PERINATAL JOURNAL

Original Article

Perinatal Journal 2026; 34(2):101-111

https://doi.org/10.57239/prn.26.03420010

NRF2 and PINK1 expression across pathological T stages of urothelial bladder carcinoma

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Abstract

Transcription factor protein called Nrf2 is important in ROS metabolism as the key to the expression of several genes including PINK1 which plays role in mitophagy, an important process in tumorigenesis for supporting metabolic plasticity of cancer cells. The role of those protein in cancer has a complex, often contradictory role, including in bladder carcinoma, the most common cancer in urinary tract. To determine the expression of Nrf2 and PINK2 and its correlation with T Stadium in urothelial bladder carcinoma. This study was an analytic observational study with cross-sectional approach. Samples were used from Formalin-Fixed Paraffin-Embedded (FFPE) preparations of urothelial carcinoma at the Anatomical Pathology Laboratory of general academic Soetomo Hospital Surabaya from January 2014 to December 2023. Samples were divided into pT1, pT2, pT3, and pT4 based on histopathological examination. Immunohistochemical examination using Nrf2 and PINK1 antibodies and differences in expression and correlation of both antibodies were analyzed using statistical tests. There was significant correlation between PINK1 expresson and T stage of urothelial bladder carcinoma (p value = 0,013). There was no correlation between Nrf2 expression and T stage (p value = 0,167). Furthermore, there was no significant correlation between Nrf2 and PINK1 expression (p value = 0.059). Expression of PINK1 plays role in T stage of urothelial bladder carcinoma and may have a potential prognostic value. Whereas expression of Nrf2 has no correlation with T Stage in this experiment.

Keywords: NRF2, PINK1, T stage, Cancer, Bladder carcinoma

Introduction

Bladder cancer is the most common malignancy of the urinary tract and ranks as the ninth most frequently diagnosed cancer worldwide (World Health Organization, 2022). Urothelial carcinoma represents the predominant histological subtype of bladder cancer (Williamson et al., 2022). In Indonesia, the five-year prevalence of urothelial carcinoma has reached 21,376 cases, with newly diagnosed cases ranking thirteenth nationwide (Global Cancer Observatory, 2022).

Histopathological diagnosis of bladder carcinoma is established most using tissue obtained from transurethral resection of bladder tumor (TURBT). However, TURBT specimens often present limitations in accurately assessing muscular invasion due to insufficient sampling depth and interpretative challenges (van Straten et al., 2023). Precise determination of pathological T stage (pT), particularly the depth of invasion into the bladder wall, is crucial, as prognosis and therapeutic strategies differ substantially between Non–Muscle-Invasive Bladder Cancer (NMIBC) and muscle-invasive bladder cancer (MIBC) (Williamson et al., 2022). NMIBC is generally managed with TURBT followed by intravesical Bacillus Calmette–Guérin (BCG) or intravesical chemotherapy, whereas MIBC requires radical cystectomy with bilateral pelvic lymph node dissection and neoadjuvant cisplatin-based chemotherapy (Degeorge et al., 2017). Despite advances in surgical and systemic therapies, bladder cancer remains challenging to treat due to its high recurrence rate, invasive behavior, and metastatic potential (Li et al., 2020).

At the molecular level, tumor invasion has been associated with dysregulated redox homeostasis, particularly involving reactive oxygen species (ROS). ROS are generated through aerobic respiration and cellular signaling pathways and exert dual effects on cancer cells, whereby moderate levels promote tumor survival, while excessive accumulation induces cell death (Kasai et al., 2020; Liu et al., 2023). Cancer cells typically exhibit elevated ROS levels compared to normal cells as a consequence of increased metabol activity (Nakamura & Takada, 2021). To counteract oxidative stress, cells activate antioxidant defense

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mechanisms regulated by nuclear factor erythroid 2–related factor 2 (Nrf2), a key transcription factor controlling the expression of genes involved in ROS detoxification and cellular survival (Kasai et al., 2020; Murata et al., 2015; Rojo de la Vega et al., 2018).

