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Dysregulation of the NRF2/KEAP1 signaling pathway and its impact on the development of bladder cancer

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ABSTRACT

Aim: To perform a comparative analysis of genetic alterations in the Nuclear factor erythroid 2-related factor 2 or NFE2L2 / Kelch-like ECH-associated protein 1 (NRF2/KEAP1) pathway and to evaluate their impact on bladder cancer pathology.

Methods: DNA sequencing was performed on 40 bladder cancer patients to identify potential mutations in the KEAP1 and NRF2 genes. The pathogenic effects of the identified mutations were predicted using PolyPhen-2 and SNAP tools. Various databases were utilized to analyze mutational profiles, mRNA expression alterations, and survival outcomes, while the STRING database was employed to examine protein–protein interactions.

Results: Experimental mutation analysis detected 9 mutations in the KEAP1 and NRF2 genes. Analysis of data from the Cancer Genome Atlas (TCGA) identified a total of 31 mutations across these two genes. In patients with high-grade bladder cancer, potentially pathogenic mutations were observed in the BTB and IVR domains of KEAP1 and in the topologically associating domain (TAD) of NRF2. Expression levels of NRF2 were significantly lower in patients compared to healthy controls ($p < 0.01$). Protein–protein interaction analysis revealed interactions with the Cullin 3 (CUL3) splicing factor, which is a core component of the E3 ubiquitin ligase complex.

Conclusion: The findings of the present study suggest that gene mutations and expression alterations within the NRF2/KEAP1 pathway may serve as potential risk factors for the development of bladder cancer.

Keywords: Bladder cancer, NRF2/KEAP pathway, mutation, expression.

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1. Introduction

Bladder cancer is a prevalent malignancy globally and is notable for its pronounced heterogeneity with substantial variation in underlying risk factors, biological characteristics and clinical outcomes among patients [1,2]. Its incidence is nearly threefold higher in men than in women with environmental exposures, aging and lifestyle-related factors playing important roles in its development. In addition, approximately 25% are present with disease that has invaded the muscular layer of the bladder, a condition that markedly elevates the risk of metastatic spread and represents a serious threat to patient survival [3,4]. Conventional therapeutic strategies including transurethral resection,

radical cystectomy and intravesical Bacillus Calmette-Guérin (BCG) therapy often prove insufficient due to cancer recurrence or progression to invasive forms. Despite advances in diagnostic and therapeutic approaches, current strategies have not yet provided satisfactory solutions for bladder cancer management [5,6]. Clinical outcome is largely determined by tumor grade, invasion depth, lymph node status, and the emergence of chemotherapy resistance, highlighting the need for more refined molecular stratification and targeted therapeutic approaches [7,8]. Accordingly, detailed characterization of the genetic mechanisms involved in bladder cancer progression, as well as the identification of molecular biomarkers, is crucial for the development of improved therapeutic strategies.

The Nuclear Factor Erythroid 2-Related Factor 2 (NFE2L2, commonly referred to as NRF2)/ Kelch-like ECH-associated protein 1 (KEAP1) signaling pathway is essential for protecting various cell types, tissues and organs from oxidative stress-related damage. This pathway has led to NRF2 being described as a "multiorgan protector" [9,10]. NRF2 regulates cellular responses to oxidative stress by controlling mechanisms that prevent damage to cellular components sensitive to redox alterations. It serves as the key regulator of the oxidant/antioxidant balance whereas KEAP1 inhibits NRF2 under basal conditions [11-12]. Upon oxidative stress, NRF2 is activated and cooperates with Maf family transcription factors to induce cytoprotective genes. The NRF2 heterodimer rapidly translocates to the nucleus via a nuclear localization signal and binds to the antioxidant response element (ARE) sequence.

Growing evidence indicates that dysregulation of the NRF2/KEAP1 signaling pathway contributes to tumor initiation and progression across multiple cancer types. Genetic alterations, including mutations and aberrant expression of pathway components, have been shown to promote persistent NRF2 activation, thereby conferring growth advantages to malignant cells [13]. Aberrant activation of the NRF2/KEAP1 signaling pathway has been shown to increase therapy resistance and confer a selective growth advantage to tumor cells through the maintenance of cytoprotective and antioxidant gene expression. Accumulating evidence indicates that malignant cells actively exploit elevated NRF2 activity to enhance survival, sustain proliferation, and adapt to metabolic and oxidative stress, thereby contributing to several hallmarks of cancer, including therapy resistance [14,15].

