Gene Expression and Regulatory Webwork of *POLR2K* in Bladder Carcinogenesis by Integrated Bioinformatics Approaches

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Abstract

BackgroundRNA polymerase II subunit K (*POLR2K*) belongs to one of the multiple subunits of RNA polymerase II (Pol II), whose biological function is to synthesize mRNA. Aberrant *POLR2K* expression is related to carcinogenesis. However, *POLR2K*'s underlying role in bladder cancer has not been explored. In the current study, we intend to analyze the function of *POLR2K* and its regulatory network within bladder cancer.

Methods: Public sequencing data was obtained from GEO and TCGA to investigate *POLR2K* expression and regulatory network within bladder cancer (BLCA) by using GEPIA and Oncomine as well as cBioPortal online tool. LinkedOmics was employed to identify genes displaying significantly differential expression patterns and to perform GO and KEGG analyses. After differential genes was assigned and ranked, GSEA analysis was performed to obtain target networks for transcription factors, miRNAs, and kinases that could regulate *POLR2K*–associated gene network. Subsequent functional webwork analyses were used to identify cancer-relevant pathways Moreover, *POLR2K* gene is verified by ChIP-seq in MCF-7 cell line with transcription factor binding evidence in the ENCODE Transcription Factor Binding Site Profiles dataset. In the end, the biological behavior of

POLR2K in BLCA was validated by cell function experiments and tissue micro-assay.

Conclusions: The current study implies that *POLR2K* gene is overexpressed and often amplified in BLCA, providing the first evidence that *POLR2K* deregulation, especially increased transcription, may promote BLCA. Knocking down *POLR2K* in BLCA 5637 and T24 cells could decrease cellular viability, reduce metastasis ability and induce G1 arrest, which might be mediated by E2F1. These findings uncover a unique expression pattern of *POLR2K* and its potential regulatory networks in BLCA, contributing greatly to study of the role of *POLR2K* in cancer development.

Background

Bladder cancer is the 11th most prevalently diagnosed cancer worldwide [1]. About 70% of patients initially diagnosed with non-muscle-invasive bladder cancer (NMIBC), need post-surgical management and appropriated surveillance, which could be not only costly but also invasive [2]. Despite high recurrence and metastasis, the development of various targeted drugs , including Erdafitinib and Nivolumab[3], has contributed to the significant improvement in the survival of patients and brought about a revolutionary breakthrough in the treatment of advanced BLCA[2, 4]. But these targeted drugs have also shown unsatisfactory results. Erdafitinib is prescribed only when abnormal fibroblast growth factor receptor (FGFR) gene is detected in advanced BLCA patients. And Nivolumab may cause serious side effects since it could mislead the immune system to attack healthy cells. The etiology of BLCA is particularly multiplex, including processes involved in signal transduction and transcriptional regulation, which reflects the accumulated physiological effects of multiple genes [5]. Screening gene networks for alterations associated with carcinogenesis and cancer proliferation could be an encouraging method to identify novel drug target for BLCA.

RNA polymerase II subunit K (*POLR2K*), also known as RPABC4, is a gene encoding one of multiple subunits of RNA polymerase II (Pol II), whose biological function is to synthesize mRNA. This subunit could be also found in another two polymerases, RNA polymerase III (Pol III) and RNA polymerase I (Pol I). *POLR2K* could be utilized to synthesize diverse function non coding RNAs, small RNAs, mRNA progenitors, and ribosomal RNA progenitors. *POLR2K* is related to DNA replication, translation synthesis and transcription [6, 7] while it participates in many tumor formation and progression-promoting cell signaling pathways [8]. Regulation of *POLR2K* was first explored in chronic hepatitis, where its association with IRF1 was clarified[9], and then its over-expression was confirmed in multiple

malicious cancers, containing bladder/breast/ovarian cancer[10, 11]. In our bioinformatics-based analysis, we found that *POLR2K* might serve as a prognostic gene in no cancers except in bladder cancer. Therefore, we want to clarify the role of *POLR2K* in BLCA and its influences on cancer development, expression patterns and regulatory networks of *POLR2K* in BLCA.

In this study, we found that *POLR2K* was overexpressed in BLCA patient samples from The Cancer Genome Atlas compared to normal liver samples. In addition, Kaplan-Meier survival analysis displayed that poor progression-free survival and poor overall survival of BLCA patients were related to *POLR2K* overexpression (Supplementary Figure 1). Therefore, we further explore the role of *POLR2K* in BLCA from various public databases. Genomic alterations and functional networks relating to *POLR2K* in BLCA were analyzed by using multi-dimensional analysis methods. Thus, these results reveal a novel function of *POLR2K* as a regulator of cancer proliferation and provide a potential strategy of treatment for BLCA. *POLR2K* expression might function as a novel therapeutic target for bladder cancer.

Methods

2.1 Oncomine analysis

Oncomine 4.5 (www.oncomine.org), one of the largest existing oncogene chip database which currently includes 264 independent datasets, containing various cancer types analysis methods[12], was used for analyzing DNA copy number and mRNA expression of *POLR2K* within bladder cancer whose publicly-accessible online database could be derived from the Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo). *POLR2K* expression was evaluated in bladder carcinoma tissues relative to normal bladder tissues where significant differences were determined as p 0.01. The validation of this method was demonstrated by Limei Zhong et al to analyze SIRP1 expression levels in breast and lung cancers, and by Shvartsur et al to investigate RKIP cross-talk in multiple myeloma [13, 14].

2.2Pathway Commons

Pathway Commons is a collective database of biological pathway database derived from multiple sources associated with interactions from various organisms and functional correlations between genes in signaling pathways[15], whose data can be derived from its partner databases and the pathway data are represented in the BioPAX file.*POLR2K* was used as seed gene to acquire the neighboring associated genes. This method was used by Sonmez and Can to compare tissue/disease specific integrated networks [16].

2.3UALCAN analysis

UALCAN is a comprehensive transcriptome database whose data is collected from the TCGA, and correlation between clinicopathological features and gene expression could be retrieved [17]. UALCAN (ualcan.path.uab.edu) is used to analyze cancer transcriptome data based on TCGA database. This online tool was deployed by Lyu et al to analyze the prognostic value of P4HB in bladder urothelial carcinoma [18].

2.4cBioPortal analysis

The platform(cbioportal.org), termed as cBioPortal, has hosted more than 200 cancer genomics studies , which is often used to explore, visualize, and analyze multi-dimensional cancer genomic and clinical data information based on its multiple analysis features[19]. The mRNA expression, CNVs, and mutation of *POLR2K* were chosen as search parameters and then the tab Network was used for visualizing its biological interaction network based on the alteration frequencies. Association between the expression of secreted phosphoprotein-related genes and prognosis

of human cancer was evaluated by this method [20]. Cytoscope was further introduced to analyze GO and KEGG pathway.

2.5LinkedOmics analysis

With abundance in multi-omics and clinical data information of various cancers from TCGA-associated multidimensional datasets[21], LinkedOmics (www.linkedomics.org/ login.php) was utilized to investigate mRNA sequence data from 408 BLCA patients within the Cancer Genome Atlas database. The gene expression difference correlated with *POLR2K* was studied by using LinkFinder module and Pearson's correlation coefficients was chosen to investigate the results. Individual gene's statistical plots are presented in heat maps or scatter plots. After the results were ranked, the pathway enrichment analysis of GO and KEGG as well as target enrichment analysis of transcription factor, miRNA and kinase was performed by GSEA. This website was used by Liang et al to analyze abnormal expression and prognostic significance of EPB41L1 in kidney renal clear cell carcinoma [22].Target enrichment of the transcription factor and miRNA was primarily derived from Molecular Signatures Database.

2.6GEPIA analysis

GEPIA, an interactive web application providing and analyzing the RNA sequencing expression data based on the GTEx and TCGA projects [23], enables users to perform various of gene expression analyses and survival analyses including overall survival and progression-free survival. For the survival analysis, *POLR2K* was input as a gene symbol; BLCA was added as the datasets; other setting, except Methods tab, was unchanged by default. This method was used to investigate the role of SRGN in the survival and immune infiltrates of skin cutaneous melanoma SKCM-metastasis patients [24], and also used in the study from Wang et al [25].

2.7GeneMANIA analysis

GeneMANIA is used for generating gene function hypotheses, analyzing gene list and then prioritizing genes for functional category assays [26]. When given a query gene list, GeneMANIA (www.genemania.org), by utilizing publically-available proteomics and genomics data information, could extend this list with functional similar and interacting genes to produce a functional related networks. GeneMANIA was also used for visualizing the gene network and predicting gene functions. This approach was used to analyze microenvironment dependent gene expression signatures in reprogrammed human colon normal and cancer cell lines [27], and by Shen et al to study 14-3-3gamma protein in uterine leiomyoma cells [28].

- **Enrichr analysis**

Enrichr is a collective annotation resource for curated human gene datasets and a search engine of accumulated multiple biological knowledge about billions of biological discoveries [29]. We use 50 neighboring genes of *POLR2K* to inquire Enrichr in Cell Types tab (amp.pharm.mssm.edu/Enrichr). This online tool was used to analyze gene expression profiles and protein-protein interaction networks in multiple tissues of systemic sclerosis [30].