The role of Nrf2 in cancer is complex and context dependent. While Nrf2 activation protects normal cells from carcinogenesis induced by oxidative damage, persistent activation in cancer cells has been shown to promote tumor progression by enhancing resistance to oxidative stress (Li et al., 2020). In bladder cancer, inhibition of Nrf2 has been reported to increase sensitivity to cytotoxic therapies and suppress tumor cell migration, suggesting a role in tumor invasion (Ciamporcero et al., 2018).

One downstream target gene regulated by Nrf2 is phosphatase and tensin homolog–induced kinase 1 (PINK1), a serine/threonine kinase involved in mitophagy, the selective elimination of damaged mitochondria (Murata et al., 2015; Vara-Perez et al., 2019). Mitophagy plays an important role in tumorigenesis by supporting metabolic plasticity, enabling cancer cells to adapt to metabolic stress through recycling of cellular components (Vara-Perez et al., 2019). Unlike normal cells, which primarily rely on mitochondrial oxidative phosphorylation, bladder cancer cells predominantly utilize aerobic glycolysis to rapidly generate ATP. This metabolic reprogramming is reflected by increased expression of genes involved in glycolysis, the pentose phosphate pathway, and fatty acid synthesis, indicating reduced mitochondrial activity (Cormio et al., 2017).

Elevated PINK1 expression has been reported in several malignancies, including non–small cell lung cancer, where it is associated with chemoresistance (Zhang et al., 2017), and papillary renal cell carcinoma compared with normal renal tissue (Simon et al., 2020). However, studies investigating PINK1 expression in urothelial carcinoma remain limited and have primarily utilized molecular techniques such as western blotting and polymerase chain reaction (PCR), rather than tissue-based analyses (Lou et al., 2021; Zhang et al., 2024).

Given the marked differences in prognosis and management between NMIBC and MIBC, identifying molecular factors associated with tumor invasion is of significant clinical importance. Previous in vitro studies have demonstrated that upregulation of Nrf2 expression enhances migration of urothelial carcinoma cells, suggesting its potential involvement in invasive behavior (Vilchez Mercedes et al., 2022). Furthermore, increased PINK1 expression has been associated with metastatic disease in ovarian cancer (Zheng et al., 2023). To date, no studies have evaluated the expression of Nrf2 and PINK1 in bladder urothelial carcinoma tissue and their correlation with muscle invasion status. Therefore, this study aims to investigate the association between Nrf2 and PINK1 expression and muscle invasion in urothelial bladder carcinoma.

Methods

Study design

This study was an analytical observational study with a retrospective cross-sectional design. No intervention was applied. The objective was to evaluate the expression of nuclear factor erythroid 2–Related Factor 2 (Nrf2) and phosphatase and tensin homolog–induced kinase 1 (PINK1) in urothelial bladder carcinoma across different pathological T stages.

Study population, samples, and sampling technique

The study population comprised Formalin-Fixed Paraffin-Embedded (FFPE) tumor tissue blocks obtained from radical cystectomy specimens of patients diagnosed with urothelial bladder carcinoma at the Anatomical Pathology Laboratory, Dr. Soetomo General Hospital, Surabaya, Indonesia, between January 2014 and December 2023.

Samples were selected using non-probability purposive sampling based on predefined inclusion and exclusion criteria. Inclusion criteria were FFPE tissue blocks derived from radical cystectomy specimens with histopathologically confirmed urothelial carcinoma and adequate representative tumor tissue for immunohistochemical analysis. Exclusion criteria included damaged, missing, exhausted, or unsectionable tissue blocks and specimens submitted for second-opinion evaluation.

A total of 49 FFPE tissue blocks were included, consisting of non–muscle-invasive bladder cancer (NMIBC, pT1) and muscle-invasive bladder cancer (MIBC, pT2–pT4) cases.