The tumor-promoting role of NRF2 was initially identified in lung cancer, where genetic alterations in the NFE2L2/KEAP1/CUL3 axis lead to constitutive activation of the pathway [16,17]. Subsequent research has shown that that dysregulated NRF2/KEAP1 signaling extends beyond lung malignancies and plays a crucial role in a variety of cancers, such as pancreatic, ovarian, liver and gallbladder tumors. In non-small cell lung cancer, both somatic mutations and epigenetic silencing of KEAP1 have been identified as common mechanisms underlying sustained NRF2 activity [18]. Furthermore, experimental evidence suggests that NRF2 mutations can occur at early stages of hepatocarcinogenesis, highlighting the contribution of NRF2 pathway deregulation not only to tumor progression but also to tumor initiation [19].

In this study, we explore mutations and variants within the NRF2/KEAP1 pathway which regulates a wide range of cellular processes and has been increasingly recognized

for its oncogenic potential. We subsequently evaluated how these alterations may contribute to tumor development and disease progression. By focusing on pathway-specific changes in bladder tumor tissue; our goal is to identify novel biomarkers that could support clinical decision making in diagnosis, prognosis and disease management.

2. Materials and methods

2.1. Study Population: The study cohort comprised 40 patients (36 males and 4 females), ranging in age from 41 to 84 years, all of whom had been diagnosed with bladder cancer and were admitted to the Urology Clinic of Nigde Omer Halisdemir University Training and Research Hospital. Blood and tissue samples were collected from the patients. Mutation analysis was performed on blood samples and tumor grading was evaluated in tissue specimens. Approval for the study protocol was granted by the Non-Interventional Clinical Research Ethics Committee at Nigde Omer Halisdemir University (Decision No. 2021/06, dated 14 January 2021).

Biopsy samples were obtained using Transurethral Resection (TUR) for bladder tumors or bladder punch biopsy methods. Histopathological evaluation of papillary urothelial carcinomas was performed to classify tumors into low-grade and high-grade categories. Low-grade papillary urothelial carcinomas were defined by regular tissue architecture and cellular morphology with evenly distributed, polarized, and tightly adherent cells. These tumors exhibited minimal but distinct atypia, including hyperchromatic nuclei, rare mitotic figures primarily in the basal region and slight variations in nuclear size and shape. In contrast, high-grade papillary urothelial carcinomas displayed larger, hyperchromatic nuclei with potentially loose cell junctions and more pronounced atypical features. Among the patient cohort, 14 individuals were diagnosed with invasive high-grade bladder carcinoma while 16 patients presented with low-grade bladder carcinoma.

2.2. Mutation Profile Analysis: Genomic DNA was extracted using the Zymo Quick-DNA Kits (Zymo Research Corp., Irvine, CA, USA). The purity and concentration of the isolated DNA were assessed spectrophotometrically. Polymerase Chain Reaction (PCR) amplification was performed targeting three exons of the KEAP1 gene and two exons of the NRF2 gene. Approximately, 200 ng of DNA was used as template for PCR amplification.

For mutation detection, Sanger DNA sequencing (ABI Prism 3100, Applied Biosystems) was employed to identify potential mutations and polymorphisms in the KEAP1 and NRF2 genes among the 40 bladder cancer patients. The raw sequencing data were processed using Chromas version 2.6.5, and mutations were identified by comparison with reference sequences retrieved from the Ensembl genome browser.

2.3. Bioinformatic Analysis of KEAP1/NRF2 Mutations: The cBio Cancer Genomics Portal (http://cbioportal.org) serves as an open-access bioinformatics platform that consolidates data from The Cancer Genome Atlas (TCGA), including somatic mutations, copy number variations, mRNA expression profiles obtained via microarray and RNA sequencing, DNA methylation patterns, and protein as well as phosphoprotein expression data [20]. For a comprehensive evaluation of NRF2 and KEAP1 gene mutations in bladder cancer samples (n = 406), bladder cancer was selected as the target disease in the cBioPortal platform, followed by mutation analysis using the OncoPrint, Cancer Types Summary, and

2.4. Mutation Analysis modules: To assess the potential pathogenicity and clinical significance of mutations identified in polarity complex genes scores from the Polymorphism Phenotyping v2 (PolyPhen-2) and SIFT databases were utilized. PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) is a publicly accessible computational tool that evaluates the likely effects of mutations and single nucleotide polymorphisms (SNPs) on protein function and stability based on structural features and evolutionary conservation of amino acid positions. PolyPhen-2 predicts the likely structural and functional effects of amino acid changes, categorizing variants as "probably damaging," "possibly damaging," "benign," or "unknown" [21]. Meanwhile, the SIFT algorithm (https://sift.bii.a-star.edu.sg/) determines the influence of amino acid substitutions on protein activity by considering sequence homology, evolutionary conservation, and the physicochemical characteristics of residues, classifying variants as either "tolerated" or "deleterious" [22].