2.9 Cell culture and transfection, antibodies and reagents

Human urinary bladder cancer 5637 and T24 cell lines were bought from the Cell Bank of the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). 5637 and T24 cells were cultured in 10% fetal bovine serum (Gibco, USA)-added RPMI-1640, supplemented with streptomycin and penicillin at a concentration of 100 U/mL respectively and maintained in a humidified CO2 (5%) incubator at 37℃. GAPDH antibody was purchased from

Abclonal#AC033, POLR2K antibody from SAB (#42880) and E2F1 antibody from Proteintech (#66515-1-Ig).The siRNA of POLR2K was purchased from Corues Biotechnology (Nanjing, China). The sequences (5'to3') of siRNA used to transfect are: POLR2Khumansi RNA1: CCAAUCAGAUGCAGAGAAUTT; AUUCUCUGCAUCUGAUUGGTT. POLR2Khumansi RNA2: GAGAAUGUGGAUACAGAAUTT; AUUCUGUAUCCACAUUCUCTT. Non-control (human): UUCUCCGAACGUGUCACGUTT; ACGUGACACGUUCGGAGAATT. The knockdown efficiency of siRNA was well above 95% at 48h. Transfection with the siRNA was performed with the help of Lipofectamine RNAiMAX Regent (Life Technologies) according to the manufacturer's instructions.

For transfection, we selected cells with good growth status and in the logarithmic growth phase, digested then into a single cell suspension and seeded theses in a 6-well plate. Then the 6-well cell plate was positioned in the incubator overnight. Upon attachment, the cells were prepared to be transfected with POLR2K-siRNA and Non control-siRNA at room temperature. Briefly, 9 μl RNAiMAX Regent was added to the 200μl RPMI 1640 medium free of FBS and then put aside for 5 min. Again, 10 μl of siRNA was added to 200μl of the medium and then set aside for another 5min, and subsequently blended with the RNAiMAX solution made in the last step. It was later positioned for 20 min. The original medium in the 6-well cell plate was replaced with 1.6 ml of the 1640 medium with 10% FBS six hours before the transfection step begins. The prepared transfection solution was finally added and kept to be maintained in the incubator.

2.10 RT-PCR assays and Western Blot

Extracted the total RNA of each siRNA-transfected and the blank controls 5637 and T24 cell lines respectively using RNA-easy kit (Vazyme) as the manufacturer's protocol then converted to cDNA by cDNA Synthesis Kit according to manufacturer's protocol. Real-time PCR was performed using CFX Connect Real-Time PCR Detection System. For real-time PCR of gene expression, the 20 μL PCR included 1 μL cDNA, 10 μL 2 × AceQ q-PCR SYBR Green Master Mix, 8 μL ddH2O and 1 μL 10 mM of primer mix. The primers for the q-PCR were as follows: POLR2K forward Primer Sequence (5’ to3’): GGAGAGTGTCACACAGAAAATGA, POLR2K reverse Primer Sequence (5’ to3’): TCGAGCATCAAAAACGACCAAT; E2F1 forward Primer (5’ to3’): CCCAACTCCCTCTACCCT, E2F1 reverse Primer Sequence (5’ to3’): CTCCCATCTCATATCCATCCTG. GAPDH forward Primer Sequence: TCGGAGTCAACGGATTTGG, GAPDH reverse Primer Sequence: TCGCCCCACTTGATTTTGGA. GAPDH was used as the internal control. Default threshold settings was used to determinate the ΔCt. and tThe threshold cycle (Ct) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. The mRNA expressions relative to the GAPDH expressions were calculated using the 2-ΔΔCt method.

Western Blot was performed as we previously did [31].

2.11 MTT assay, Colony formation, Wound healing, Transwell and Cell cycle analysis

MTT assay was applied to determine cell viability. Before being seeding in the 96 holes, the cells were transfected with POLR2K-siRNA and NC-siRNA in the disk and cultured for 12 hours. And then transfected-cells and blank control cells were seeded into 96-well culture plates; the time for cells to adhere to the holes is defined as starting point. To determine cell viability, the absorbance values were checked before and 1 to 3 days. Before detecting the absorbance values, MTT at 20 μL of 5 mg/mL was added to cells, then cells were incubated at 37 °C for another 4 h. After the supernatant was discarded, DMSO of 200μl of was added into 96-well plates, and oscillated to dissolve the crystalline matter. Absorbance (A) of the final formazan was detected at 570 nm using Universal Microplate Readern (BioTek Instruments, USA).

Colony formation was performed to evaluate the growth ability of bladder cancer cells. Twenty-four hours after transfection, the 5637 and T24 cells were digested and transfered to a 6-well culture plate and maintained in the incubator. The growth status of the cells was observed every 3 days. Every other three days, we re-transfected 5637 and T24 cells with siRNA to maintain the low level of POLR2K. After 12 days, the colonies were fixed with formaldehyde, stained with 0.1% crystalline purple, washed and dried at room temperature. The picture was taken by phone and processed by Photoshop.

The effects of POLR2K knockdown on the cell cycle were detected by flow cytometer with PI staining. We cultured siRNA-transfected 5637 and T24 cells in RPMI-1640 medium for 48 h. Next, the cells were harvested and suspended with PBS. Centrifuged the sample at 1000 rpm for 5 min, discarded the supernatant liquid, and slowly combined with 75 % ice-cold ethanol and incubated overnight at 4 °C. The cells were washed twice at 1000 rpm, re-suspended in PI/RNase Staining Buffer (KeyGen Biotechnology) and incubated for 30 min at 4 °C in the dark and detected by flow cytometer (Becton Dickinson). Data were analyzed by FlowJoy version 10.

Wound healing experiments were performed to assess cell migration ability. Cells were cultured in 6-well plates 3 × 105 cells/well and maintained in RPMI-1640 supplemented 10 % FBS for 24 h until they reached confluence. 200-μl pipette tip was used to create a wound in the adherent cells. Then serum-free 1640 medium was deployed to maintain the cells. The changes in wound area were pictured using an inverted microscope.

Before Transwell experiment, the cells were seeded into disk, and transfected with siRNAs. Twenty-four hours later, cells were digested, centrifuged. After discarding the supernatant and re-suspensed with the 1640 media, the cells was centrifuged and rinsed again. It was then re-suspended with the 1640 medium. After calculating the cell numbers, and the cells were prepared into 400μl cell suspension with 1640 medium and then placed in the upper chamber. In the lower chamber of the Transwell, 600μl of the RPMI culture somution supplemented with 10% FBS was added and maintained at 37 °C. The cells that penetrats the membranes were used to evaluate the invasive ability of cancer cells. Twenty-four hours later, the chamber was removed, the remaining cells wiped with a cotton swab, and dried at room temperature. The sample was fixed with 4% paraformaldehyde and stained with 0.1% crystalline purple. The filter membrane was dried with a cotton swab, and the sample was photographed.

2.12 Tissue microarrays (TMAs)

BLCA tissues and matched noncancerous bladder tissues were analyzed using TMAs purchased from OUTDO BIOTECH (Shanghai, China). 39 tissue pairs in the TMAs were subjected to immunohistochemistry to assess POLR2K and E2F1 expression. Immunohistochemical staining was performed as Bin et al described [32]. Briefly, before the tissue micro-assay slide was probed with the desired primary antibody, such as POLR2K and E2F1 in a humidified chamber overnight at 4 °C, goat serums were used to block in a humidified chamber for 1 h. Then the section was washed by newly-made PBS and hybridized with the secondary antibody. Hematoxylin was used to counterstain. ZSGB-BIO (Histostain-SP Kits, cat: SPN-9001) provides all reagents. The TMAs were independently evaluated by two investigators who were blind to clinical data of the patients. Expression level of PORL2K and E2F1 was assessed by Histochemistry score (H-SCORE). H-SCORE is a scoring method for tissue immunohistochemical results, which mirrors the positive intensity and the positive ratio. The formula is: H-SCORE=$\sum$ (PI × I) = (percent of cells of strong intensity ×3) + (percent of cells of moderate intensity ×2) + (percent of cells of weak intensity ×1). In the formula, I means the intensity of staining and PI stands for the percent of positive cells to the total number of cells in this position.

2.13 Statistical analysis

All experiments were repeated for at least three times. Experimental values were expressed as the means ± standard deviation (SD) of the number of experiments indicated in the legends. Data processing among multiple groups and analysis of variance (ANOVA) was performed using GraphPad Prism software. All comparisons were made relative to untreated controls and significance of difference is designated as *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

Results

3.1 *POLR2K* expression in bladder cancer

POLR2K, as an important gene in the RNA process machinery, is the only subunit common to all three polymerases that dose dependently improves the assembly of the Pol III pre-initiation complex [33, 34]. As Pol III products are required for protein synthesis and cancers have unusually high levels of Pol III activity [35], it is possible that the up-regulation of *POLR2K* could facilitate Pol III assembly and thus contribute to cell proliferation and cancer development [11].Inspired by this ,we used GEPIA to perform survival analysis for *POLR2K* in various types of cancers, and found that *POLR2K* overexpression was significantly related to poor survival(Supplementary Figure 1). Therefore, we want to further clarify the role of *POLR2K* in BLCA and understand the influences on cancer development, expression patterns and regulatory networks of *POLR2K* in BLCA.

