BOLA family genes are the drivers and potential biomarkers of survival in kidney renal clear cell carcinoma patients

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ABSTRACT

الأهداف: تهدف هذه الدراسة إلى تحليل الإمكانات التشخيصية والتنبؤية لعائلة جين BOLA في سرطان خلايا الكلى الصافية (KIRC).

المنهجية: هذه الدراسة هي دراسة مراقبة. أجريت الدراسة بأكملها في جامعة الأمير سطام بن عبدالعزيز، المملكة العربية السعودية من يناير إلى نوفمبر 2023م. نظراً لأنها تتضمن طرقاً حاسوبية (in silico) وفي المختبر (in vitro)، لم تكن الموافقة الأخلاقية مطلوبة. تم استخدام تقنيات تجريبية حاسوبية وجزيئية مختلفة في هذه الدراسة.

النتائج: لوحظت زيادة كبيرة في التنظيم وانخفاض في ميثيل الجينات BOLA1 و BOLA2 و BOLA3 عبر 12 سلالة من خلايا KIRC. أكد التحليل الاسترجاعي ل the cancer genome atlas program (TCGA)-KIRC cohorts انخفاض مثيلة هذه الجينات في أنسجة KIRC. أظهر تحليل cBioPortal تغييرات جينية ضئيلة، وكان التضخيم هو الأكثر شيوعاً. أظهرت بيانات Kaplan-Meier plotter أن تعبير BOLA1 و BOLA2 و BOLA3 العالي مرتبط ب shorter overall survival و relapse-free survival في KIRC. ربط تحليل Tumor-immune system interactions database التعبير عن BOLA1 بالأنواع الفرعية المناعية والجزيئية. أدى إسكات التعبير الجيني ل BOLA1 و BOLA2 و BOLA3 في خلايا 786-0 إلى تقليل نمو الخلايا وانتشارها، مما أدى إلى تعزيز قدرة التئام الجروح.

الخلاصة: يمكن أن تعمل جينات BOLA كعلامات تشخيصية وتنبؤية في KIRC، مما يوفر رؤى حول الأهداف العلاجية وتطور المرض.

Objectives: To analyze the diagnostic and prognostic potential of the BOLA gene family in kidney renal clear cell carcinoma (KIRC).

Methods: This study is an observational study. The whole study was carried out at Prince Sattam bin Abdulaziz University, Al-Kharj, Saudi Arabia from January to November 2023. As it involves in silico and in vitro methods, ethical consent was not required. Various in silico and molecular experimental techniques were employed in this study.

Results: Significant up-regulation and hypomethylation of BOLA1, BOLA2, and BOLA3 were observed across 12 KIRC cell lines. Retrospective analysis of the cancer genome atlas program (TCGA)-KIRC cohorts confirmed hypomethylation of these genes in KIRC tissues. The cBioPortal analysis showed minimal genetic alterations, with amplification being the most common. Kaplan-Meier plotter data revealed that high BOLA1, BOLA2, and BOLA3 expression correlated with shorter overall survival and relapse-free survival in KIRC. Tumor-immune system interactions database analysis linked BOLA1 expression to immune and molecular subtypes. Gene silencing of BOLA1, BOLA2, and BOLA3 in 786-0 cells reduced cell growth and proliferation, enhancing wound healing capacity.

Conclusion: BOLA genes may serve as diagnostic and prognostic markers in KIRC, offering insights into therapeutic targets and disease progression.

Keywords: BOLA genes, carcinoma, prognostic biomarkers, gene expression regulation, molecular targeted therapy

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Ranked as the eighth most prevalent cancer, kidney renal clear cell carcinoma (KIRC) constitutes a predominant subset among malignant kidney tumors.[1] Risk factors of KIRC include lifestyle choices such as smoking and obesity, along with other conditions, including high blood pressure, chronic kidney disease, and inherited disorders like Von Hippel-Lindau syndrome.[2] Occupational exposure to toxins like cadmium and trichloroethylene also heighten risk. Men, older individuals, and those with a family history of kidney cancer face elevated risks.[3] Understanding these factors is crucial for targeted prevention and early detection strategies, ultimately improving outcomes for KIRC patients.[1] Notoriously linked to resistance against radiotherapy and chemotherapy, metastatic KIRC poses a formidable challenge, with less than a 20% 2-year survival rate for affected patients.[1,4] Recognizing the potential for a favorable prognosis through early detection and surgical intervention, it becomes imperative to delve into the genomic alterations and inherent molecular mechanisms of KIRC. These investigations are vital for advancing our understanding, ultimately paving the way for enhanced early diagnosis and treatment strategies.

The human BOLA gene family comprises BOLA1, BOLA2, and BOLA3 members.[5] In humans, the BOLA gene family plays crucial roles in various cellular functions, particularly those related to mitochondrial integrity, iron homeostasis, and oxidative stress response. The BOLA proteins, such as BOLA1 and BOLA2, are involved in the biogenesis and maintenance of iron-sulfur (Fe-S) clusters, which are essential cofactors for numerous enzymes involved in cellular metabolism and DNA repair.[5] By regulating Fe-S cluster assembly, BOLA proteins ensure proper mitochondrial function and energy production. Additionally, BOLA proteins contribute to the cellular response to oxidative stress. They help protect mitochondria from oxidative damage by maintaining the balance of reactive oxygen species and supporting antioxidant defenses. This protection is critical in preventing mitochondrial dysfunction, which is associated with various neurodegenerative diseases and aging.[5]

Although BOLA family members, such as BOL2 and BOLA3, have been implicated as assembly factors for mitochondrial Fe-S cluster proteins with roles in cancer cell biology, the specific functions of BOLA1, BOLA2, and BOLA3 in cancer remain unclear.[6]

Previous research has highlighted the crucial involvement of BOLA3 in the development and progression of cardiovascular diseases.[7] BOLA2 has been closely associated with hepatocellular carcinoma tumor growth and progression.[6] BOLA1 plays a critical role in regulating mitochondrial morphology and is involved in the onset of various diseases.[8] In ovarian cancer, elevated expression levels of BOLA2 and BOLA3 have been observed, indicating their potential as biomarkers for the disease.[9] Furthermore, in lung adenocarcinoma, BOLA3 expression correlates with immune cell infiltrates.[9]

The expression patterns, prognostic implications, and immune properties of the BOLA family in KIRC are areas that have not been explored in the existing literature. This emphasizes the need for further investigation to elucidate the nuanced role of BOLA family members in KIRC and to unravel their potential as markers and therapeutic targets. In this study, the role of the BOLA gene family in KIRC was investigated through a combination of in silico analyses and molecular experiments, aiming to elucidate its potential functions and contributions in KIRC.