2.5. Gene Expression Dynamics and Survival Analysis: GEPIA (http://gepia.cancer-pku.cn/) represents an extensive database comprising gene expression profiles from 9,736 tumor specimens and 8,587 normal tissue samples [23]. The expression patterns of KEAP1 and NRF2 were examined through graphical outputs provided by the platform, integrating data from The Cancer Genome Atlas (TCGA) and the Genotype-Tissue Expression (GTEx) projects, including 404 bladder cancer samples and 28 normal tissue specimens. The prognostic significance of high versus low mRNA expression levels of the target genes was assessed using the GEPIA platform. Transcript levels were expressed as transcripts per million (TPM). To perform differential expression analysis, data were log2-transformed [log2(TPM + 1)], and log2 fold change (log2FC) was calculated by comparing median expression levels between tumor and normal tissues. Genes exceeding the predefined thresholds for both log2FC and q value were defined as differentially expressed. Survival analysis revealed that patients with high risk scores had significantly reduced recurrence-free survival and worse prognostic outcomes compared with those in the low-risk group. Survival analyses based on differential gene expression levels were performed using a web-based platform.

2.6. Protein–protein interaction analysis: STRING (http://string-db.org) is a widely used database for constructing protein–protein interaction (PPI) networks, providing confidence scores for each interaction between proteins [24]. It is a high-confidence and integrative bioinformatics resource that combines diverse interaction evidence, including experimental data, computational predictions, text mining and curated database information. STRING is specifically designed to map global networks of protein interactions, encompassing both direct (physical) and indirect (functional) associations. Using this platform the predicted interactions between KEAP1 and NRF2 proteins were analyzed given their pivotal role in regulating cellular oxidative stress responses and cancer-associated pathways. The database offered insights into both known and predicted interactions facilitating a deeper understanding of how NRF2/KEAP1 mutations may alter cellular networks and downstream signaling mechanisms.

2.7. Statistical analysis: All statistical evaluations related to the in silico analyses were performed using the GEPIA platform. Differences in gene expression levels were

assessed using one-way ANOVA. Overall survival was analyzed through Kaplan–Meier survival plots. For hypothesis testing, GEPIA employs the Log-rank (Mantel–Cox) test which was also used to compare gene expression between high- and low-expression cohorts. For all analyses, results with p-values < 0.05 were regarded as statistically significant.

3. Results

3.1. Results of Mutation Profile Analysis: Mutation analysis was carried out using Sanger sequencing on genomic DNA isolated from blood samples of bladder cancer patients. In total, nine variants were detected in the KEAP1 and NRF2 genes, including five missense mutations, one frameshift mutation, and three single nucleotide polymorphisms (SNPs). All identified variants had been previously documented in the Human Gene Mutation Database (HGMD). The identified variants exhibited a heterozygous genotype and are illustrated in the electropherograms shown in Figure 1.

The most frequently altered gene was KEAP1, in which 8 mutations were identified. Among these, 4 were missense mutations, 3 were SNPs and 1 was a frameshift mutation. The p.D256G, p.L115Q, p.Q178L and p.I137Mfs*33 mutations detected in KEAP1 were observed in 4 different patients diagnosed with high-grade bladder carcinoma. The p.L115Q and p.Q178L mutations are located within the BTB domain whereas the p.D256G and p.T277M mutations are situated in the IVR domain. The IVR domain of KEAP1 contains a conserved nuclear export signal sequence essential for the cytoplasmic localization of the protein. Therefore, the p.D256G and p.T277M mutations, being positioned within a region involved in intracellular signaling, are considered particularly significant.

The p.I137Mfs*33 frameshift mutation causes a shift in the reading frame, potentially leading to premature termination of the polypeptide chain, making it a mutation of high functional importance. In addition, three distinct SNPs located within intronic regions were identified in the study cohort.