Using TCGA and the Gene Expression Omnibus (GEO) database, we firstly assessed *POLR2K* transcription levels in BLCA studies. Analysis of the Oncomine database revealed an increase in DNA copy number variation (CNV) and mRNA expression of *POLR2K* in BLCA tissues compared with normal tissues (p 0.01). *POLR2K* ranked within the top 17% of transcriptome profile and within the top 9% of DNA CNVs (Figure 1), when the fold differences were adjusted to 2. In addition, correlation analysis of *POLR2K* with clinic pathological features of 408 bladder cancer patients from the Cancer Genome Atlas database was conducted by UALCAN website. And the results consistently suggested high transcription levels of *POLR2K* in BLCA. Compared with healthy people, the level of transcription for *POLR2K* was much higher in bladder cancer patient based on the sub-group such as disease stages, gender, smoking condition, histological subtypes, and nodal metastasis status (Figure 2). Furthermore, Kaplan-Meier survival analysis displayed that high levels of *POLR2K* was positively related to poor progression-free survival (PFS) and overall survival (OS) in bladder cancer patients (Supplementary Figure 1).Therefore, *POLR2K* expression could become a potential diagnostic indicator in BLCA.

3.2 Alterations of *POLR2K* in bladder cancer

3.2.1 *Type and frequency of POLR2K alterations in bladder cancer*

POLR2K is a gene encoding one of multiple subunits of RNA polymerase II (Pol II), whose biological function is to synthesize mRNA. This subunit could be also found in another two polymerases, RNA polymerase III (Pol III) and RNA polymerase I (Pol I). *POLR2K* could be utilized to synthesize diverse function non coding RNAs, small RNAs, mRNA progenitors, and ribosomal RNA progenitors. In order to investigate the type and frequency of *POLR2K* changes within bladder cancer, we then used the ciBioPortal to analyze sequence data from BLCA patient tissues in the TCGA database. Compared with normal healthy tissues, *POLR2K* level was altered in 141 of 408 (35%) BLCA patient tissues (Figure 3a). These alterations includes mRNA up-regulation in 139 cases (34.5%), amplification in 59 cases (14.7%), multiple alterations in 55 cases (13.7%) mRNA and down-regulation in 2 cases (0.49%) (Supplementary Fig 2). Therefore, amplification is identified in BLCA, as the most prevalent type of *POLR2K* CNV.

3.2.2 *Interaction network of POLR2K alterations in bladder cancer*

To investigate biological interaction network of *POLR2K* in BLCA, we used Common Pathways to obtain *POLR2K*–neighboring genes, and then used Cbioportal to inquire the alteration frequencies of these genes. (Figure 3b and Supplementary Fig 2). The neighboring genes of *POLR2K* make KDMSA (35%), KMT2D (33%) together with INTS8 (32%) as one of the most frequent alterations in BLCA. With the help of Cytoscape, analysis of GO category annotations implied these genes encoding proteins localized primarily to, or functioned at, RNA polymerase complex, the spliceosomal complex, and MLL3/4 complex. These proteins are fundamentally related to posttranscriptional gene expression processes (i.e. snRNA and dsRNA processing, transcription-coupled nucleotide-excision repair, etc), but they were also enriched such as “U2-type spliceosomal complex” and “basal transcription machinery binding” (Figure 4a–4c). As expected, KEGG pathway analysis indicated that *POLR2K* is connected to Spliceosome, RNA polymerase, mRNA surveillance and Basal transcription factors (Figure 4d). As a result, the interaction network of *POLR2K* alterations is related to several RNA metabolic processes and the gene expression regulation.

3.3 Enrichment analysis based on *POLR2K*functional network in bladder cancer

3.3.1 *GO annotation and KEGG enrichment analysis for co-expression genes related to POLR2Kin bladder cancer*

Using LinkedOmics online tool, we took advantage of TCGA database to explore mRNA sequencing data comprising 408 patients of BLCA. From the volcano plot depicted in Figure 5a, significant negative correlation with *POLR2K* was suggested among 2395 genes (dark green dots), while significant positive correlation was indicated among 2635genes (dark red dots) (P-value<0.01, false discovery rate FDR< 0.05). The heat map displayed 50 significant genes whose mRNA expression positively or negatively correlated with *POLR2K* in BLCA (Figure 5b, 5c). This result demonstrated that *POLR2K* had an extensive influence on the transcriptome. Supplementary Figure 3a–3c show scatter plots for each gene. Significantly, *POLR2K* expression is positively related to the expression of ZNF706 (positive rank #1, Pearson correlation = 0.7887, p = 8.055e-88), YWHAZ (Pearson correlation = 0.6695, p = 2.224e-54), and COX6C(Pearson correlation = 0.6638, p = 3.552e-53), which mirrors alterations in mitochondrial composition as well as apoptosis, DNA repair, and transcriptional regulation. Further exploration by GSEA analysis demonstrated differentially expressed genes were primarily related to mitochondrial protein complex, spliceosome complex, replication fork and condensed chromosome, whereas those genes are involved mainly in mitochondrial gene expression, mRNA/ncRNA processing, DNA replication and cell cycle. These genes also serve as constituent of mitochondria and ribosomes (Figure6a–6c and Supplementary Tables 1–3).

Interestingly, GSEA analysis implied that these genes were also located in MHC protein complex, secretory granule membrane, cell leading edge and extracellular matrix, where they participated in the positive regulation of cell motility, antigen binding and immune response-regulating signaling pathway. They were also involved in response to type I interferon, I-kappaB kinase/NF-kappaB signaling, Toll-like / NOD-like receptor signaling pathway and JAK-STAT signaling pathway, all of which were associated with tumor immune escape. Also, GSEA analysis of KEGG pathway showed highly significant enrichment in cell cycle, DNA replication, ribosome and spliceosome, as well as in Natural killer cell mediated cytotoxicity and Cytokine-cytokine receptor interaction (Figure 6d, 6e and Supplementary Table 4). Taken together, *POLR2K* function networks were mainly responsible for gene expression, mRNA surveillance, cell cycle, DNA replication while also involved in tumor immune response and survival, which demonstrated significant deregulation of cancer related pathways.

3.3.2 *POLR2K networks of transcription factor, miRNA and kinase targets and in bladder cancer*

Transcription factor, miRNA and Kinase target network of associated gene dataset created by GSEA were used to examine targets of *POLR2K* in BLCA. The significant kinase-target networks were associated mainly with mitogen-activated protein kinase 6 (Kinase_MAPK6), homeodomain interacting protein kinase 2Kinase_HIPK2and mitogen-activated protein kinase 7 (Kinase_MAPK7) (Table 1 and Supplementary Tables 5–7); the miRNA-target network was generally related to AACTGGA_MIR145, TTGCCAA_MIR202, ACTTTAT_MIR507, GTGCAAT_MIR25_MIR32_MIR92_MIR363_MIR367 and TATTATA_MIR374; the transcription factor-target network was involved fundamentally in the cell cycle regulation factors including V$E2F1_Q6, V$FOXO1_02 and V$CDC5_01, whereas also involved in the IRF Transcription Factor (IRF) family including V$IRF7_01, V$IRF1_01, and V$ISRE_01. Correlation among genes for the Kinase MAPK7, miR-145 and E2F1_Q6 respectively was uncovered by subsequent protein-protein interaction networks created by GeneMANIA. These genes enriched for transcription factor E2F1 have been linked primarily to modulating cell cycle checkpoint, DNA repair and DNA replication, while associated with MCM complex, G1/S transition of mitotic cell cycle and telomere maintenance (Figure 7). Furthermore, genes enriched for MAPK7 are related mainly to the regulation of innate immune response, toll-like receptor family signaling and stress-activated protein kinase signaling cascade (Supplementary Figure 4). And genes enriched for miRNA-374 can be related primarily to modulation of Notch signaling pathway, activating transcription factor binding and modulation of transcription in other organism involved in symbiotic interaction (Supplementary Figure 5)

4. Validation by Experiments

4.1 In vitro *knockdown of POLR2Kinhibits bladder cancer cell proliferation and progression*

Since increased *POLR2K* level is related to malignant proliferation, *POLR2K* might have critical effects on one or multiple steps of bladder cancer progression. To validate the effects of *POLR2K* on cell proliferation in bladder cancer cell, siRNA was used to knockdown *POLR2K* in bladder cancer 5637 and T-24 cells. To avoid the off target effects of *POLR2K*-siRNA, we used siRNA1 and siRNA2 to knockdown *POLR2K*, in the following experiments. The knockdown efficiency was assessed by RT-qPCR, and Western blotting (Figure. 8a-8c).The MTT experiment indicated that the viability of 5637 and T24 cells were significantly reduced after *POLR2K* knockdown (Figure. 8d-8e).In addition, Colony formation was performed to evaluate the growth capability of siRNA-transfected 5637 and T24 cancer cells. The results showed that there were significantly fewer colonies in the *POLR2K* knockdown group than in the Non-control group (Figure. 8f-8g), demonstrating that decreased expression of *POLR2K* could slow down bladder cancer cell growth. Taken together, these results indicated that in vitro knockdown of *POLR2K* suppressed bladder cancer cell proliferation and progression.