Pathological evaluation

Pathological diagnosis and T staging were determined according to the WHO Classification of Tumours of the Urinary Tract (2022) and the American Joint Committee on Cancer (AJCC) staging system. Tumor invasion was categorized as pT1, pT2, pT3, or pT4 based on the depth of invasion into bladder structures. Histopathological evaluation was performed independently and simultaneously by two observers using a binocular light microscope (Olympus CX31).

Immunohistochemical analysis

Immunohistochemical staining was performed on FFPE tissue sections (<4 µm). Nrf2 expression was assessed using a monoclonal anti-Nrf2 antibody (bsm-52179R; dilution 1:200; Bioss Antibodies), with positive expression defined as nuclear staining. PINK1 expression was evaluated using a monoclonal anti- PINK1 antibody (bsm-51265M; dilution 1:200; Bioss Antibodies), with positive expression defined as cytoplasmic staining.

Both markers were evaluated semi-quantitatively using the histochemical scoring (H-score) method, calculated as: H-score = (0 × % unstained cells) + (1 × % weakly stained cells) + (2 × % moderately stained cells) + (3 × % strongly stained cells), resulting in scores ranging from 0 to 300. Assessments were performed independently by two observers using a binocular light microscope (Olympus CX31).

Equipment and reagents

Key equipment included a Leica microtome, Olympus CX31 binocular light microscope, Olympus DP2-BSW digital imaging system, Leica paraffin bath, and standard histopathology laboratory instruments. Reagents included monoclonal antibodies against Nrf2 and PINK1 (Bioss Antibodies), xylene, phosphate- buffered saline, trypsin, diaminobenzidine (DAB), and chromogen solutions.

Study setting and period

The study was conducted at the Anatomical Pathology Laboratory, Dr. Soetomo General Hospital, Surabaya, Indonesia. Laboratory procedures and data analysis were performed between January and June 2024.

Statistical analysis

Statistical analysis was performed using SPSS software. Descriptive statistics summarized clinicopathological characteristics, including pathological diagnosis, T stage, sex, and age. Data normality was assessed prior to inferential analysis. Differences in Nrf2 and PINK1 expression across invasion stages were analyzed using the Kruskal–Wallis test. Correlations between Nrf2 and PINK1 expression were evaluated using Spearman's rank correlation test, as appropriate. A p-value < 0.05 was considered statistically significant.

Result

This study evaluated the expression of nuclear factor erythroid 2–related factor 2 (Nrf2) and phosphatase and tensin homolog–induced kinase 1 (PINK1) in urothelial bladder carcinoma tissues to analyze their association with muscle invasion status. Population data were obtained from medical records at the Anatomical Pathology Laboratory, Dr. Soetomo General Hospital, Surabaya. Descriptive analyses were performed to summarize clinicopathological characteristics, while inferential analyses assessed differences and correlations between pathological T stage and Nrf2 and PINK1 expression.

The age of patients ranged from 33 to 70 years. The youngest patient (33 years) was diagnosed with pT4 disease, while the oldest patient (70 years) belonged to the pT1 (NMIBC) group. The mean age of the study population was 55.24 years. The most frequently observed ages were 55 and 60 years, each occurring in 4 patients (8.0%). When stratified into 10-year age intervals, the highest proportion of cases was observed in the 51–60-year age group (42.9%). NMIBC cases were most frequently observed in the 61–70-year age group, whereas MIBC cases were most commonly observed in patients aged 61–70 years (Table 1).