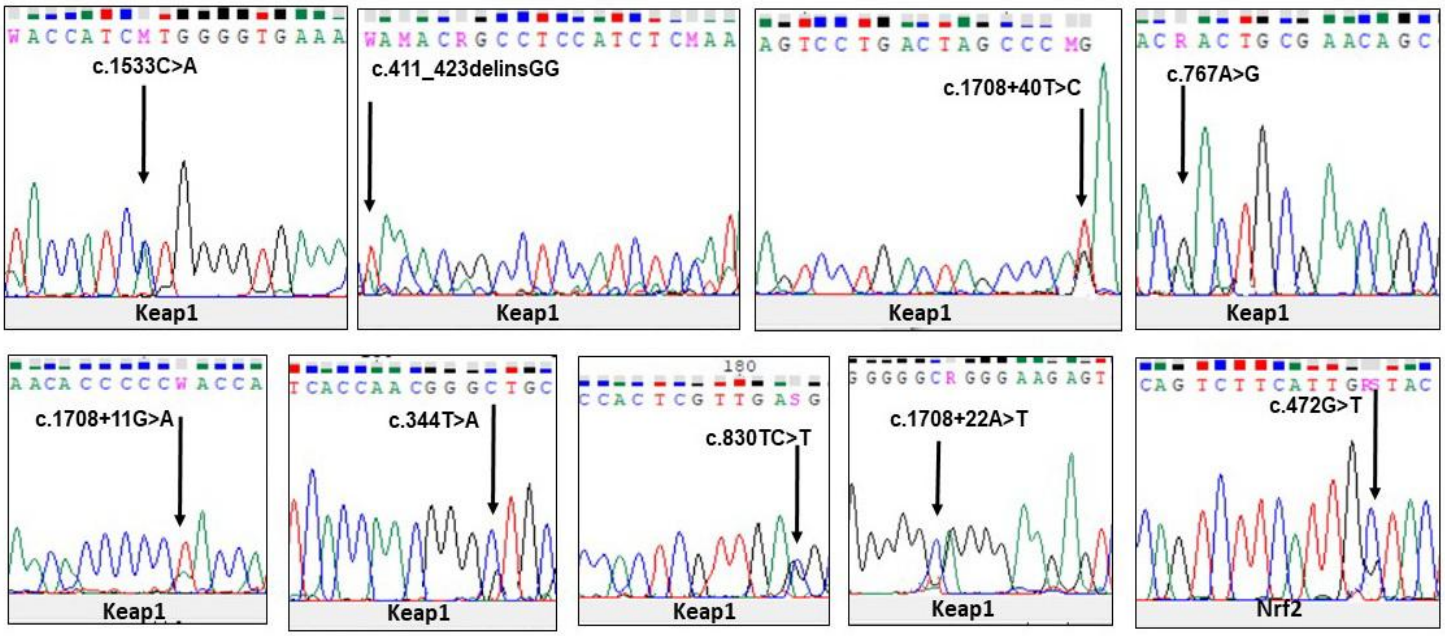

Figure 1. Sequencing electropherograms of KEAP1 and NRF2 gene mutations and variants. Arrows indicate localizations of the mutation and variant. KEAP1, Kelch-like ECH-associated protein 1; NRF2, nuclear factor erythroid-2-like 2.

Human NRF2 belongs to the Cap 'n' Collar (CNC) family of transcription factors and is a 605–amino acid protein with an approximate molecular mass of 67.7 kDa that is essential for maintaining cellular homeostasis. Comparative analysis across species indicates that human NRF2 is highly conserved and contains seven functionally important domains, designated Neh1–Neh7, which exhibit strong evolutionary conservation.

In the present study, one missense mutation in the NRF2 gene was identified p.A158P located within the transactivation domain of the NRF2 protein. This mutation was detected in a patient with high-grade bladder carcinoma.

The mutations identified through both experimental and in silico analyses of KEAP1 and NRF2 are summarized in Table 1 (Supplement 1) and their positions within the respective protein domains are schematically illustrated in Figure 2 (A–B).

3.2. Results of Bioinformatic Analysis of KEAP1/NRF2 Mutations: In the bladder cancer patient cohort, alterations in the KEAP1 and NRF2 genes were examined using data obtained from the cBioPortal platform. It was determined that approximately 11% of patients exhibited at least one genetic alteration including missense, nonsense, frameshift mutations, deletions, or gene amplifications in