4.2 In vitro *knockdown of* POLR2K *decreased bladder cancer cell invasion and migration*

Since POLR2K overexpression is positively related with metastasis stage in BLCA patients based on our bioinformatics analysis (Figure 2f), we want to validate whether POLR2Kcould have an influence on invasion and migration in BLCA cells in vitro. Therefore, Wound Healing and Transwell experiments were performed in siRNA-transfected 5637 and T24 cell lines, results showing that migration and invasion ability of the POLR2K knockdown group was remarkably suppressed compared with that of Non-control group (8h-8j).Taken together, these results indicated that in vitro knockdown of POLR2K suppressed bladder cancer cell invasion and migration.

4.3 In vitro *knockdown of*POLR2K *lead to decreased E2F1 level and G1 arrest*.

Based on analysis of *POLR2K* functional network in bladder cancer, we have found that *POLR2K* is related with cell cycle (Figure 6e) and may be involved fundamentally in the V$E2F1_Q6 dataset. To validate this, q-PCR and Western Blot were used to examine E2F1 level after knocking down *POLR2K*.The results showed that upon *POLR2K* knockdown, E2F1 is diminished both in mRNA and protein level at 48 hours (Figure 8k-8m). In consistence with this, Flowjo analysis showed that G1 arrest occurs after knocking down *POLR2K* according to the PI staining (Figure 8n-8o). These findings show that *POLR2K* could modulate the expression of E2F1 in BLCA. Interestingly, we found that E2F1 could in return regulate *POLR2K* expression according to HA-E2F1_MCF-7_hg19_1 Gene Set in ENCODE Transcription Factor Binding Site Profiles(https://maayanlab.cloud/Harmonizome/gene_set/HA-E2F1_MCF-7_hg19_1/ENCODE+Transcription+Factor+Binding+Site+Profiles). The corresponding binding sequence was shown in Figure 8p. Furthermore, TCGA database showed a significantly positive correlation between *POLR2K* and E2F1 (Figure 8q), whose result is similar with ours based on tissue micro-assays (Supplementary Figure 6). Taken together, these results suggests that the *POLR2K* may interact with E2F1 in a positive loop where malignant phenotypes of bladder cancer cells could be aggregated.

4.4 PORL2K *is a positive indicator in BLCA*

To validate the clinical relevance of our bioinformatics findings, we detected POLR2K and E2F1 expression patterns in bladder cancer patients by immune-staining of a tumor tissue microarray. Consistent with our bioinfomatics findings in bladder cancer, the level of E2F1 and POLR2K was significantly increased compared with the matched non-cancerous tissues (Figure 8s-8u), and the expression of E2F1 was related to POLR2K expression in bladder cancer (Supplementary Figure 6.). These results indicate a hint that POLR2K could be a tumor promoter within the bladder cancer.

Discussion

DNA-directed RNA polymerases (Pol I, Pol II and Pol III) are differentially expressed and deregulated in multiple cancers [36]. *POLR2K*, a subunit of these polymerases, participates in multiple steps of transcription [37].In this paper, we initially discovered that *POLR2K* was overexpressed in 408 BLCA samples from the TCGA and that high *POLR2K* expression may serve as an indicator of poor survival. Subsequently, further studies of the expression of *POLR2K* and its regulatory network will be necessary to obtain additional insight into the possible function of *POLR2K* in BLCA. Therefore, we conducted bioinformatic analyses of sequence data and cell function experiments, hoping to motivate the future research of *POLR2K* in bladder cancer.

It has been a wearing problem for years since early detection approach of BLCA eluded many clinicians. Cystoscopy and urine cytology are currently employed to diagnose BLCA [38]. However, cystoscopy is an invasive method with low sensitivity for bladder carcinoma in situ. Urine cytology is a non-invasive and has a higher specificity but lower sensitivity for low-grade urothelial tumors. In spite of the search for urinary biomarkers for the early and non-invasive detection of BLCA, no available biomarkers are currently employed in clinical practice. Thus, potential BLCA biomarkers are urgently required to increase the early diagnosis of BLCA. We also demonstrated that CNVs and mRNA expression of *POLR2K* are much higher in bladder cancer tissues compared with normal tissues by analyzing transcriptional sequence data from clinical patient tissues. We discovered that *POLR2K* overexpression exists in many cases of bladder carcinogenesis. We think that *POLR2K* research could move the field of liquid biopsy biomarkers forward, which deserves additional clinical and research attention. And it needs to be verified whether *POLR2K* could be detectable in liquid biopsy.

It has been reported that CNVs could directly influence gene expression and have drastic phenotypic consequences due to altering gene dosage or disrupting coding sequences. [39, 40]. This work discovered that amplification was the main type of *POLR2K* change and *POLR2K* copy number was augmented, which was related to poor survival including overall survival (OS) and disease-free survival (DFS) (Supplementary Figure 1). In light of these findings, we inferred that alterations in chromosomal structure might be involved in the altered *POLR2K* expression as well as *POLR2K* dysfunction in BLCA. *POLRAK* genetic change could lead to alterations in numerous downstream signals that may ultimately result in carcinogenesis for *POLR2K* plays a critical role in multiple biological functions. In fact, neighboring gene networks in close proximity to *POLR2K* display amplification with varying strengths in BLCA. Meanwhile, associated functional network is found to be related to spliceosome signaling, mRNA surveillance and ribosome signaling. Therefore, the network of *POLR2K* alterations is related to posttranscriptional modulation, which is involved in protein translation as well as RNA splicing, in consistence with several other published results about the biological roles of *POLR2K* [41, 42]. Furthermore, related functional networks are also involved in positive regulation of cell motility, antigen binding and immune response-regulating signaling pathway. Therefore, the network based on *POLR2K* genetic change is also associated with the tumor immune response, which is closely related to the mechanism by which tumor escapes from host immune system.

To reveal critical network of transcription factors, miRNAs and target kinases, GSEA analysis is performed. Our results imply that the functional network of *POLR2K* is involved generally in the ribosome, spliceosome, mRNA/ncRNA processing, cell cycle and DNA replication. Just the same as the mutation webwork, the functional association network that integrates the effects of *POLR2K* transcription alteration participates in RNA metabolic processes and gene expression regulation. As represented above, we conclude that *POLR2K* has a profound influence on the maintenance of short introns splicing [43, 44].

Oncogenic kinases play a critical role in coupling intracellular signaling pathways with extracellular signals, which promote cancer progression in all stages [45]. We discovered that *POLR2K* in BLCA is related to a network of kinases including Kinase_MAPK7, Kinase_MAPK6 and Kinase_HIPK2. All of these kinases regulate mitosis, gene expression and apoptosis. Indeed, HIPK2 has been recognized as a signaling molecule which acts in various signal transduction pathways and cellular processes such as cell proliferation and apoptosis, transcriptional regulation and antiviral responses[46, 47] while MAPK7 could facilitate tumor escape from immune surveillance [48, 49] . Deregulated activity of HIPK2 may affect the genome integrity, leading to cancer development [50]. In BLCA, *POLR2K* might modulate DNA replication, repair, and gene expression via HIP2 kinase.

“Continuous proliferation” has been proposed as top one of 10 hallmark features of tumors [51]. One crucial explanation is that cell cycle-associated proteins, if aberrantly expressed, could contribute to cell cycle disorder in tumor cells, which results in decreased differentiation, abnormal proliferation and rapid progression in cancer cells[52]. E2F1 is among key links in cell cycle modulation web-work. Abnormal E2F1 expression proactively is related to the tumor formation and progression of BLCA [53]. One study has published that elevated levels of E2F1 is involved in shorter survival of bladder cancer patients [54], and another study showed that the *POLR2K* network of transcription factor targets is related to E2F1[55]. Thus, these analyses suggest that E2F1 may be a critical target of *POLR2K* which modulates cell cycle and cancer progression in BLCA patients by acting through E2F1 transcription factor. Interestingly, according to known transcription factor binding site motifs from the TRANSFAC, we found that E2F1 could directly bind the promoter region of *POLR2K* likely to modulate the expression of *POLR2K* (http://amp.pharm.mssm.edu/Harmonizome/gene_set/E2F1/TRANSFAC+Predicted+Transcription+Factor+Targets). More convincingly, *POLR2K* gene is verified, by ChIP-seq in MCF-7 cell line , with transcription factor binding evidence in the HA-E2F1_MCF-7_hg19_1 transcription factor binding site profile from the ENCODE Transcription

Factor Binding Site Profiles dataset .Thus, these analyses indicate that E2F1 could interact with *POLR2K* to modulate the survival of cancer cells.

Cancer and chronic inflammation could be linked together by activating innate immune responses through NLR and TLR signals [56]. Chronic inflammation, mainly due to aberrant inflammasome or NF-κB activation, is closed coupled with cancer through TLRs-involved cytokine production[57]. The IFN-regulatory factor family proteins are transcription factors with varying biological functions, which might render a microenvironment for immune evasion and tumor progression [58]. Therefore, our analysis could represent that *POLR2K* might play a vital role in tumor microenvironment where *POLR2K* act as a chronic inflammation keeper and an immune surveillance regulator.