Table 1. Characteristics of sample

Characteristics	T1 Stage	T2 Stage	T3 Stage	T4 Stage	Total n (%)
Sex					
Male	4	17	9	16	46 (93,8%)
Female	0	1	0	2	3 (6,2%)
Age					
31-40	0	3	0	1	4 (8,16%)
41-50	1	1	3	5	18 (36,7%)
51-60	1	6	5	9	9 (18,4%)
61-70	2	8	1	3	18 (36,7%)
Total	4	18	9	18	49

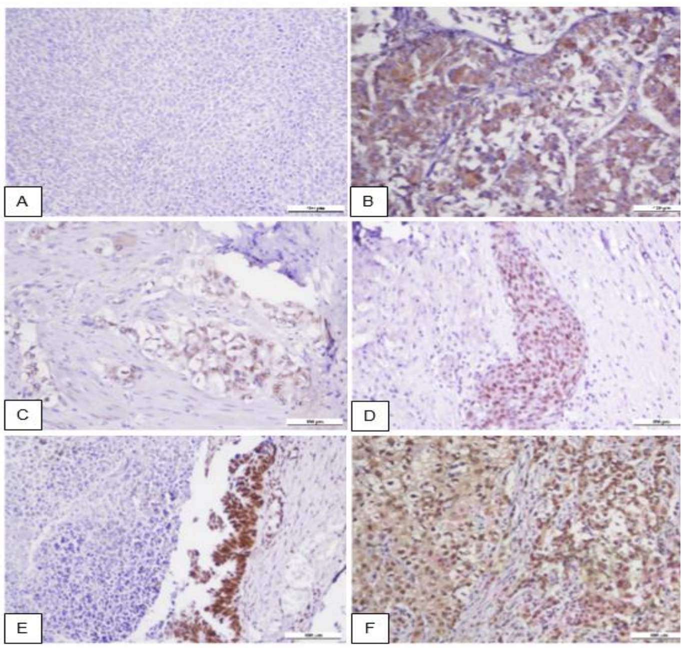

Figure 1. Nrf2 expression in urothelial carcinoma tumor cells. (A) Negative expression; (B) Cytoplasmic Nrf2 expression; (C) Weak nuclear expression; (D) Moderate nuclear expression;
(E) Strong nuclear expression; (F) Combined nuclear and cytoplasmic expression

Clinicopathological characteristics: A total of 49 patients with urothelial bladder carcinoma were included in this study. Based on pathological T stage, 4 cases (8.2%) were classified as pT1, 18 cases (36.7%) as pT2, 9 cases (18.4%) as pT3, and 18 cases (36.7%) as pT4. Accordingly, 4 cases were categorized as non–muscle-invasive bladder cancer (NMIBC), whereas 45 cases were classified as muscle-invasive bladder cancer (MIBC).

The study population was predominantly male, with 46 male patients (93.8%) and 3 female patients (6.2%). Male patients were distributed across all pathological stages, including 4 cases at pT1 (8.2%), 17 cases at pT2 (34.7%), 9 cases at pT3 (18.4%), and 16 cases at pT4 (32.7%). Female patients were observed only in pT2 (1 case, 2.0%) and pT4 (2 cases, 4.1%) stages. No female patients were identified in the pT1 or pT3 groups; consequently, all NMIBC cases in this study occurred in male patients.

Nrf2 and PINK1 expression according to pathological T stage

Nrf2 expression in urothelial bladder carcinoma was predominantly negative, with 26 cases showing no nuclear staining. Statistical analysis demonstrated that Nrf2 expression was not normally distributed (Kolmogorov–Smirnov test, $p < 0.001$). Comparison across pathological T stages using the Kruskal–Wallis test revealed no significant difference in Nrf2 expression among stages ($p = 0.149$). Consistently, Spearman's rank correlation analysis showed no significant association between Nrf2 expression and pathological T stage ($\rho = 0.201$, $p = 0.137$) (Table 2).

