM staging of the genes in the study population

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The expression of our target genes in the Ta stage *CLC4* (2.82 folds) was upregulated. In stage T1 *CLC4* (8.26 folds was upregulated. In stage T2a *CLC4* (23.40 folds), was upregulated. In stage 3 *CLC4* (4.91 folds), was upregulated of the tumour is shown in (Fig 6). Li et al. (2021) examined colorectal cancer ANO1 expression. ANO1 expression is higher in T3 and T4 than Ta, T1, and T2. 41 of 54 stage T3 patients and 5 of 6 stage T4 patients expressed positive. He et al., 2022, also observed that ANO1 expression was high in TNM high-grade stages such T3 and T4, but not in Ta, T1, and T2.

Grade of Tumor in study population

Fig 7 shows that expression of the target genes in high-grade and low grade tumor.

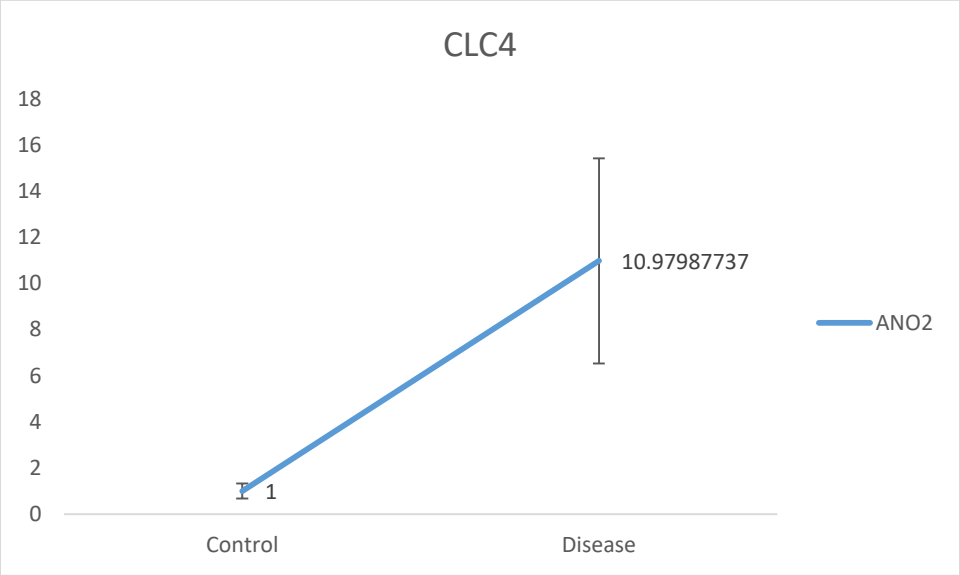

Fig 1 relative expression of CLC4 gene in cases and controls.

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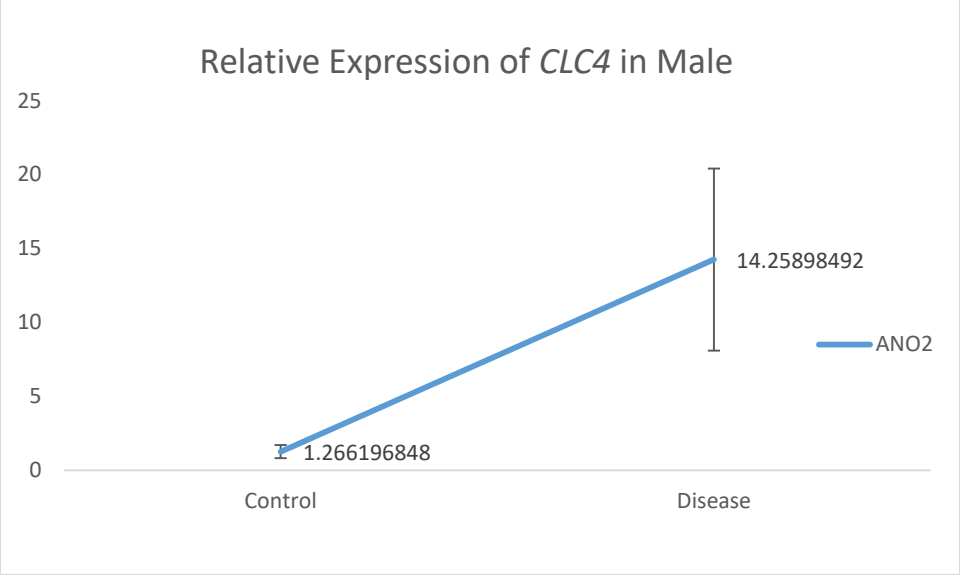