Methods. This observational study was carried out at Prince Sattam bin Abdulaziz University, Al-Kharj, Saudi Arabia, from January to November 2023. As it involves in silico and in vitro methods, ethical consent was not required.

In this study, 12 KIRC cell lines were obtained from the American Type Culture Collection (USA), and utilized: ACHN, Caki-1 (CRL-1611), 786-O (CRL-1932), A-498 (HTB-44), SN12C (CRL-2181), 769-P (CRL-1933), Caki-2 (HTB-47), UO-31 (HTB-73), RCC4 (CRL-1614), TK-10 (CRL-2396), and RXF-393 (CRL-1912). These cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C with 5% carbon dioxide (CO_2). Additionally, 5 normal kidney cell lines were also purchased and employed: HK-2, RPTEC/TERT1, HK-11, HEK 293, and LLC-PK1. These were cultured in different media including Dulbecco's modified eagle medium (DMEM), DMEM/F-12, and Eagle's minimum essential medium (EMEM), all supplemented with 10% FBS and 1% penicillin-streptomycin, under the same conditions of 37°C with 5% CO_2.

The DNA extraction was carried out utilizing the organic method, ensuring efficient isolation of genomic DNA from the cell lines.[10] For RNA extraction, the TRIzol method was employed to extract high-quality RNA, enabling downstream molecular analyses with optimal integrity and purity.[11]

SYBR green-based real-time quantitative polymerase chain reaction (RT-qPCR) was carried out following the previously described protocols.[12] The RT-qPCR primers are as follow:

β-actin forward 5′-CCCAGCACAATGAAGATCAA-3′ and reverse 5′-ACATCGCTGGAAGGTGGAC-3′: BOLA1 forward 5′-AGGTGCTAGAGCTTCGCAACGA-3′ and reverse 5′- TGGACCAGCCGGTGTCGTTGT-3′: BOLA2 forward 5′-TGGAACTCAGCGCCGAATACCT-3′ and reverse 5′-ACTCGGAAGCTACAGGAGCAAC-3′: BOLA3 forward 5′-GAAAAGTTTCCACGAGCTACAGC-3′ and reverse 5′-CATCTGGTGCTGCTGGACAGTT-3′.

The DNA methylation profiling of BOLA genes across 12 KIRC and 5 cell lines was carried out at the DKFZ Genomics and Proteomics Core Facility in Heidelberg, Germany. The Illumina Infinium HumanMethylation450K BeadChip (Illumina, San Diego, USA) was utilized following the manufacturer's guidelines.

OncoDB and UALCAN are specialized cancer database that compiles comprehensive information on cancer-associated genes and their genomic alterations.[13,14] These databases serve as valuable resources for researchers, providing curated data on gene expression, methylation, and clinical associations. In this study, OncoDB and UALCAN were used for validating the promoter methylation levels of BOLA family genes in TCGA-KIRC datasets.

To evaluate the prognostic significance, mRNA expression levels of the BOLA family genes were examined using Kaplan-Meier plotter.[15] Patient cohorts were stratified into 2 groups (high vs. low expression), and the analysis assessed overall survival (OS) and relapse-free survival (RFS) between these groups.

cBioPortal is a versatile and user-friendly platform extensively utilized for comprehensive exploration of genetic alterations in various cancers.[16] Using cBioPortal, our study carried out detailed genetic alteration analysis of the BOLA family genes in KIRC.

The TISIDB consolidates diverse data sources in oncoimmunology, providing a comprehensive platform for in-depth investigations into interactions between tumors and the immune system.[17] In our study, the TISIDB database played a pivotal role in dissecting the expression patterns of BOLA genes across diverse immune and molecular subtypes of KIRC.

The DAVID furnishes an extensive suite of tools, empowering researchers to decipher the biological functions of extensive gene lists.[18] The BOLA family genes underwent gene enrichment analysis via the DAVID database.

The siRNAs designed to target BOLA genes were synthesized by the OBiO company (Shanghai, China). To achieve silencing of BOLA genes in 786-O cells, siRNA transfection was carried out using the INTERFERin transfection reagent (Polyplus, Illkirch, France). Cells were transfected with siRNA at a final concentration of 50 nM, following the manufacturer's protocol. After incubation for 6 hours at 37°C, the transfection medium was replaced, and cells were harvested for analysis at 48 hours post-transfection.

To evaluate the efficiency of gene knockdown, RT-qPCR for the BOLA genes was carried out under the previously outlined conditions, and Western blot analysis was carried out.[5]

Cell proliferation was assessed using the CCK-8 kit (Dojindo, Kumamoto, Japan) as per the manufacturer's instructions. Cells were seeded at 1×10^3 cells/well in 96-well plates and monitored every 24 hours. For colony formation, transfected cells were seeded at 5×10^2 cells/well in 6-well plates. After 14 days, colonies were stained with 0.1% crystal violet.

Cells were grown to 90% confluence, and wounds were created by scratching the monolayer with a sterile pipette tip. After rinsing with serum-free media, wound closure was observed and imaged at 0 and 24 hours using a phase-contrast microscope. The percentage of wound closure was calculated for analysis.

DrugBank is a comprehensive bioinformatics database providing information on drugs, their targets, and interactions.[19] In the present study, DrugBank database was used to predict BOLA genes inhibitory drugs.

Statistical analysis. Statistical analysis and data visualization were carried out using R (version 4.2.3) and The Statistical Package for the Social Sciences, version 21.0 (IBM Corp., Armonk, NY, USA). Group comparisons were carried out using independent sample t-tests, while Pearson correlation analysis was used to explore relationships between groups. Cox regression analysis and Kaplan-Meier curves were employed for prognostic evaluation. Diagnostic performance was assessed with receiver operating characteristic (ROC) curves. Statistical significance was set at $p<0.05$, $p<0.01$, and $p<0.001$.