In contrast, PINK1 expression was detected in all cases and exhibited a non-normal distribution (Kolmogorov–Smirnov test, $p = 0.023$). Kruskal–Wallis analysis demonstrated a significant difference in PINK1 expression across pathological T stages ($p = 0.043$). Furthermore, Spearman's rank correlation analysis revealed a significant positive correlation between PINK1 expression and pathological T stage ($\rho = 0.353$, $p = 0.013$), indicating higher PINK1 expression in more advanced tumors (Table 2). Correlation analysis between Nrf2 and PINK1 expression showed no statistically significant association ($\rho = 0.271$, $p = 0.059$) (Table 2).

Table 2. Correlation analysis among Nrf2 Expression, PINK1 expression, and pathological T stage in urothelial carcinoma of the urinary bladder

Variables	Spearman's ρ	p value
Nrf2 expression vs Pathological T stage	0.215	0.137
PINK1 expression vs Pathological T stage	0.353	0.013
Nrf2 expression vs PINK1 expression	0.271	0.059

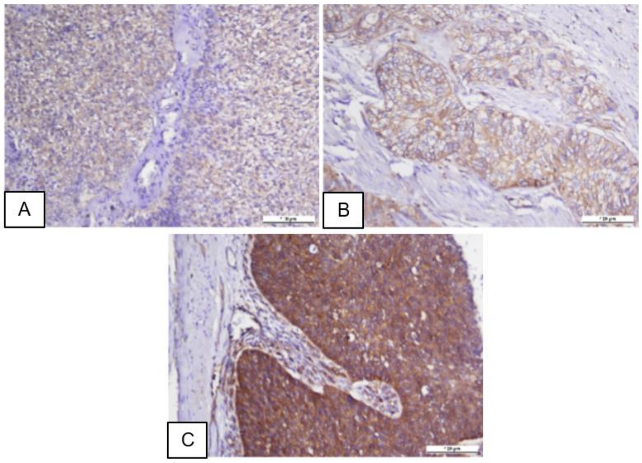

Figure 2. PINK1 expression in urothelial carcinoma tumor cells. (A) Weak positive expression; (B) Moderate positive expression; (C) Strong positive expression

Discussion

Clinicopathological characteristics of the study population

Bladder cancer is the tenth most frequently diagnosed malignancy worldwide, with the highest incidence observed in developing countries (Bray et al., 2018). Urothelial carcinoma represents the predominant histological subtype of bladder cancer (Williamson et al., 2022). In the present study, all samples were derived from radical cystectomy specimens and were histologically confirmed as urothelial carcinoma, ensuring homogeneity of tumor subtype.

A large meta-analysis reported that approximately 75% of bladder cancer cases are diagnosed at stage T1 or classified as non–muscle-invasive bladder cancer (NMIBC) (Catto et al., 2021). In contrast, NMIBC accounted for only 8.2% of cases in this study. This discrepancy is primarily attributable to the inclusion criteria, as only specimens obtained from radical cystectomy were analyzed. Radical cystectomy is generally indicated for muscle-invasive disease (≥ pT2), whereas NMIBC is commonly managed with transurethral resection followed by intravesical therapy, such as Bacillus Calmette–Guérin (BCG) or chemotherapy (Compérat et al., 2022). Consequently, early-stage tumors were underrepresented in this cohort.

Sex distribution

The study population demonstrated a marked male predominance, with males accounting for 93.8% of cases. This finding is consistent with epidemiological data reported by Siegel et al. (2019), which showed that approximately three-quarters of newly diagnosed bladder cancer cases occur in men (Siegel et al., 2019). However, the male predominance observed in this study was more pronounced than that reported in population-based studies.

Globally, age-standardized male-to-female incidence ratios for bladder cancer range from approximately 2:1 to 6:1, depending on geographic region and environmental exposure (Ferlay et al., 2021; Jubber et al., 2023). The male-to-female ratio observed in this study (15.3:1) exceeds these estimates, likely reflecting referral bias and the predominance of advanced-stage disease requiring radical surgical management.