Fig 2 Relative expression of gene in Males

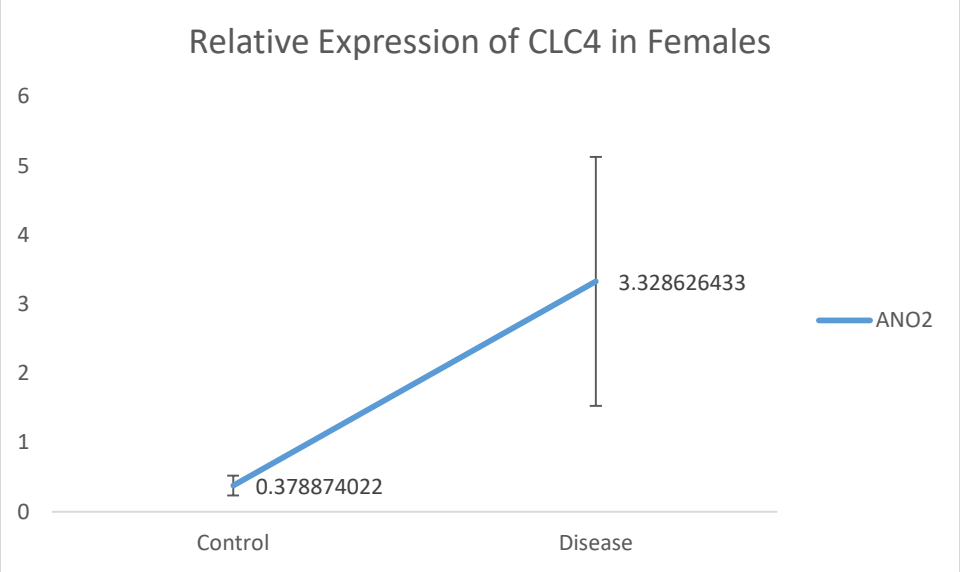

Fig 3 Relative expression of CLC4 gene in Females

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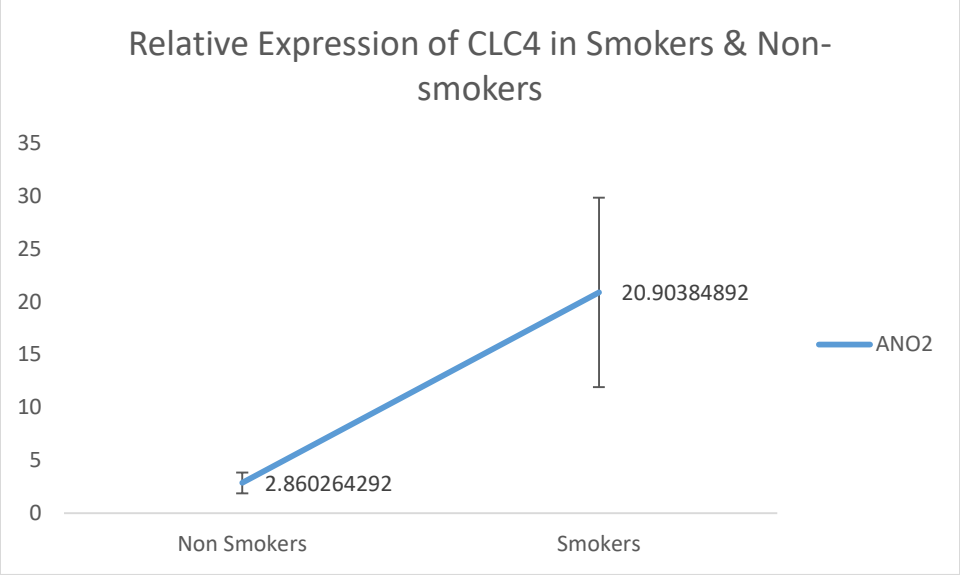