After POLR2K knockdown, cell viability, growth ability, invasion and metastasis ability of 5637 and T24 cells were decreased and G1 arrest induced. Combined with our bioinformatics analysis, we suspected this could be caused by E2F1 down-regulation after POLR2K knockdown, which was verified by q-PCR and Western Blot. Actually, many studies have reported that decreased E2F1 could induce G1 arrest [59-61].E2F1-POLR2k pathway is the critical pathway identified in our study, which has not been reported in any animal models or cell lines before, although there have been many experimental studies about E2F1[62-64] and POLR2K [11, 65]in various cancers respectively. We are the first team to report the close relationship between POLR2K and E2F1.We find that POLR2K could interact with E2F1 in a positive feedback loop to progress bladder cancer. We hypothesize that E2F1 could bind POLR2K promoter to promote the latter transcription in BLCA because E2F1 ChIP-seq has demonstrated that POLR2K promoter could be bound with E2F1 in breast cancer MCF-7 cell lines. And up-regulated POLR2K could directly or indirectly increase E2F1 mRNA level, for which one possible explanation is that POLR2K is able to help form stable open complexes and efficiently extend a dinucleotide on a pre-melted template or RNA on an elongation scaffold during E2F1 mRNA formation. These results indicates that POLR2K could interact with E2F1 in a positive loop. Therefore, it is reasonable to speculate that POLR2K might function as a novel therapeutic target. TCGA database has also showed a significantly positive correlation between POLR2K and E2F1($R = 0.42$; $p-value = 2.6e-18$).In consistence with this, tissue micro-assay showed that the expression levels of E2F1 and POLR2K were increased compared with the matched adjacent normal tissues, and the expression of E2F1 was positively related to POLR2K expression in bladder cancer. However, whether E2F1 could bind the promoter of POLR2K to enhance the latter's transcription in bladder cancer as in breast cancer still need to be verified by experiments such as ChIP-seq and Luciferase Reporter assay in BLCA. Specific mechanisms of how POLR2K interacts with E2F1 require further investigations.

Furthermore, using bioinformatics methods, we have identified that miRNA-145 and HIPK2 could also interact indirectly with POLR2K to promote bladder cancer progression, which are supposed to be validated by experiments. Although we have clarified how E2F1 could modulate POLR2K expression, how miRNA-145 and HIPK2 interact with POLR2K requires further study to understand regulatory network of POLR2K.Using TargetScan and BRENDA database, we found that miRNA-145 and HIPK2 have not direct effects on POLR2K. We infer that they could engage with POLR2K in an indirect way, which demands further studies.

Our data mining also recognized some miRNAs that were related to *POLR2K*. These miRNAs are short non-coding RNA molecules that post-transcriptional regulate protein expression. Distinct miRNAs alterations could be used to characterize BLCA [66, 67]. The particular miRNAs in this paper are associated with cancer occurrence, metastasis and invasion. Indeed, miR-145, miR-202 and miR-374 has been proposed as diagnostic, prognostic or therapeutic marker of BLCA [68, 69] .miR-202 and miR-347 participate in invasion, metastasis and cancer progression[69, 70], while miR-145 modulates *suppressor of cytokine signaling 7* (*socs7*) to enhance IFN-β expression, thus contributing

to BLCA apoptosis. We speculate that deregulation of these miRNAs would be in consistence with the phenotype of *POLR2K* overexpression in BLCA, which should be further verified by experiments.

Our study presents striking evidence for the significance of *POLR2K* in urinary bladder carcinogenesis and demonstrate its potential as an early indicator in BLCA. This study implies that *POLR2K* overexpression in BLCA has profound impacts on tumor immune surveillance and on multiple steps of gene expression and of the cell cycle, thus contributing to immune surveillance evasion ultimately. *POLR2K* is particularly associated with some tumor-related transcription factors such as E2F1 and IRF family, miRNAs such as miRNA-145 and kinases such as HIPK2. Our study deploys current online websites to conduct bioinformatics analysis of bladder cancer. This strategy has superiorities in terms of simplicity and large sample size, which enables us to perform more large-scale *POLR2K* genomics research and functional studies free of charge, compared with classic chip screening.

Meanwhile, by using the TCGA database, we also face some limitations. The first one is that the BLCA samples in TCGA database include three ethnic group (Caucasian, African-american and Asian). Different genetic background of BLCA patients can influence gene expression profiles. The second limitation is that the BLCA samples is relatively small in stage 1(e.g., the relatively low number of severe cases), which may have limited the statistical significance of some correlations. Thus, adequate inclusion of each ethnic group and BLCA stages should be included in further prospective studies to know more about the generalisability of this study. The third one is that transcriptional sequencing could directly present information about protein level or protein activity, which should be tackled in subsequent studies with experiments. The fourth limitation is that the infiltration of tumor-associated normal cells has a profound influence on the analysis of clinical tumor samples by genomic methods, such as copy number data or gene expression profiles, and therefore, biological interpretation of the analysis results deserves considerable attention considering sample heterogeneity, which could be the reason why the 50 most frequently altered neighbor genes enriched in the CD56+NKCells and 721_B_lymphoblasts (Supplementary Table 8).

Conclusion

Based on bioinformatics and experiments, this work reveals PORL2K could interact with E2F1 in a positive feedback loop to progress bladder cancer, and POLR2K overexpression is closely related with poor survival. It is reasonable to speculate that POLR2K could serve as a potential prognostic biomarker or a therapeutic target in BLCA.

Declarations

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Not applicable.

Authors ’ contributions

LY and KW designed and performed the research, analyzed and interpreted data. LY and WG drafted the manuscript. KW and XC participated in data analysis and figure preparation. YZ, QG and LW reviewed the manuscript. LY and KW conceived the study and participated in research design and data interpretation. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets during and/or analyzed during the current study available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Declarations

The authors declare that they have no competing interests.

Abbreviations

BLCA Bladder Carcinoma POLR2K RNA polymerase II subunit K TCGA The Cancer Genome Atlas CNVs Copy number variations E2F1 E2F Transcription Factor 1 GEO Gene Expression Omnibus GO Gene Ontology GEPIA Gene Expression Profiling Interactive Analysis GSEA Gene Set Enrichment Analysis IFN Interferon-α/β receptor OS Overall Survival DFS Disease-free Survival socs7 Suppressor of Cytokine Signaling 7 NLRs NOD-Like Receptors TLRs Toll-like Receptors ANOVA: Analysis of variance

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Table

Table 1

The Kinase, miRNA and transcription factor-target networks of POLR2K in bladder urothelial carcinoma (LinkedOmics).

Enriched Category	Geneset	LeadingEdgeNum	FDR
Kinase Target	Kinase_HIPK2	6	0.0468
	Kinase_MAPK7	12	0.0025
	Kinase_MAPK6	8	0.0380
miRNA Target	AACTGGA_MIR145	73	0.0286
	TTGCCAA_MIR507	47	0.0097
	ATAGGAA_MIR202	34	0.0243
	GTGCAAT_MIR25_MIR32_MIR92_MIR363_MIR367	82	0.0184
	TATTATA_MIR374	106	0.0185
Transcription Factor Target	V$E2F1_Q6	74	0.0312
	V$CDC5_01	101	0.0095
	V$FOXO1_02	93	0.0134
	V$IRF_Q6	74	0.0016
	V$ISRE_01	92	0
	V$IRF7_01	74	0.0078
	V$IRF1_01	81	0.0069

Abbreviations: LeadingEdgeNum, the number of leading edge genes; FDR, false discovery rate from Benjamini and Hochberg from gene set enrichment analysis (GSEA). V$, the annotation found in Molecular Signatures Database (MSigDB) for transcription factors (TF).