Age distribution

Previous studies have reported a wide age range for urothelial bladder carcinoma, spanning from adolescence to advanced age. In contrast, the age range in this study was narrower (33–70 years), with patients under 40 years of age comprising only 4% of the cohort. This proportion is lower than that reported by Aliramaji et al. (10.9%) but comparable to findings by Gupta et al. (5.19%) (Aliramaji et al., 2015; Gupta et al., 2009).

The mean age of patients in this study was younger than that reported in large epidemiological cohorts, such as the study by Yang et al., which reported mean ages of 66 and 71 years for female and male patients, respectively (Yang et al., 2013). The most common age group in the present study was 51–60 years, which is younger than the peak age groups reported in previous studies. This difference may reflect regional variations, lifestyle-related risk factors, or selection bias toward surgically treated patients.

Association of Nrf2 expression with pathological T stage

Nuclear factor erythroid 2–related factor 2 (Nrf2) is a key transcription factor involved in cellular defense against oxidative stress. Under physiological conditions, Nrf2 is weakly expressed in the cytoplasm and undergoes rapid degradation through its interaction with Kelch-like ECH-associated protein 1 (Keap1) (Wu et al., 2024). Upon oxidative stress, Nrf2 dissociates from Keap1, translocates into the nucleus, and activates the transcription of antioxidant and cytoprotective genes via the antioxidant response element (ARE) (Rojo de la Vega et al., 2018).

Although Nrf2 plays a protective role in normal tissues, its function in cancer is complex and context dependent. While Nrf2 activation may suppress carcinogenesis in early stages, sustained or aberrant activation can promote tumor progression, chemoresistance, angiogenesis, and metastasis (Wu et al., 2019, 2024). Constitutive activation of Nrf2 in cancer has been linked to genetic alterations affecting KEAP1, CUL3, or Nrf2 itself, resulting in impaired negative regulation (Baird et al., 2022).

In this study, Nrf2 expression was evaluated using nuclear immunoreactivity and quantified using H-score analysis. The results demonstrated no significant difference in Nrf2 expression across pathological

T stages and no significant correlation between Nrf2 expression and muscle invasion. These findings contrast with studies by Bocci et al. and Mercedes et al., which suggested that Nrf2 promotes aggressive invasion and migration in bladder cancer through partial epithelial–mesenchymal transition (EMT) (Bocci et al., 2019; Vilchez Mercedes et al., 2022).

Previous evidence indicates that invasive urothelial carcinoma cells exhibit higher Nrf2 activity compared to normal urothelial cells. Hayashi et al. reported that inactivation of the tumor suppressor gene GULP1 leads to increased nuclear accumulation of Nrf2 in invasive bladder cancer (Hayashi et al., 2020). However, existing data do not demonstrate consistent differences in Nrf2 expression among various pathological T stages once invasion has occurred. Therefore, Nrf2 may contribute to the acquisition of invasive potential but may not discriminate between degrees of tumor invasion in advanced disease.

Association of PINK1 expression with pathological T stage

Mitochondrial homeostasis plays a crucial role in cancer biology, and several anticancer agents exert their effects by disrupting mitochondrial function through apoptosis, autophagy, or metabolic inhibition (Wang et al., 2022). PTEN-induced putative kinase 1 (PINK1) is a central regulator of mitophagy, facilitating the selective removal of damaged mitochondria to maintain intracellular homeostasis (Wang et al., 2023; Yao et al., 2019).

PINK1 exhibits dual roles in cancer, acting as either a tumor suppressor or tumor promoter depending on tumor type and cellular context. High PINK1 expression has been associated with favorable outcomes in certain cancers, such as hepatocellular and renal carcinoma, whereas elevated PINK1 levels correlate with poor prognosis in breast, lung, ovarian, glioma, and melanoma (Wang et al., 2022).

In this study, PINK1 expression was detected in the cytoplasm of tumor cells and quantified using H-score analysis. All cases demonstrated positive PINK1 expression. Importantly, PINK1 expression differed significantly across pathological T stages and showed a positive correlation with muscle invasion, suggesting an association between higher PINK1 expression and advanced tumor stage.