Fig 4 Relative expression of genes in smokers and non-smokers

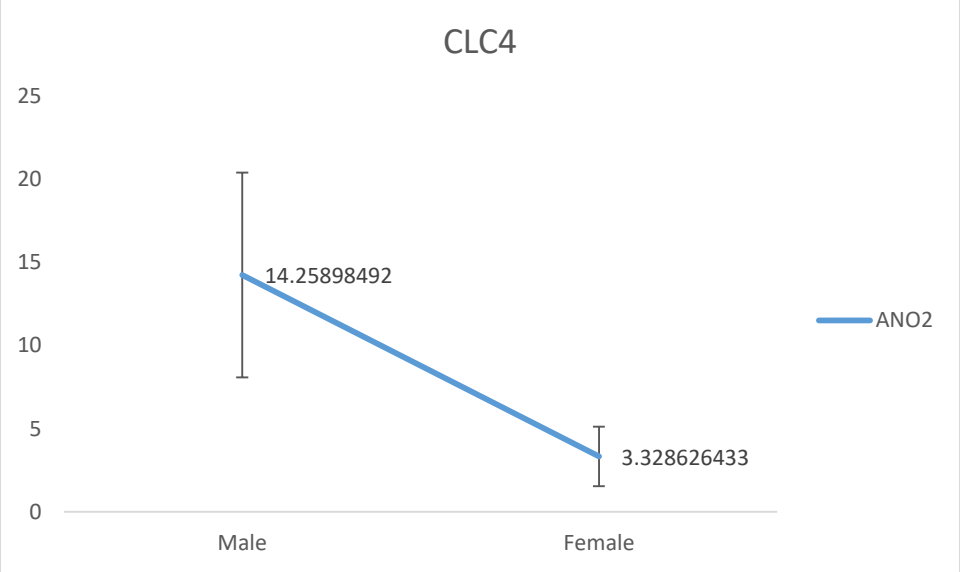

Fig 5 Relative expression of CLC4 gene in Males and Females

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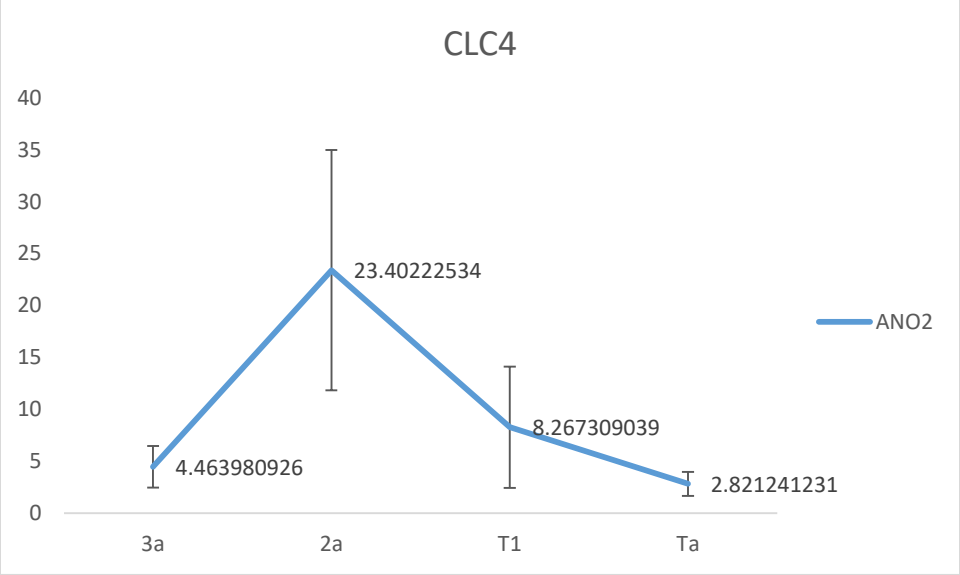