Figures

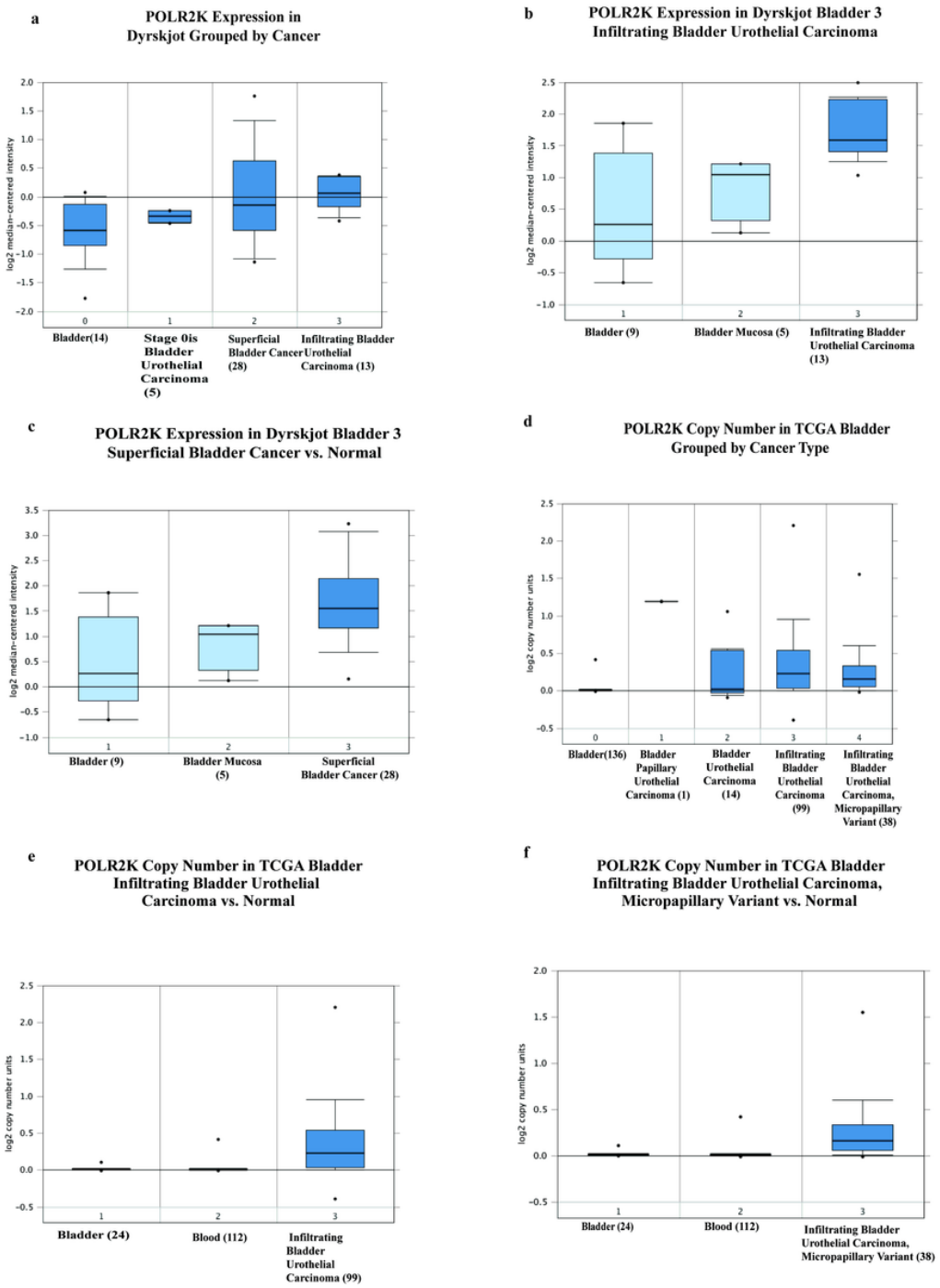

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Figure 1

POLR2K transcription in bladder cancer (Oncomine). Levels of POLR2K mRNA and DNA copy number were remarkably higher in bladder urothelial carcinoma than in normal tissues. (a) Box plot showing POLR2K mRNA levels in Dyrskjot dataset. Threshold by: p-value 10e-4, fold change 2, gene rank top 10%. (b-c) Box plot showing POLR2K mRNA levels of either Infiltrating Bladder Urothelial Carcinoma or Superficial Bladder Cancer versus normal bladder tissue. For figure b: p-value 3.72e-5, fold change 2.211, gene rank top 8%; for figure c: p-value 6.43e-5, fold change 2.178, gene rank top 17%. (d) Box plot showing POLR2K copy number in The Cancer Genome Atlas (TCGA) Bladder datasets. Threshold by: p-value 10e-4, fold change 2, gene rank top 10%. (e-f) Box plot showing POLR2K copy number of either Infiltrating Bladder Urothelial Carcinoma or Infiltrating Bladder Urothelial Carcinoma,

Micropapillary Variant versus normal bladder tissue. For figure e: p-value 5.11e-12, fold change 1.218, gene rank top 4%; for figure f: p-value 1.23e-5, fold change 1.179, gene rank top 9%.

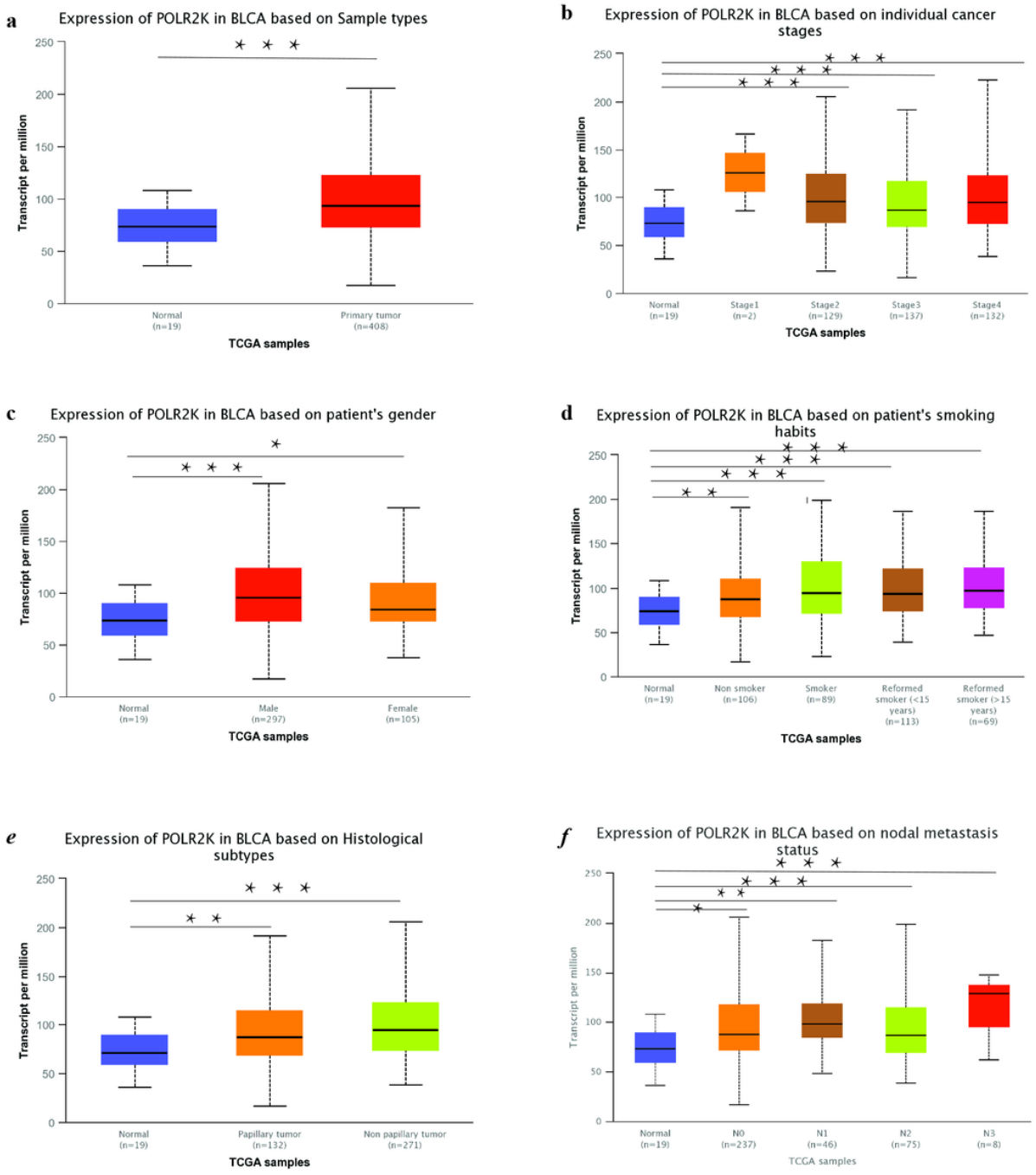

Figure 2

POLR2K transcription in subgroups of patients with bladder cancer, stratified based on disease stage, gender and other criteria (UALCAN). (a) Boxplot displaying relative level of POLR2K in normal and BLCA tissue. (b) Boxplot displaying relative level of POLR2K in normal individuals or in BLCA patients in stages 1, 2, 3 or 4. (c) Boxplot displaying relative level of POLR2K in normal individuals of either gender or male or female BLCA patients. (d) Boxplot displaying relative levle of POLR2K in normal individuals or in BLCA patients of Non-smoker, Smoker, Reformed smoker (<15 years) and Reformed smoker (>15 years). (e) Boxplot displaying relative levle of POLR2K in normal individuals or in BLCA patients of papillary and non-papillary tumor. (f) Boxplot displaying relative levle of

POLR2K in normal individuals or BLCA patients with nodal metastasis N0, N1, N2 and N3 status. Data are mean ± SE. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