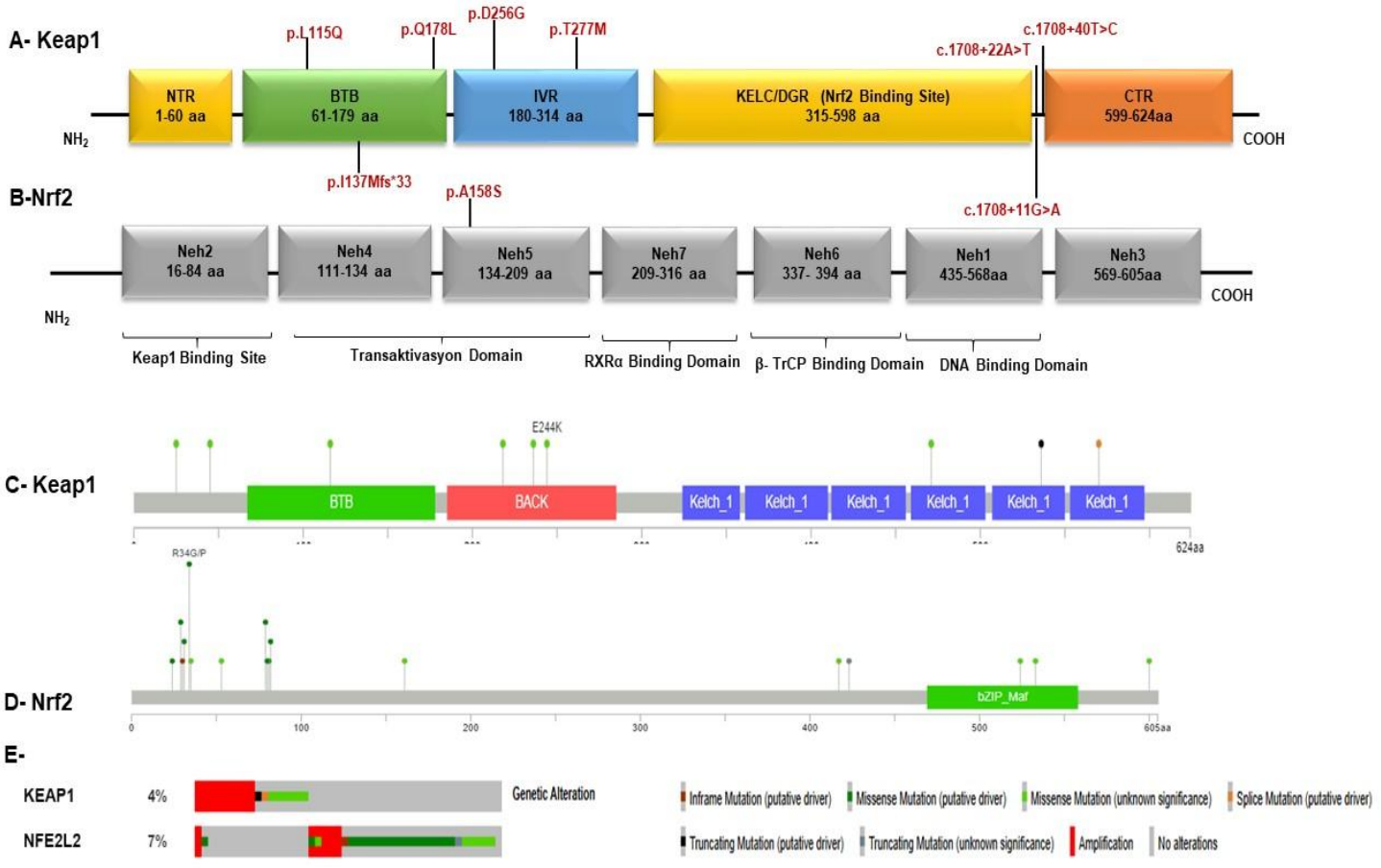

Figure 2. Schematic representation of domain architecture of the KEAP1 and NRF2 proteins and mutations detected in bladder cancer patients. Human KEAP1 is a polypeptide comprising 624 amino acids. Human NRF2 is a polypeptide comprising 605 amino acids, which contains seven Neh domains (A-B). Lollipop diagram displaying the mutations detected on the protein domains of the KEAP1 and NRF2 proteins and mutations detected in TCGA bladder cancer patient's cohort (C-D). Distribution of mutations in KEAP1 and NRF2 genes in bladder cancer patients obtained from cBioPortal. Percentages of overall mutations for each gene are given on the left (E).

the target genes. In total, 31 distinct mutations were identified across the two genes: 27 missense mutations, 1 nonsense mutation, 1 splice site mutation and 2 frameshift mutations. Among these, the gene with the highest mutation frequency was NRF2 (7%). In addition gene amplification events were observed in both KEAP1 and NRF2. The distribution of these mutations within the corresponding protein domains, as identified in the TCGA bladder cancer dataset, is illustrated in Figure 2 (C–D).

Functional predictions generated by PolyPhen-2, SIFT and SNAP analyses indicated that the experimentally identified missense mutations in KEAP1 (p.L115Q, p.Q178L, p.D256G and p.T277M) and in NRF2 (p.A158S) exhibited high pathogenicity scores (approaching 1.0), suggesting their classification as potentially deleterious variants.

To further evaluate the potential functional impact of these mutations, the "*Multiple Sequence Alignment*" feature within PolyPhen-2 was used to compare the affected amino acid residues across different species. This comparative analysis revealed that the KEAP1 mutations (p.L115Q, p.Q178L, p.D256G, and p.T277M) occur at evolutionarily conserved amino acid positions, underscoring their