Fig 6 Relative expression of CLC4 in Tumor stage

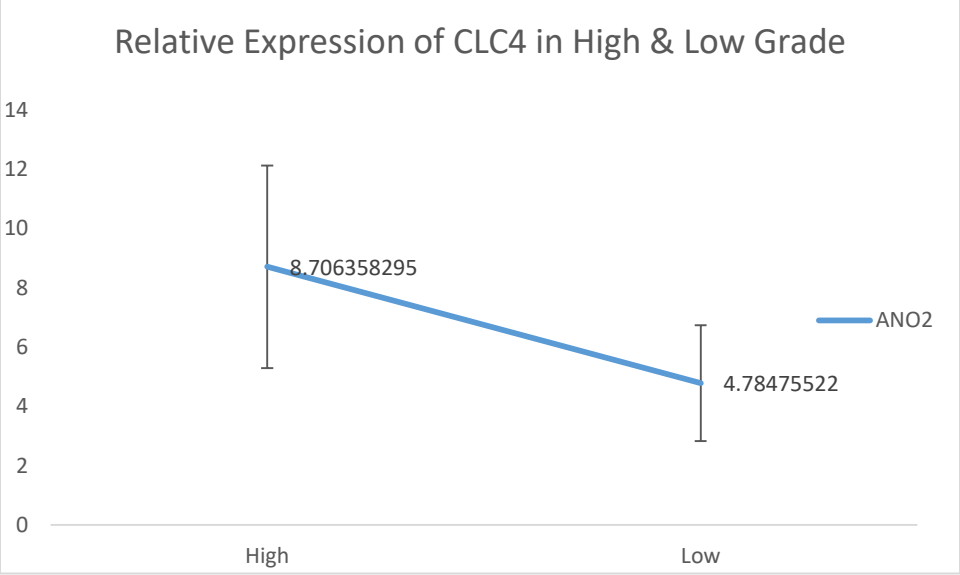

Fig 7 Relative expression of CLC4 in tumor grade

Discussion

Bladder cancer, also known as urological cancer, is one of the most common types of cancer worldwide. *ANO1 - 10* and *TMEM16A - K* are involved in a wide range of physiological functions. CaCCs are involved in olfaction, phototransduction, smooth muscle contraction, nociception, cell proliferation, and excitability modulation, among other things. EGF receptor (*EGFR*) and calmodulin-dependent protein kinase II (*CAMKII*) activation was lowered by

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knocking down or reducing its chloride-channel function. These key players have been recognized by scientifc studies to modulate signaling, infammatory and tumorigenic functions during the pathogenesis of associated carcinomas. Plasma levels of *CACCs* have been considered as a diagnostic biomarker for bladder carcinoma. It has been suggested that by knowing expression of these genes can improve the prognosis and drug designing. The variations in the expression and activity of these genes have been related to various infammatory mediators.

Long-term tobacco use, such as cigarette smoking, is the most common risk factor, accounting for more than half of newly diagnosed bladder cancer cases. Hydrocarbons, tryptophan metabolites, aromatic amines, and chemicals used in the rubber, electric, cable, paint, and textile sectors have all been linked to an increased risk of bladder cancer.

Ion channels and pumps regulate not just membrane potential, ion homeostasis, and electrical signalling in excitable cells, but also cell proliferation, migration, apoptosis, and differentiation (Litan and Langhans, 2015). Although cancer is not yet classified as channelopathy, the non-traditional involvement of ion channels and pumps is becoming more widely acknowledged. Many ion channels and pumps have been discovered as potential players in cancer start, control, and progression. *TMEM-16A (ANO1)* is a member of the *TMEM-16* family that is elevated in several malignancies (Kashyap et al., 2009). *TMEM-16A* was discovered to be amplified in oral cancer, head and neck squamous cell carcinoma (HNSCC), gastrointestinal stromal tumour, breast cancer, and other cancers before being identified as a calcium activated chloride channel (Duvvuri et al., 2012, Jia, Liu et al., 2015).

CLC4, also known as transmembrane protein and is a second protein structural component of the calcium-activated chloride channel (*CaCCs*). CaCCs regulates neuronal transmission in the central nervous system, specifically the hippocampus, inferior olive, olfactory epithelium, retina, and thalamus. The gene silencing reduced the growth of PC-3 xenograft tumours in nude mice and increased apoptosis in tumour cells. Those whose tumours expressed high amounts of *ANO1* had a poorer overall survival.

We obtained 20 matched samples of bladder cancer patients in this investigation, including tumour (post biopsy) and normal (surrounding unaffected tissue analysed microscopically) tissue samples. These samples were collected from two different Islamabad hospitals. Following RNA extraction, cDNA synthesis, and expression analysis, qPCR was performed. Real-time PCR is an

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effective, precise, cost-effective, and dependable method for quantifying gene expression in biological materials. The Levak method (2delta delta Ct) was used to assess the qPCR findings for relative expression.

Data from statistical analysis show that all of the genes under investigation, as well as other factors, are implicated in the development of bladder cancer. Data with p values of 0.05 is significant, and data with p values of 0.01 is extremely significant. By comparing the results of males, females, smokers, and non-smokers, it is found to be substantially elevated in cancer patients. Males are more affected than females when comparing males and females.

We discovered an increase in mRNA expression of *CLC4* in tumours when compared to controls using relative expression analysis. *CLC4* expression study revealed a 4.2 fold increase in tumour samples when compared to control samples. Furthermore, regulation has been demonstrated at various stages of bladder cancer. To understand the molecular mechanism of chloride channels, more research and a larger sample size are required. Increased understanding of the molecular mechanisms of chloride channels will aid in the development of novel anticancer medicines that target the individual chloride channels responsible for cancer cell initiation, invasion, and metastasis.

Conclusion

The levels of *CLC4* in bladder cancer tissue were measured in the current study. The results were strikingly different from what had previously been reported in other malignanciesThe genes involved in blood cancer are significantly elevated and are also responsible for other illnesses. To rule out this tumor-causing condition and discover a better cure, new technologies and medication therapies are required. In conclusion, our work demonstrates the critical significance of chloride channels in cancer formation.

RECOMMENDATIONS

Further research is required to validate our findings. Future research with large cohort studies will open up a new channel for investigating the role of the relevant gene in cancer production and aid in the development of innovative anticancer medicines**.**

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