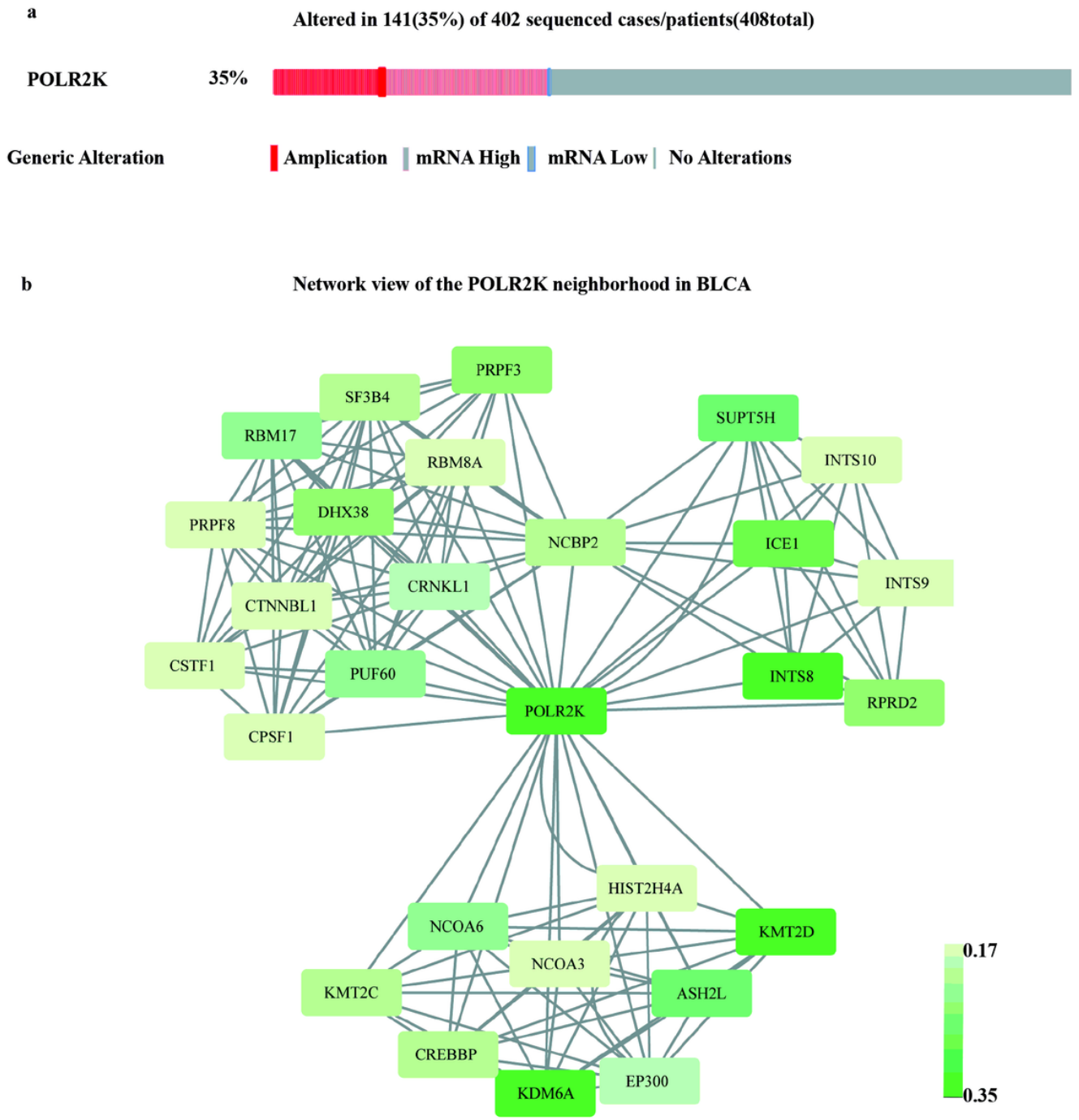

Figure 3

Visual summary of POLR2K alterations and biological interaction webwork in BLCA. (a) OncoPrint of POLR2K alterations in BLCA. The OncoPrint overviews genomic alterations in POLR2K influencing individual samples (columns) in BLCA from the TCGA. The distinct categories of genetic alterations are featured in distinct colours. (b) Network view of the POLR2K neighborhood in BLCA. To visualize the neighborhood gene of POLR2K, Pathway Commons (https://www.pathwaycommons.org) and Cytoscape were used. POLR2K is used as a seed gene, and all other genes are automatically identified as altered in BLCA. Darker blue indicates increased frequency of alteration in BLCA.

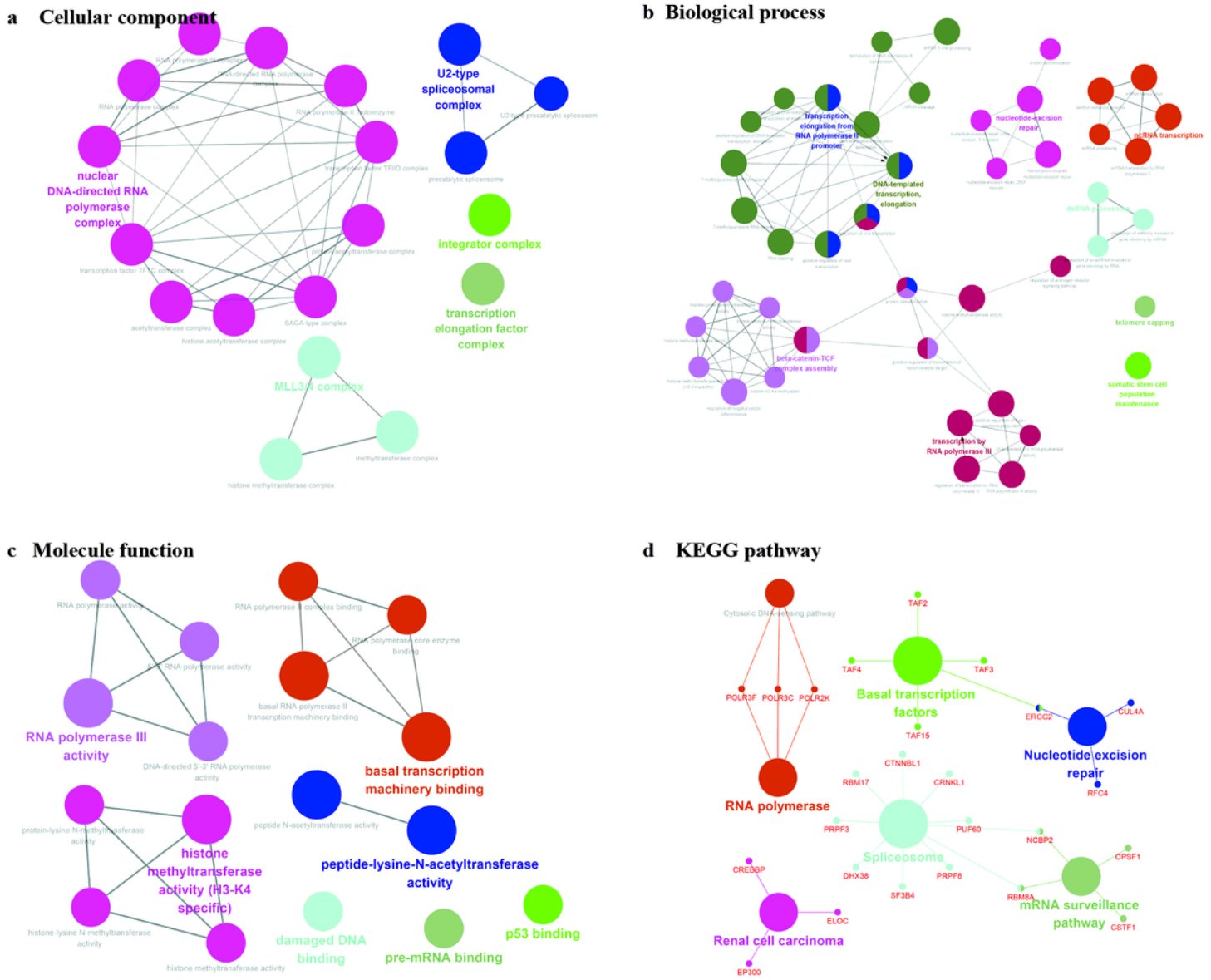

Figure 4

Enrichment analysis of the genes altered in the POLR2K neighborhood BLCA. With the help of Cytoscape, the colorful pictures display the enrichment results of the top 50 genes altered in the POLR2K neighborhood in BLCA. (a) Cellular components. (b) Biological processes. (c) Molecular functions. (d) KEGG pathway analysis. Nodes represent ontology terms or KEGG pathways that were significantly enriched for the predicted target genes. Edges represent connections within the ontology tree and colors highlight terms correlated in meaning. The most significant terms of the group showing a biological meaning in association with the system under analysis are captioned. A p-value of < 0.01 was considered statistically significant.

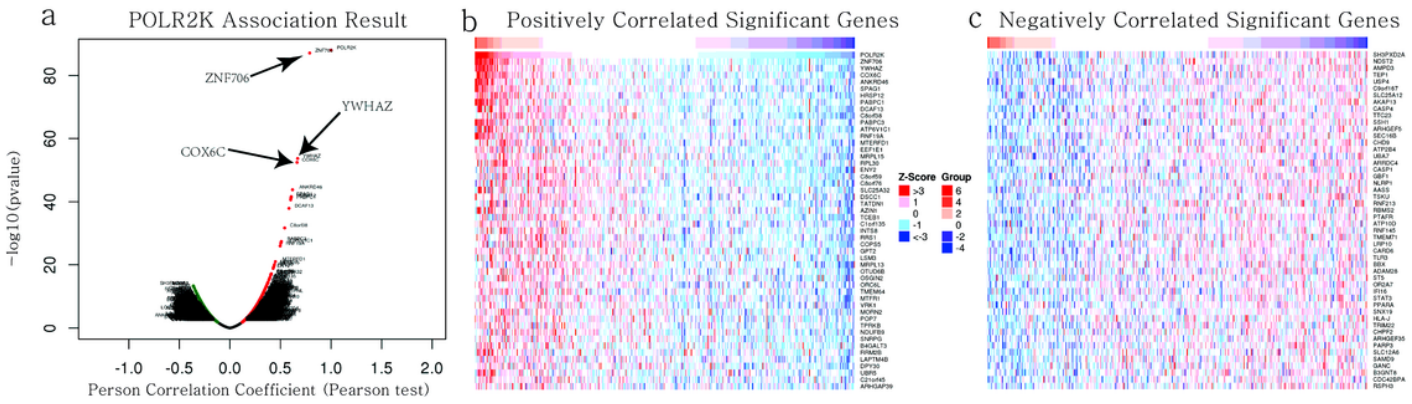

Figure 5

Genes differentially expressed in correlation with POLR2K in BLCA (http://linkedomics.org/). (a) A Pearson test was deployed to analyze relationships between POLR2K and genes differentially expressed in BLCA. (b–c) Heat maps dispalying genes positively and negatively correlated with POLR2K in BLACA (TOP 50). Red indicates positively correlated genes and green indicates negatively correlated genes.