Results. To investigate expression differences among the BOLA family members, we first carried out RT-qPCR to assess the mRNA levels of each BOLA gene in KIRC cell lines. As depicted in **Figure 1A**, BOLA1 (p=3.48x10-10), BOLA2 (p=1.04x10-8), and BOLA3 (p=2.96x10-7) genes exhibited a significant up-regulation in KIRC cell lines (n=12) compared to control cell lines (n=5, **Figure 1B**).

Moreover, bisulfite sequencing analysis indicated significant hypomethylation of BOLA1 (p=3.44x10-11), BOLA2 (p=2.91x10-9), and BOLA3 (p=1.29x10-9) genes in KIRC cell lines (n=12) compared to control

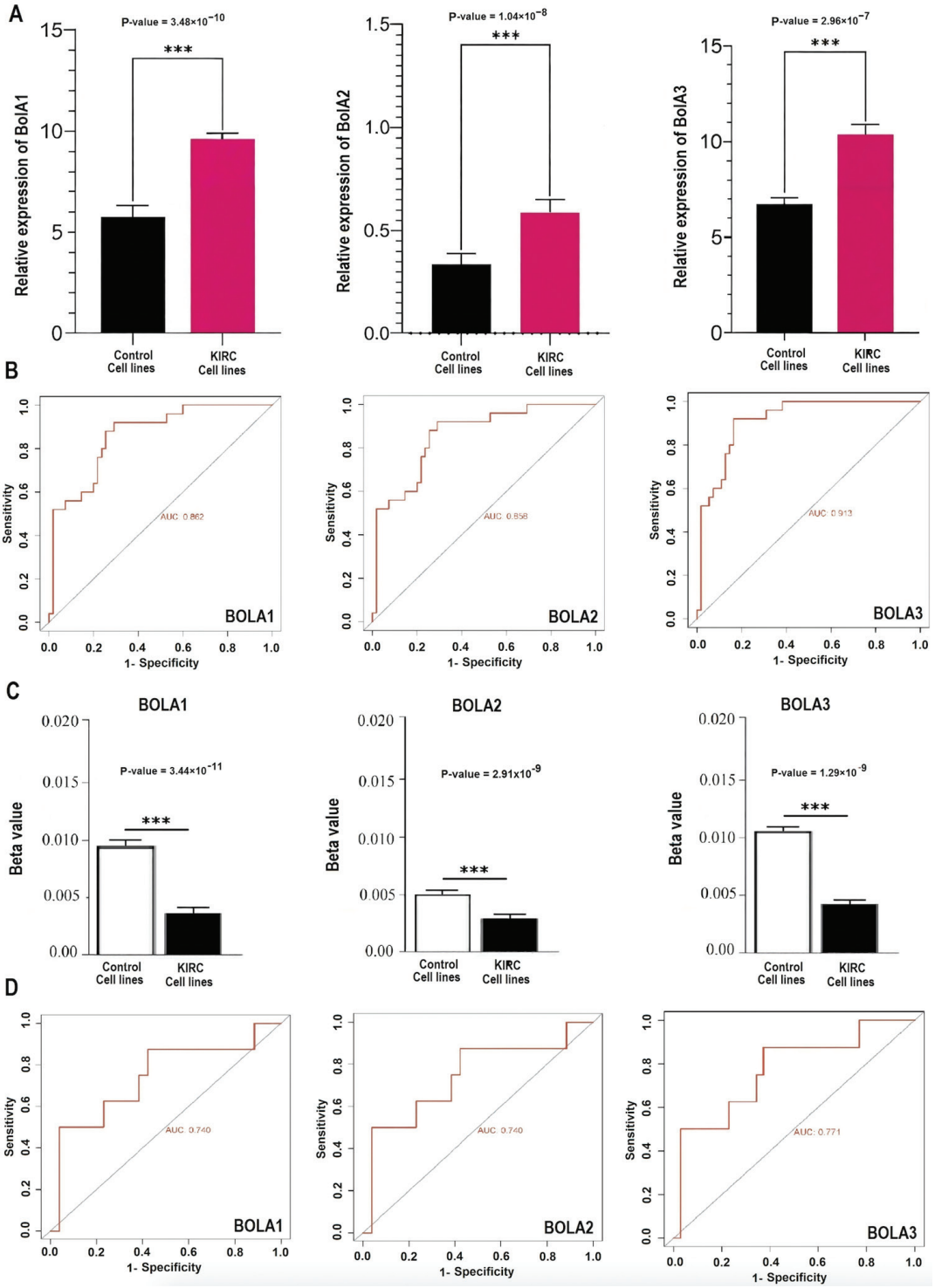

Figure 1 - Profiling the expression, promoter methylation, and receiver operating characteristic (ROC) analysis of BOLA1, BOLA2, and BOLA3 genes in kidney renal clear cell carcinoma (KIRC) and control cell lines. **A**) Real-time quantitative polymerase chain reaction-based expression profiling of BOLA1, BOLA2, and BOLA3 genes in KIRC and control cell lines. **B**) ROC curves based on gene expressions of BOLA1, BOLA2, and BOLA3. **C**) Bisulfite sequencing-based promoter methylation profiling of BOLA1, BOLA2, and BOLA3 genes in KIRC and control cell lines. **D**) ROC curves based on promoter methylation level of BOLA1, BOLA2, and BOLA3. ***$p<0.001$. KIRC: kidney renal clear cell carcinoma, AUC: area under the curve

cell lines (n=05, Figure 1C). The hypomethylated BOLA1, BOLA2, and BOLA3 genes exhibited robust diagnostic capabilities in distinguishing KIRC patients from normal individuals (Figure 1D).

To verify the potential mechanisms behind the abnormal up-regulation, promoter methylation analysis of BOLA family genes in the KIRC TCGA datasets was carried out using OncoDB, UALCAN, and GSCA databases. The results indicated significant hypomethylation of BOLA1 (p=1.188070E-02), BOLA2 (p=1.791820E-02), and BOLA3 (p=1.086020162E-02) genes in TCGA KIRC samples compared to normal samples (Figure 2A-C).