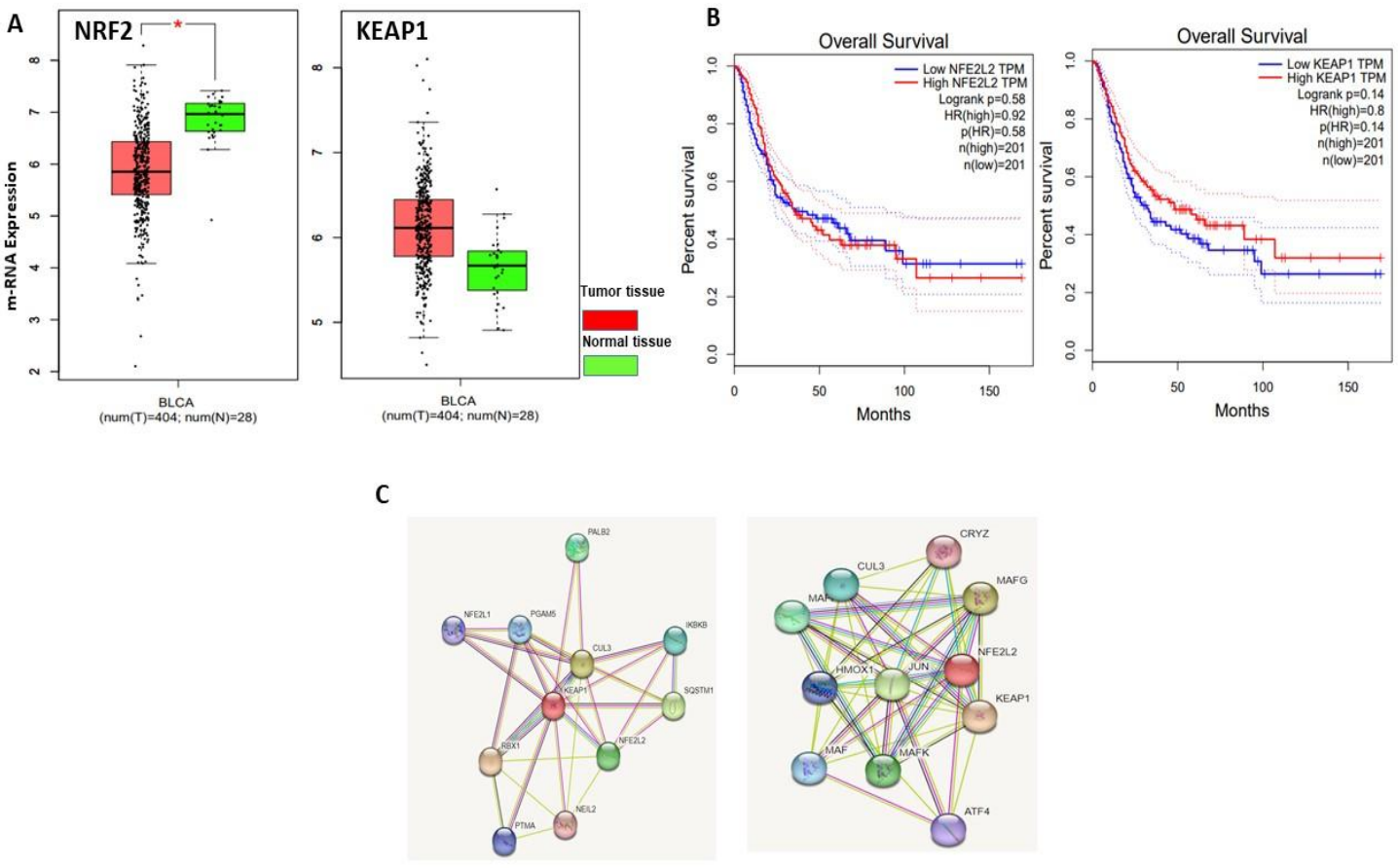

Figure 3. Comparative analysis of m-RNA expression values of tumor and healthy individuals in KEAP1 and NRF2 genes ($p < 0.05$) [Red box indicates the high expressions of m-RNA: Blue box indicates the low expressions of m-RNA] (A). Comparison of Kaplan–Meier survival curves of the high and low expressions of KEAP1 and NRF2 genes in TCGA bladder cancer patients cohort ($p < 0.05$). [Red line indicates the high expressions of m-RNA: Blue line indicates the low expressions of m-RNA] (B). Protein-protein interaction analysis images for KEAP1 and NRF2 (C).

possible functional significance. Similarly, the p.A158S missense mutation in NRF2 demonstrated a pathogenicity score close to 1 and was categorized as "affected" indicating a strong likelihood of pathogenic potential. Consistent with this the p.A158S substitution was also found to disrupt a highly conserved residue across multiple species, suggesting a possible detrimental effect on NRF2 protein function.

3.3. Results of Gene Expression Dynamics and Survival Analysis: For this study, transcriptomic datasets from bladder cancer patients and 91 normal tissue samples retrieved from TCGA were analyzed to characterize the m-RNA expression patterns of KEAP1 and NRF2. The results revealed a significant decrease in NRF2 expression in tumor tissues compared with normal controls ($p < 0.01$; Figure 3A). Conversely, Kaplan–Meier survival analysis, which divided patients based on high versus low gene expression levels, indicated no significant correlation between KEAP1 or NRF2 expression and overall survival outcomes (Figure 3B).