Although the role of PINK1 in bladder cancer remains poorly understood, previous studies have suggested that reduced PINK1/Parkin signaling may suppress bladder cancer progression (Lou et al., 2021; Zhu et al., 2020). Conversely, Zheng et al. demonstrated that elevated PINK1 expression promotes metastasis and chemoresistance in ovarian cancer through the PINK1–PTEN pathway and induction of EMT via downregulation of E-cadherin (Zheng et al., 2023). Similar pro-invasive effects of PINK1 have been reported in lung cancer models (Liu et al., 2018). Collectively, these findings support a potential pro-tumorigenic role of PINK1 in advanced urothelial carcinoma.

Relationship between Nrf2 and PINK1 expression

Nrf2 regulates the expression of multiple antioxidant genes through binding to ARE sequences, which have been identified in the promoter region of PINK1, suggesting that PINK1 may be a downstream target of Nrf2 (Murata et al., 2015). Contrary to this hypothesis, the present study did not demonstrate a significant correlation between Nrf2 and PINK1 expression.

This lack of correlation may be explained by the fact that PINK1 expression is regulated by multiple transcription factors beyond Nrf2. Nuclear respiratory factor 1 (NRF-1), FOXO3a, NF-κB, and mitochondrial proteins such as DARS-2 have all been implicated in the regulation of PINK1 expression through distinct molecular mechanisms (Duan et al., 2014; Gupta et al., 2022; Li et al., 2025; Lu et al., 2020). Therefore, Nrf2 may not serve as the dominant regulator of PINK1 expression in invasive urothelial carcinoma.

Furthermore, although Nrf2-mediated regulation of PINK1 has been demonstrated in normal cells, evidence linking this pathway directly to tumor invasion or pathological staging in urothelial carcinoma remains limited. Thus, the absence of correlation observed in this study suggests that Nrf2 and PINK1 may influence bladder cancer progression through independent or context-specific mechanisms.

Study limitations

This study has several limitations. The sample distribution was weighted toward advanced pathological stages due to the inclusion of radical cystectomy specimens, resulting in underrepresentation of NMIBC cases. Consequently, the findings may not be generalized to early-stage bladder cancer. Additionally, the cross-sectional design precludes causal inference, and further molecular and functional studies are warranted to elucidate the mechanistic roles of Nrf2 and PINK1 in urothelial carcinoma progression.

Conclusion

This study shows that PINK1 expression is significantly associated with pathological T stage and muscle invasion in urothelial carcinoma of the urinary bladder, while Nrf2 expression is not significantly associated with tumor stage. These findings suggest that PINK1 may play a more relevant role in tumor progression and invasiveness compared to Nrf2. The absence of correlation between Nrf2 and PINK1 indicates that these pathways may act independently in muscle-invasive disease. Further studies are needed to clarify the molecular mechanisms underlying PINK1-mediated tumor progression and its potential clinical relevance.

Acknowledgements

The authors would like to thank the laboratory staff of the Department of Anatomical Pathology, Faculty of Medicine Universitas Airlangga Surabaya, for their technical assistance in the preparation of immunohistochemical specimens.

Funding

The authors received no financial support for the research, authorship, and/or publication of this article.

Conflict of interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Author contribution

K.I.: Conceptualization, Data curation, Writing-Original draft preparation; A.S.R: Conceptualization, Methodology, writing – Review & Editing; G.M.: Supervision, Writing – Review & Editing.

Data availability statement

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

Institutional review board statement

This study was conducted in accordance with the ethical principles of the Declaration of Helsinki. Ethical approval was obtained from the Health Research Ethics Committee of Dr. Soetomo General Academic Hospital, Surabaya, Indonesia, with approval number 3076/120/4/VIII/2024. Informed

Consent statement

Not applicable.

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