The genetic alteration landscape of BOLA1, BOLA2, and BOLA3 genes in TCGA KIRC patients was investigated using the cBioPortal web resource. The results indicated that approximately 0.4% of BOLA1, 0.2% of BOLA2, and 0% of BOLA3 in KIRC samples included in cBioPortal exhibited genetic alterations (Figure 2D&E). Amplification was the predominant genetic alteration among BOLA1, BOLA2, and BOLA3 genes (Figure 2D&E). Additionally, survival curves based on genetic alterations revealed that the KIRC patient group with genetic alterations in BOLA1 and BOLA2 genes had significant good OS (p=0.046) compared to KIRC patients without mutations in these genes (Figure 2F).

We employed the Kaplan-Meier plotter web server to assess the prognostic significance of BOLA family genes in TCGA-KIRC patients. As depicted in Figure 3A&B, the up-regulations of BOLA1, BOLA2, and BOLA3 were significantly linked to shorter OS and RFS in KIRC patients.

The impact of BOLA1, BOLA2, and BOLA3 expression on molecular and immune subtypes of KIRC was explored using the TISIDB website. Results indicated associations between BOLA1, BOLA2, and BOLA3 expression and different immune subtypes (C1: wound healing; C2: IFN-gamma dominant; C3: inflammatory; C4: lymphocyte depleted; C5: immunologically quiet; and C6: TGF-b dominant) in KIRC (Figure 3C). Additionally, BOLA1, BOLA2, and BOLA3 expression displayed variability among molecular subtypes in KIRC (Figure 3D).

Results of the gene enrichment analysis showed that BOLA1, BOLA2, and BOLA3 genes are associated with Fe-S cluster assembly complex, Beta catenin destruction complex, and Wnt signalosome. The CC terms included Gamma catenin-binding, microtubule plus end binding, dynein complex binding, and 2 iron 2 sulfur (2-Fe-2S) cluster binding, and more (Figure 4A). The MF terms included 2-Fe-2S cluster assembly, positive reg. of protein localization on centrosome, and Fe-S cluster assembly, and more (Figure 4B). The BP terms included endometrial cancer, based cell carcinoma, colorectal cancer, TGF-beta signaling pathway, and signaling pathways regulating pluripotency of the stem cell, and more (Figure 4C&D).

To investigate the functional roles of BOLA1, BOLA2, and BOLA3 in KIRC, we employed gene silencing techniques in 786-O cells. Following the transfection of 786-O cells, mRNA levels of BOLA1, BOLA2, and BOLA3 were evaluated after 24 hours using RT-qPCR and Western blot analysis. The results indicated a reduction in the expression levels of BOLA1, BOLA2, and BOLA3 (Figure 5A&B). Subsequently, we assessed the impact of inhibiting BOLA1, BOLA2, and BOLA3 on the growth, proliferation, and wound healing capacity of 786-O cells in Ctrl-780-O-control and si-786-O-BOLA1, si-786-0-BOLA2, and si-786-O-BOLA3 cells. The findings revealed that inhibiting BOLA1, BOLA2, and BOLA3 led to growth inhibition and an increased ability for wound healing in 786-O cells (Figure 5C-F).

In order to anticipate drugs that regulate the protein expression of BOLA1, BOLA2, and BOLA3, we carried out an inquiry in the DrugBank database. This investigation unveiled a few important drugs with the capabilities to reduce the mRNA expressions of BOLA genes (Table 1).

Discussion. Kidney renal clear cell carcinoma is one of the common cancers with a high mortality rate.[20] Existing diagnostic methods often detect KIRC at advanced stages, hindering timely intervention and compromising patient outcomes. Furthermore, the lack of specificity and sensitivity in current biomarkers leads to diagnostic inaccuracies and unnecessary procedures. Addressing this gap is essential for enhancing early detection, improving diagnostic accuracy, and guiding personalized treatment strategies for KIRC patients. Therefore, there is a pressing need to explore and validate novel biomarkers that can fill this crucial gap in KIRC research and clinical practice.

In this study, the role of the BOLA gene family in KIRC was investigated through a combination of in silico analyses and molecular experiments, aiming to elucidate its potential as biomarkers and contributors of KIRC.

In cancer, the BOLA family genes have been implicated in diverse molecular pathways that influence key hallmarks of cancer, including proliferation, survival, migration, and metastasis.[9] One of the primary