3.4. Results of Protein–protein interaction analysis: To investigate potential functional interactions between KEAP1 and NRF2 within cellular signaling networks, a protein–protein interaction (PPI) analysis was analyzed using the STRING platform. Computational analysis indicated that both proteins possess multiple shared interaction partners, particularly splice variants of CUL3, a cullin family protein integral to polyubiquitination pathways. Moreover, NRF2 has been shown to interact with small Maf proteins, a group of transcription factors containing a basic leucine zipper (bZIP) domain.

Among NRF2's downstream effectors, HMOX1 (heme oxygenase 1) was identified as a key cytoprotective enzyme whose altered expression or activity may present therapeutic potential in cancer contexts. The comprehensive interaction network of KEAP1, NRF2 and their associated partners is illustrated in Figure 3C.

4. Discussion

NRF2 functions as a key transcription factor, regulating the expression of a wide array of genes implicated in cellular defense mechanisms. Alongside its negative regulator KEAP1, NRF2 establishes a tightly regulated signaling axis that allows cells to respond to oxidative, electrophilic, and xenobiotic stresses [25,26]. Under normal physiological conditions this pathway contributes to cellular homeostasis; however increasing evidence shows that its dysregulation plays a critical role in cancer pathogenesis. Persistent activation of NRF2 signaling enables cancer cells to adapt to oxidative stress and preserve redox homeostasis, thereby promoting cell survival and proliferative capacity [13,14]. Several studies have demonstrated that aberrant NRF2/KEAP1 signaling promotes resistance to anticancer therapies by sustaining the continuous expression of cytoprotective genes [9,10]. In this context, NRF2 has emerged as a key regulator of cancer cell metabolism, connecting cellular stress responses with metabolic reprogramming and tumor progression [15,28].

Despite these insights, the clinical application of NRF2/KEAP1-related biomarkers remains limited. In bladder cancer, only a small number of molecular biomarkers have been validated for routine clinical use emphasizing the need for new biologically meaningful markers to improve diagnosis and treatment strategies [11]. In this study, we therefore focused on NRF2 and KEAP1, two

central regulators of cytoprotective signaling to investigate their potential roles as biomarkers and to assess their contribution to bladder cancer pathogenesis.

Mutations in KEAP1 and NRF2, key components of the cytoprotective signaling pathway, were assessed in 40 bladder cancer cases and compared with 10 healthy control samples. Initially, genotyping of the critical domains of the target genes was performed using DNA sequencing. Mutations were primarily detected within sequences encoding the functionally important domains of these genes. In total, 9 mutations were identified comprising 5 missense mutations, 1 frameshift mutation and 3 SNPs all of which had been previously documented in the HGMD database. Many mutations were identified in the KEAP1 gene (8 mutations). Among these, 4 were missense mutations, 3 were SNPs and 1 was a frameshift mutation. The p.L115Q and p.Q178L missense mutations are located within the BTB domain. The BTB domain mediates KEAP1 dimer formation, and its deletion has been shown to disrupt KEAP1 interaction with CUL3, emphasizing the importance of this domain for KEAP1–CUL3 binding. The BTB domain mediates NRF2 inhibition by promoting its ubiquitination, which targets NRF2 for proteasomal degradation [29,30].

Missense mutations within the BTB domain were detected in two male patients diagnosed with high-grade bladder carcinoma. In the present study, these BTB domain missense mutations are suggested to potentially cause persistent inhibition of NRF2 expression. Additionally, a p.I137Mfs*33 frameshift mutation was identified within the same domain. The mutation is predicted to generate a frameshift that may cause premature truncation of the encoded polypeptide. Notably, the p.I137Mfs*33 frameshift deletion was identified in a male patient with high-grade bladder carcinoma representing a mutation of biological significance.

Mutations leading to abnormal NRF2 activation can increase cytoprotective enzyme expression and alter cellular metabolism, thereby supporting therapy resistance and the development of aggressive tumor phenotypes. In addition, the p.D256G and p.T277M missense mutations were mapped to the IVR domain, which harbors a conserved nuclear export signal sequence crucial for KEAP1 cytoplasmic localization [31].

Four reactive cysteine residues have been identified within the IVR domain Cys257, Cys273, Cys288 and Cys297 which function as sensing sites for oxidative stress in the cellular environment. Although the missense mutations detected in this study were not located on these specific residues, they were classified as pathogenic. Therefore, the p.D256G and p.T277M mutations are considered significant due to their positions within regions involved in intracellular signaling. The p.D256G missense mutation was identified in a male patient with high-grade bladder carcinoma. In addition, three distinct SNPs located within the intron regions were identified in the study cohort.

The human NRF2 protein, a transcription factor with a molecular weight of 67.7 kDa and composed of 605 amino acids, plays a crucial role in maintaining cellular homeostasis and belongs to the Cap ‘n’ Collar (CNC) transcription factor family. It consists of seven highly conserved NRF2-ECH homology (Neh) domains, designated Neh1 to Neh7 [15,31].