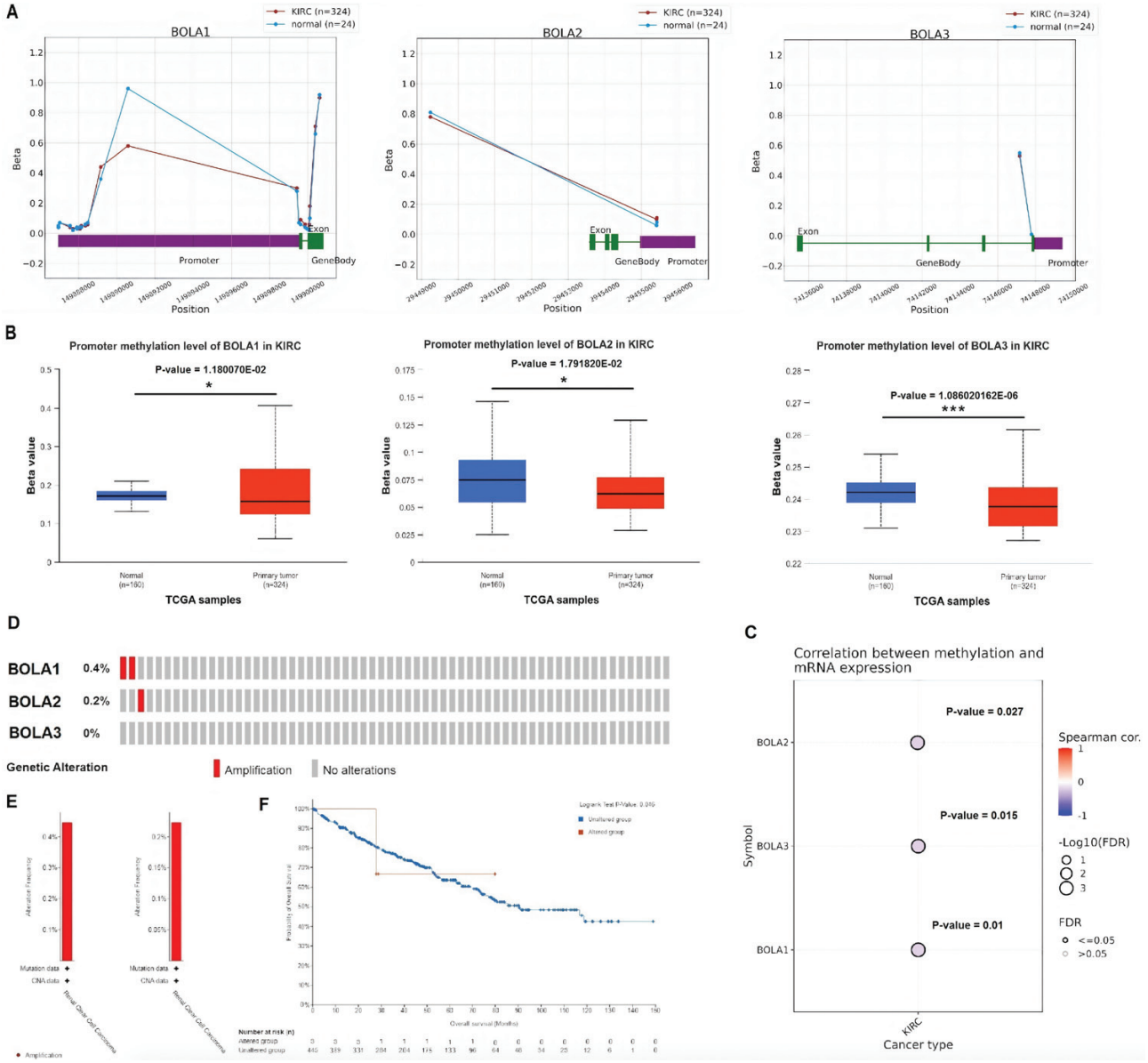

Figure 2 - Validation of BOLA1, BOLA2, and BOLA3 promoter methylation levels and mutational analysis in extended kidney renal clear cell carcinoma (KIRC) cohorts via the OncoDB, UALCAN, GSCA, and cBioPortal databases. **A)** Methylation analysis via the OncoDB database. **B)** Methylation analysis via the UALCAN database. **C)** Methylation analysis via the GSCA database. **D-E)** Frequency of genetic mutations in BOLA1, BOLA2, and BOLA3 across KIRC patients. **F)** Effect of the genetic mutations on the overall survival of the KIRC patients. *p<0.05.

roles of BOLA genes in cancer development lies in their regulatory functions over cellular proliferation and survival pathways.[9] BOLA proteins have been found to modulate cell cycle progression by interacting with cyclins, cyclin-dependent kinases (CDKs), and other cell cycle regulators.[8] Dysregulation of BOLA expression levels or activity can perturb the delicate balance of cell cycle checkpoints, leading to uncontrolled cell proliferation, a hallmark feature of cancer.[9] Dysregulated expression of BOLA genes can disrupt metabolic homeostasis, fueling the metabolic demands of rapidly proliferating cancer cells and promoting tumor progression.[21] Furthermore, BOLA genes have been implicated in the regulation of epithelial-mesenchymal transition (EMT), a critical process underlying cancer metastasis and invasion.[21] BOLA proteins interact with key EMT regulators, such as Snail, Twist, and Slug, to promote the acquisition of mesenchymal traits

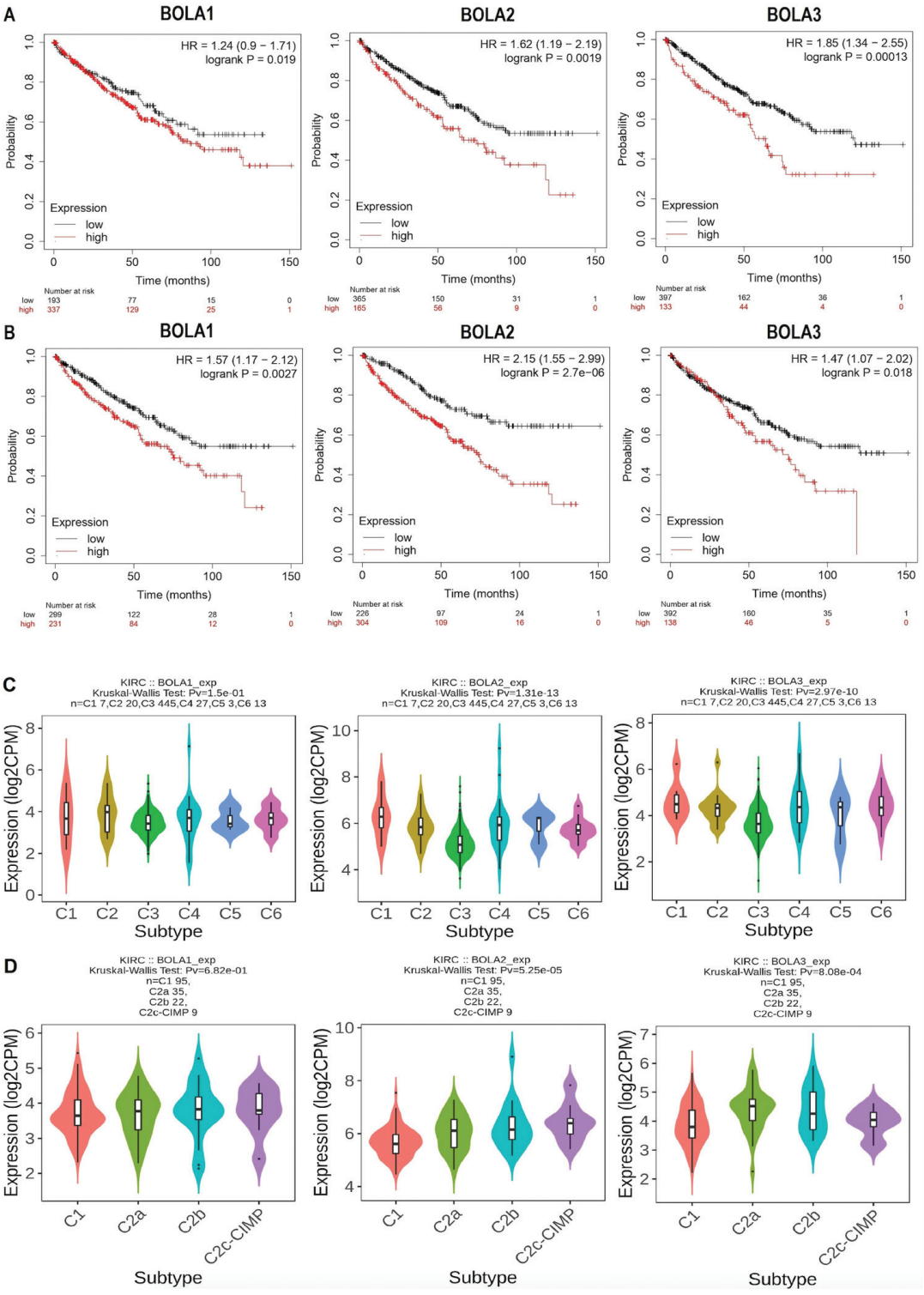

Figure 3 - Survival analysis and correlation of BOLA1, BOLA2, and BOLA3 expression with diverse immune and molecular subtypes of kidney renal clear cell carcinoma (KIRC). **A**) Effect of the up-regulated BOLA1, BOLA2, and BOLA3 on the overall survival (OS) of KIRC patients. **B**) Effect of the up-regulated BOLA1, BOLA2, and BOLA3 on the relapse-free (RFS) survival of KIRC patients. **C**) Correlation of BOLA1, BOLA2, and BOLA3 expression with diverse immune subtypes of KIRC. **D**) Correlation of BOLA1, BOLA2, and BOLA3 expression with diverse molecular subtypes of KIRC. *P*<0.05.

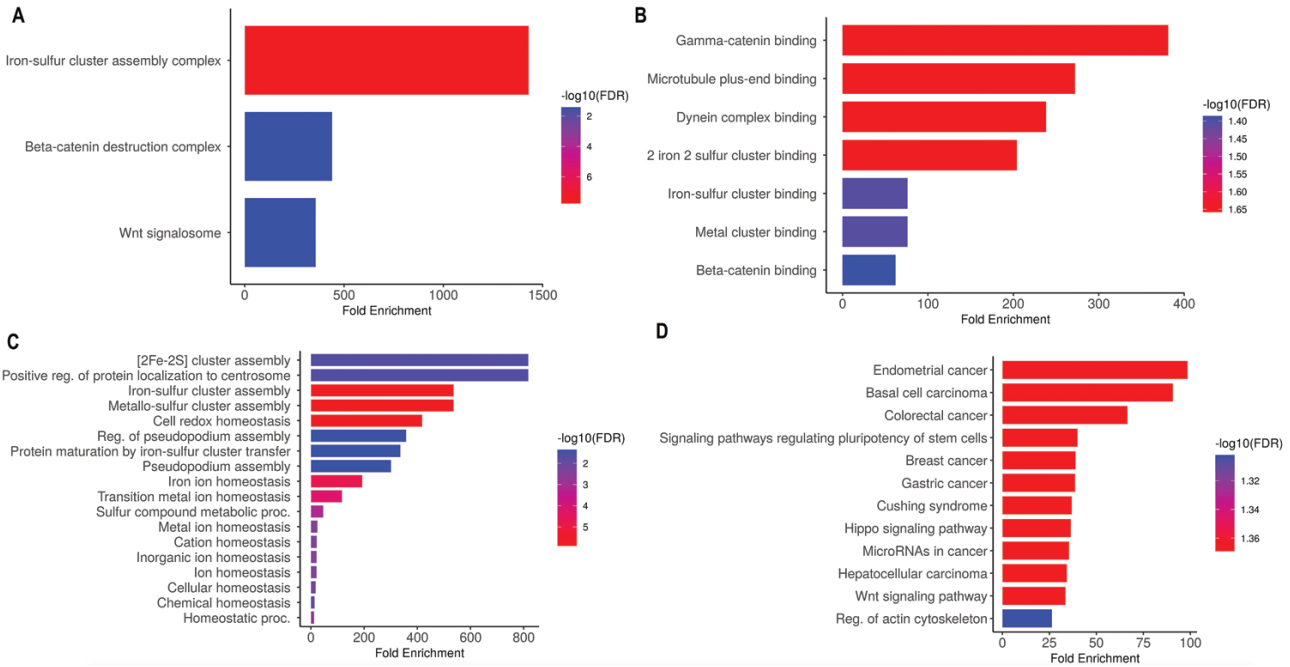

Figure 4 - Gene enrichment analysis of BOLA1, BOLA2, and BOLA3 using the DAVID tool. **A**) Cellular component (CC) terms. **B**) Biological process (BP) terms. **C**) Molecular Function (MF) terms. **D**) Kyoto encyclopedia of genes and genomes (KEGG) terms. $P<0.05$.

in cancer cells, facilitating their ability to migrate, invade surrounding tissues, and disseminate to distant organs.[21] Through their involvement in EMT, BOLA genes contribute to the metastatic cascade, driving tumor progression and therapeutic resistance in advanced cancers.[22] Yet, a comprehensive examination of the connection between expression levels and KIRC prognosis remains unexplored.

The up-regulation of BOLA genes in KIRC cell lines and tissue samples suggests their involvement in KIRC pathogenesis and their potential as biomarkers. The observed hypomethylation of BOLA1, BOLA2, and BOLA3 genes across cell lines and TCGA datasets sheds light on potential regulatory mechanisms contributing to their abnormal up-regulation in KIRC. The significant hypomethylation of BOLA1, BOLA2, and BOLA3 promoters in clinical and TCGA KIRC samples compared to normal samples suggests an epigenetic alteration that may influence gene expression levels. This finding implies a potential link between hypomethylation events and the increased expression of BOLA1, BOLA2, and BOLA3 genes in KIRC, emphasizing the importance of epigenetic regulation in KIRC pathogenesis. Similarly, previous investigations have documented similar epigenetic modifications in various genes associated with tumorigenesis in KIRC.[23-25]