In this study, a missense mutation (p.A158P) was detected in the NRF2 gene and was localized to the transactivation (Neh5) domain of the NRF2 protein. The Neh4 and Neh5 domains, which are positioned in the N-terminal region, possess distinct transactivation

properties and are known to interact with CBP and BRG1 to promote transcriptional activation (32). Furthermore, the Neh3, Neh4, and Neh5 domains constitute critical transcriptional activation regions that interact with histone acetyltransferases and coactivators, such as cyclic AMP-responsive element-binding proteins, thereby facilitating NRF2-mediated transcriptional regulation [32].

Based on functional pathogenicity analyses, the p.A158P missense mutation within the Neh5 domain of NRF2 was classified as malignant. This mutation was identified in a male patient diagnosed with high-grade bladder carcinoma. To validate our experimental findings, in silico analyses were conducted using bioinformatics databases. According to these analyses, a comprehensive mutation profile of the KEAP1 and NRF2 genes was examined using genome sequencing data from 406 bladder cancer patients available in the TCGA database. A total of 31 distinct mutations were identified across these two genes, including 27 missenses, 1 nonsense, 1 splice-site and 2 frameshift mutations. Among them, the NRF2 gene was the most frequently mutated, with a mutation frequency of 7%.

Additionally, gene amplification anomalies were detected in both genes. The NRF2 gene p.E423 nonsense mutation represents a truncating variant predicted to introduce a premature stop codon at position 423, leading to incomplete NRF2 protein synthesis. Moreover, in silico analysis the p.E524Q missense mutation in the Neh1 domain of the NRF2 gene. The Neh1 domain harbors a conserved CNC-bZIP region which is essential for DNA binding and the formation of heterodimers with small Maf (sMaf) proteins. This domain harbors a nuclear localization signal (NLS) crucial for NRF2 nuclear import and can interact with UbcM2, an E2 ubiquitin-conjugating enzyme, thereby playing a role in modulating NRF2 stability.

Given its location within the NLS signaling region the p.E524Q missense mutation may disrupt NRF2's transcriptional activation, constitutive expression of detoxification-related genes or subcellular localization. NRF2 displays context-dependent effects in cancer, acting as either a tumor suppressor or a tumor-promoting factor according to the cellular and molecular environment. Under physiological conditions, NRF2 plays a cytoprotective role. However, in this study NRF2 expression was significantly downregulated in the bladder cancer patient cohort compared to normal tissue samples. The lack of a important correlation between KEAP1 or NRF2 expression and overall survival may reflect the complex regulation of this pathway, as NRF2 activity is influenced not only by mRNA levels but also by post-translational modifications, KEAP1-mediated degradation, and cellular stress responses. Additionally, its dual role together with patient heterogeneity may obscure associations with survival outcomes. Nevertheless, reduced NRF2 expression is still known to increase cancer cell resistance to oxidative stress and diminish the efficacy of chemotherapy and radiotherapy [33–35].

Protein–protein interaction analysis performed using STRING revealed that NRF2 interacts with HMOX1, a key cytoprotective enzyme regulated by NRF2. The expression of HMOX1 is regarded as a critical determinant of cellular defense and modulation of its expression or enzymatic activity may offer therapeutic potential [36]. Heme oxygenase-1 (HO-1) possesses antioxidant, anti-inflammatory, and anti-apoptotic functions and plays a critical role in safeguarding tissues against injury induced by oxidative stress. Therefore, disrupted NRF2 activation in

bladder cancer may lead to altered HMOX1 expression or function, contributing to impaired cellular defense mechanisms [37].

Bladder cancer is the most frequently diagnosed malignancy of the urinary tract and is commonly associated with substantial recurrence rates and the development of chemotherapy resistance after surgical resection. The NRF2/KEAP1 pathway has been extensively characterized as a major regulator of cellular protective mechanisms under different stress conditions. Recent studies suggest that NRF2, a key regulator within this pathway, may act as an oncogenic factor, supporting the preservation of malignant cellular phenotypes [38,39].

Although this study provided molecular profiling analyses of mutations potentially responsible for the disruption of NRF2/KEAP1 signaling several limitations should be acknowledged. The study was conducted on a small sample cohort, and further investigations involving larger patient populations are required to better elucidate the epidemiological significance of these genetic alterations in bladder cancer. To enhance the robustness of our findings, we complemented our experimental data with analyses from TCGA database. Alterations in the NRF2/KEAP1 pathway with oncogenic potential represent attractive candidates for therapeutic targeting, although additional functional studies are needed to better define their roles in tumorigenesis. Moreover, the integration of multi-omics approaches including transcriptomic and proteomic analyses could provide a more comprehensive understanding of NRF2/KEAP1 pathway dysregulation. Taken together, these findings could aid in the discovery of new biomarkers and the development of targeted therapies, thereby supporting the progression of personalized treatment approaches for individuals with bladder cancer.

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