The exploration of BOLA1, BOLA2, and BOLA3 expression and its impact on molecular and immune subtypes of KIRC sheds light on the intricate interplay between BOLA family genes and the tumor microenvironment. Further findings of this study reveal intriguing associations between BOLA1, BOLA2, and BOLA3 expression levels and distinct immune subtypes within KIRC. The observed associations between BOLA1, BOLA2, and BOLA3 expression and immune subtypes provide valuable insights into the potential immunomodulatory roles of BOLA proteins in KIRC. The identification of specific immune subtypes, such as the IFN-gamma dominant or inflammatory subtypes, exhibiting differential expression patterns of BOLA1, BOLA2, and BOLA3, suggests that BOLA proteins may play a role in shaping the immune landscape of KIRC tumors. For instance, BOLA-mediated regulation of cytokine signaling pathways or immune cell recruitment mechanisms could contribute to the establishment of distinct immune microenvironments within KIRC tumors, with implications for disease progression and therapeutic response. Furthermore, the variability of BOLA1, BOLA2, and BOLA3 expression among molecular subtypes of KIRC highlights the heterogeneous nature of BOLA gene regulation in cancer. The differential expression of BOLA1, BOLA2, and BOLA3 across molecular subtypes may reflect

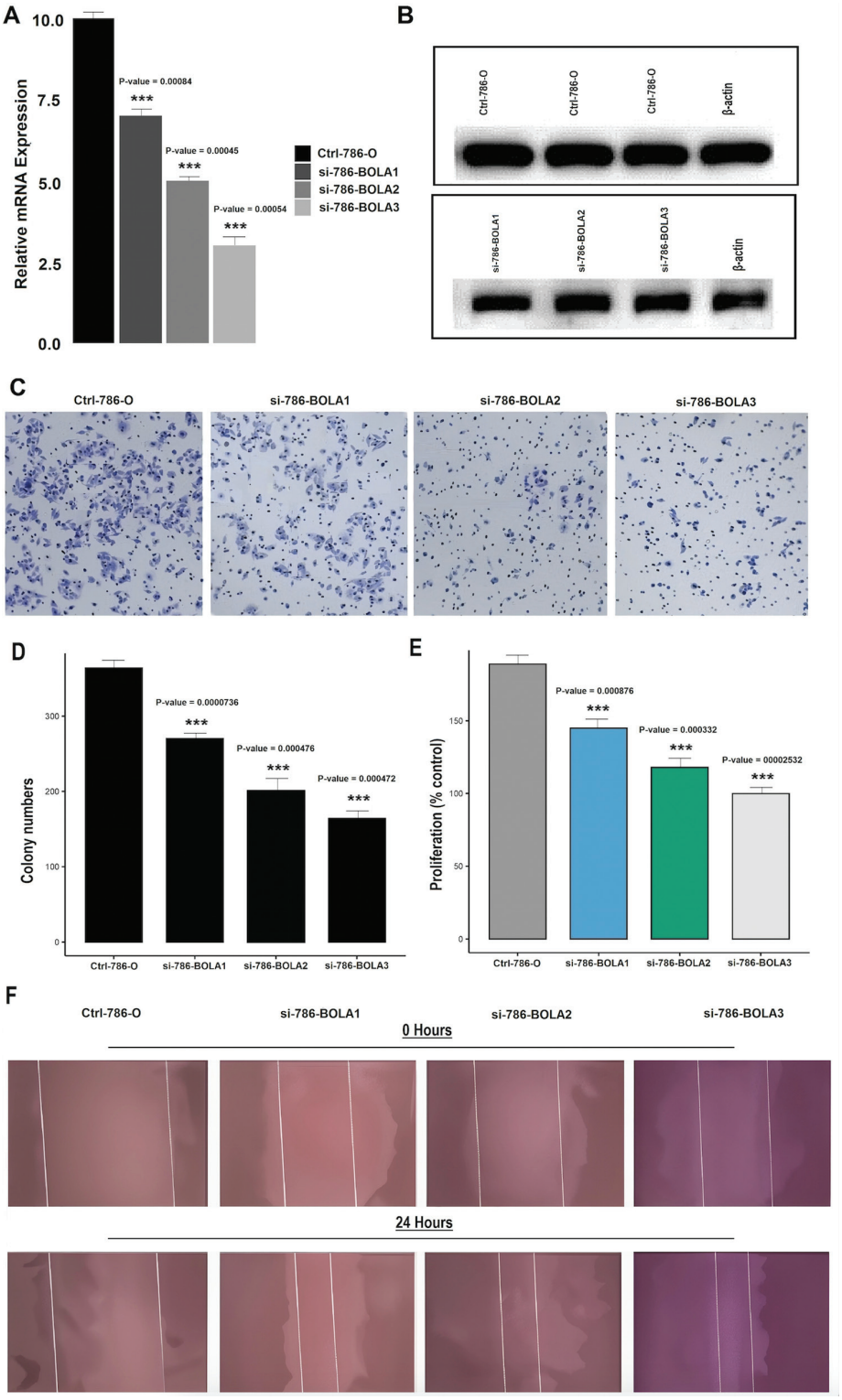

Figure 5 - Knockdown of BOLA1, BOLA2, and BOLA3 impairs the growth and metastatic potential and enhances wound healing ability of the 786-O cells. **A-B**) The transfection efficiency of BOLA1, BOLA2, and BOLA3 was checked using real-time quantitative polymerase chain reaction (RT-qPCR) and Western blot analyses. **C**) 786-O control and transfected cells were analyzed for colony formation. **D-F**) Proliferation and wound healing assays. ***P-value of <0.001.

Table 1 - Drugs associated with BOLA genes that were sourced from DrugBank.

Genes	Drugs	Effects	References	Groups
BOLA1	Estradiol Cyclosporine Quercetin	Reduce expression of BOLA1 mRNA	A21133 A21092 A23748	Approved
BOLA2	Dasatinib Cyclosporine	Increase expression of BOLA2 mRNA	A21899 A21092	Approved
BOLA3	Cyclosporine Acetylcysteine Cannabidiol	Decrease expression of BOLA3 mRNA	A21092 A20441 A21508	Approved

mRNA: messenger ribonucleic acid

underlying molecular alterations or signaling pathways specific to each subtype. Understanding the molecular basis of BOLA expression variability in KIRC could provide valuable insights into the molecular mechanisms driving tumor heterogeneity and facilitate the development of subtype-specific therapeutic strategies.

The gene enrichment analysis reveals a multifaceted landscape of associations for BOLA1, BOLA2, and BOLA3 genes, spanning critical cellular components, molecular functions, biological processes, and disease pathways. Notably, BOLA1, BOLA2, and BOLA3 genes exhibit connections with pivotal cellular complexes such as the Fe-S cluster assembly complex, implicating their involvement in essential processes like iron metabolism and redox regulation. Their association with the Beta catenin destruction complex and Wnt signalosome suggests potential roles in Wnt signaling pathway regulation, impacting cellular proliferation, differentiation, and cancer development. At the molecular level, BOLA1, BOLA2, and BOLA3 genes display interactions with proteins involved in cytoskeletal dynamics and organelle biogenesis, indicating their contributions to cellular organization and function. Furthermore, their enrichment in pathways related to cancer and signaling cascades underscores their potential significance in disease pathogenesis and cellular signaling networks. These findings provide a comprehensive framework for understanding the diverse roles of BOLA1, BOLA2, and BOLA3 genes in cellular physiology and disease, laying the groundwork for future mechanistic studies aimed at targeting these genes for disease management.

Following our investigation into drugs targeting BOLA1, BOLA2, and BOLA3, we have identified a compelling array of 6 anti-tumor agents, namely estradiol, cyclosporine, quercetin, dasatinib, acetylcysteine, and cannabidiol. Estradiol, a potent form of estrogen, has garnered attention for its potential anticancer properties, particularly in the context of hormone receptor-positive breast cancer.[26] Notably, the modulation of BOLA1, BOLA2, and BOLA3 expression introduces intriguing avenues for exploring its efficacy in cancer therapy. Cyclosporine, known primarily as an immunosuppressive medication, reveals additional anti-tumor potential through its inhibition of calcineurin signaling, thereby disrupting key pathways involved in tumorigenesis.[27-29] Quercetin, a natural flavonoid abundant in various fruits and vegetables, emerges as a promising candidate for cancer prevention and treatment, leveraging its antioxidant and anti-inflammatory attributes to target BOLA1, BOLA2, and BOLA3-mediated pathways.[30-32] Dasatinib, a potent tyrosine kinase inhibitor, offers targeted intervention against specific cancer-related pathways, potentially intersecting with BOLA1, BOLA2, and BOLA3-associated mechanisms.[30,33,34] Acetylcysteine, recognized for its antioxidant properties and role as a glutathione precursor, presents a complementary approach to cancer therapy by modulating BOLA1, BOLA2, and BOLA3 expression and associated pathways.[35] Additionally, cannabidiol, derived from cannabis, represents a novel avenue in cancer research, with ongoing exploration into its potential antitumor effects and interaction with BOLA1, BOLA2, and BOLA3 signaling.[36] Together, these findings not only expand our understanding of BOLA1, BOLA2, and BOLA3 biology but also underscore the diverse therapeutic potential of targeting BOLA1, BOLA2, and BOLA3 with existing anti-tumor agents, offering promising prospects for future cancer treatment strategies.

Study strengths & limitations. This study introduces several novel insights into the field of KIRC research. Firstly, it identifies the BOLA gene family (BOLA1, BOLA2, and BOLA3) as novel biomarkers for KIRC, highlighting their dysregulated expression in cell lines and tissue samples. This finding suggests that BOLA genes could be utilized for early diagnosis, prognostic evaluation, and as potential therapeutic targets in KIRC,

addressing a critical gap in the specificity and sensitivity of current biomarkers. Secondly, the study uncovers a significant epigenetic component in the regulation of these genes, demonstrating that the hypomethylation of BOLA1, BOLA2, and BOLA3 contributes to their abnormal up-regulation in KIRC. This epigenetic alteration presents a new avenue for exploring the mechanisms of BOLA gene regulation in KIRC pathogenesis. Additionally, the study provides insights into the role of BOLA genes in shaping the immune microenvironment, suggesting their involvement in immunomodulatory processes within KIRC tumors. Finally, the identification of existing anti-tumor agents that target BOLA gene expression offers promising therapeutic potential, broadening the scope for personalized treatment strategies. The limitations of this study include several important aspects that could influence the generalizability and robustness of the findings. Firstly, the experiments were carried out using a specific cell line (786-O cells) which may not fully capture the heterogeneity observed in primary tumors of renal cancer patients. As different cell lines exhibit distinct molecular characteristics and responses to gene knockdown or treatments, the results may not necessarily translate across other models or patient samples. Therefore, further validation of the findings across multiple renal cancer cell lines or patient-derived samples is essential to confirm the broader applicability of the results. Additionally, the study did not explore the potential role of the tumor microenvironment or other external factors, such as immune response and stromal cell interactions, which can significantly influence cancer progression and gene regulation in vivo. Future studies should incorporate in vivo models, such as patient-derived xenografts or organoid models, to better understand how BOLA gene silencing affects tumor growth and behavior in a more physiologically relevant setting. Furthermore, examining the potential post-translational modifications of BOLA genes and their interaction with other cellular pathways may reveal more regarding their role in cancer progression. Lastly, the use of in vitro assays alone limits the study's ability to capture the complexity of cancer biology. Expanding the study to include clinical samples and patient data would be beneficial to further corroborate the in vitro findings and assess the prognostic and therapeutic relevance of BOLA gene silencing in KIRC.

In conclusion, the findings from this study demonstrate significant up-regulation and hypomethylation of BOLA gene family members across KIRC cell lines, indicative of their potential as biomarkers for distinguishing KIRC patients from normal individuals. Retrospective analyses of TCGA-KIRC cohort's further support these observations, with BOLA1, BOLA2, and BOLA3 demonstrating substantial overexpression and hypomethylation in KIRC compared to normal tissues. Additionally, genetic alteration analyses reveal associations between BOLA genes and patient survival outcomes, with BOLA1 and BOLA2 mutations linked to shorter OS. Functional assays further underscore the importance of BOLA1, BOLA2, and BOLA3 in KIRC progression, as their knockdown inhibits cell growth and wound healing. Lastly, drug inquiry unveils potential therapeutic candidates targeting BOLA expression. These findings collectively suggest that BOLA1, BOLA2, and BOLA3 may serve as valuable prognostic biomarkers and therapeutic targets for KIRC.

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