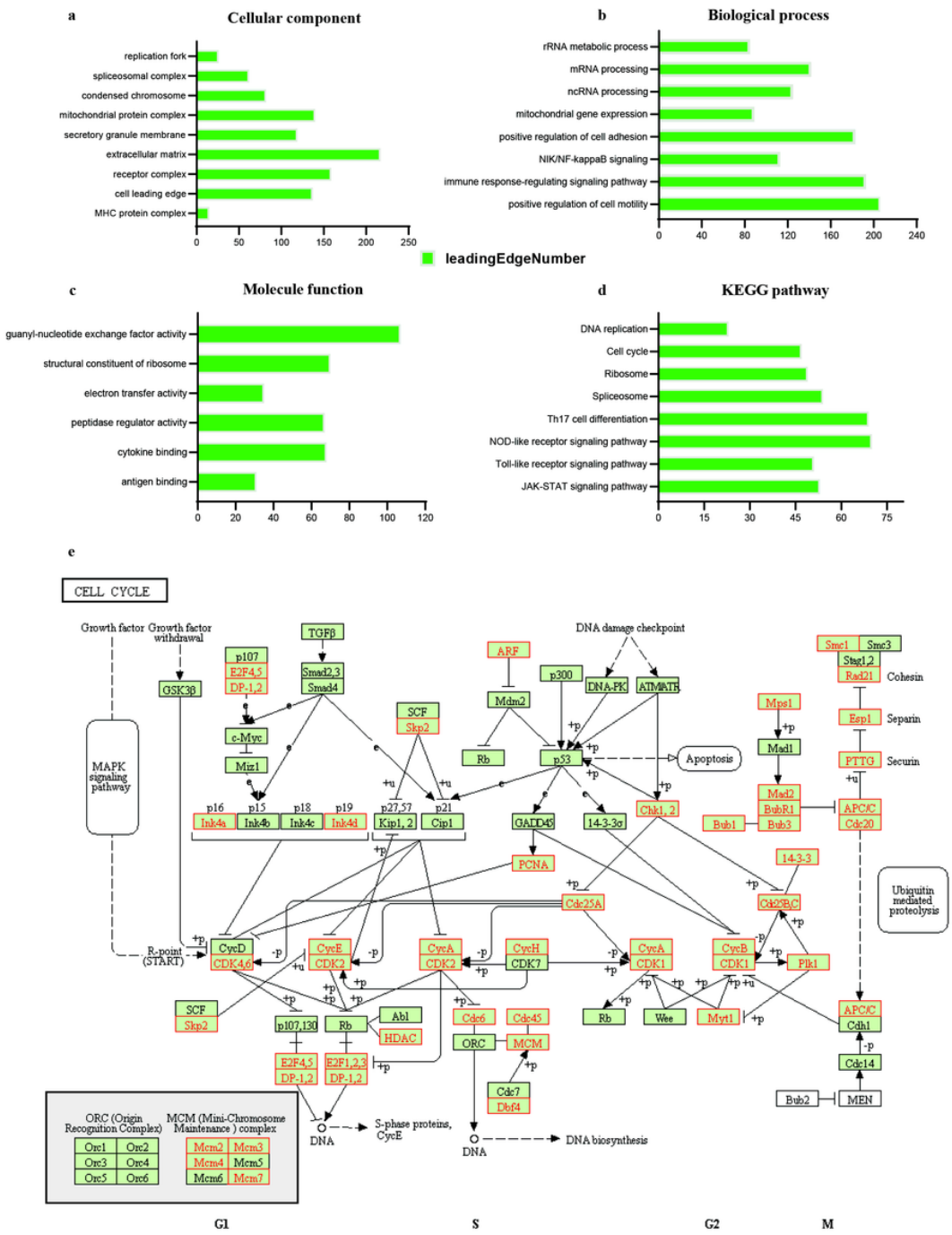

Figure 6

Significantly enriched GO annotations and KEGG pathways of POLR2K in BLCA. The enriched GO annotations and KEGG pathways of POLR2K co-expression genes in BLCA were obtained by GSEA. (a) Cellular components. (b) Biological processes. (c) Molecular functions. (d) KEGG pathway analysis. The blue column represents the LeadingEdgeNum, and the FDR< 0.05. (e) KEGG pathway annotations of the cell cycle pathway obtained from https://www.genome.jp/kegg. Red marked nodes are associated with the LeadingEdgeGene.

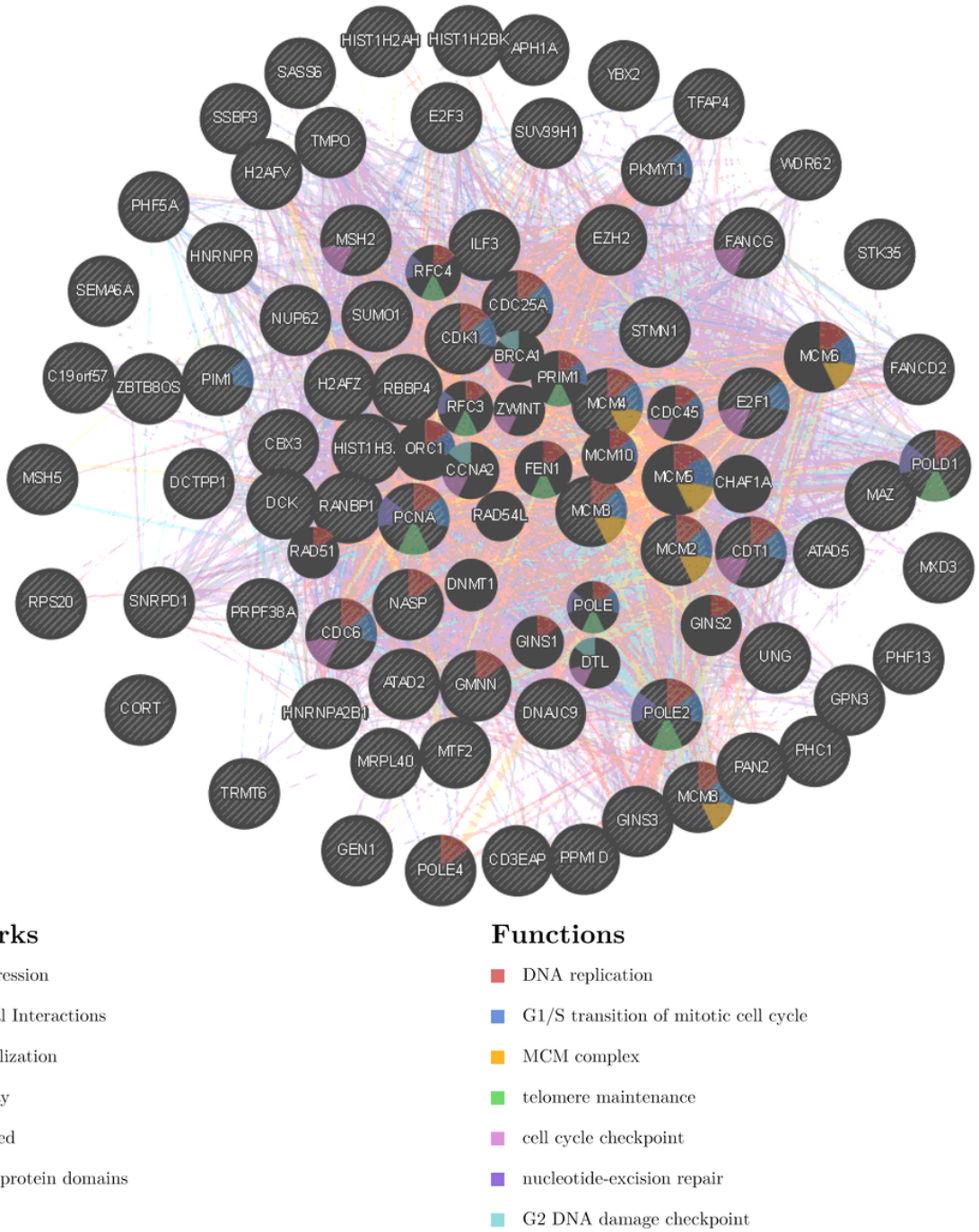

Figure 7

Protein-protein interaction network of transcription factor E2F1-target networks (GeneMANIA). Protein-protein interaction (PPI) network and functional analysis indicating the gene set that was enriched in the target network of transcription factor E2F1. Different colors of the network edge indicate the bioinformatics methods applied: co-expression, website prediction, pathway, physical interactions and co-localization. The different colors for the network nodes indicate the biological functions of the set of enrichment genes.

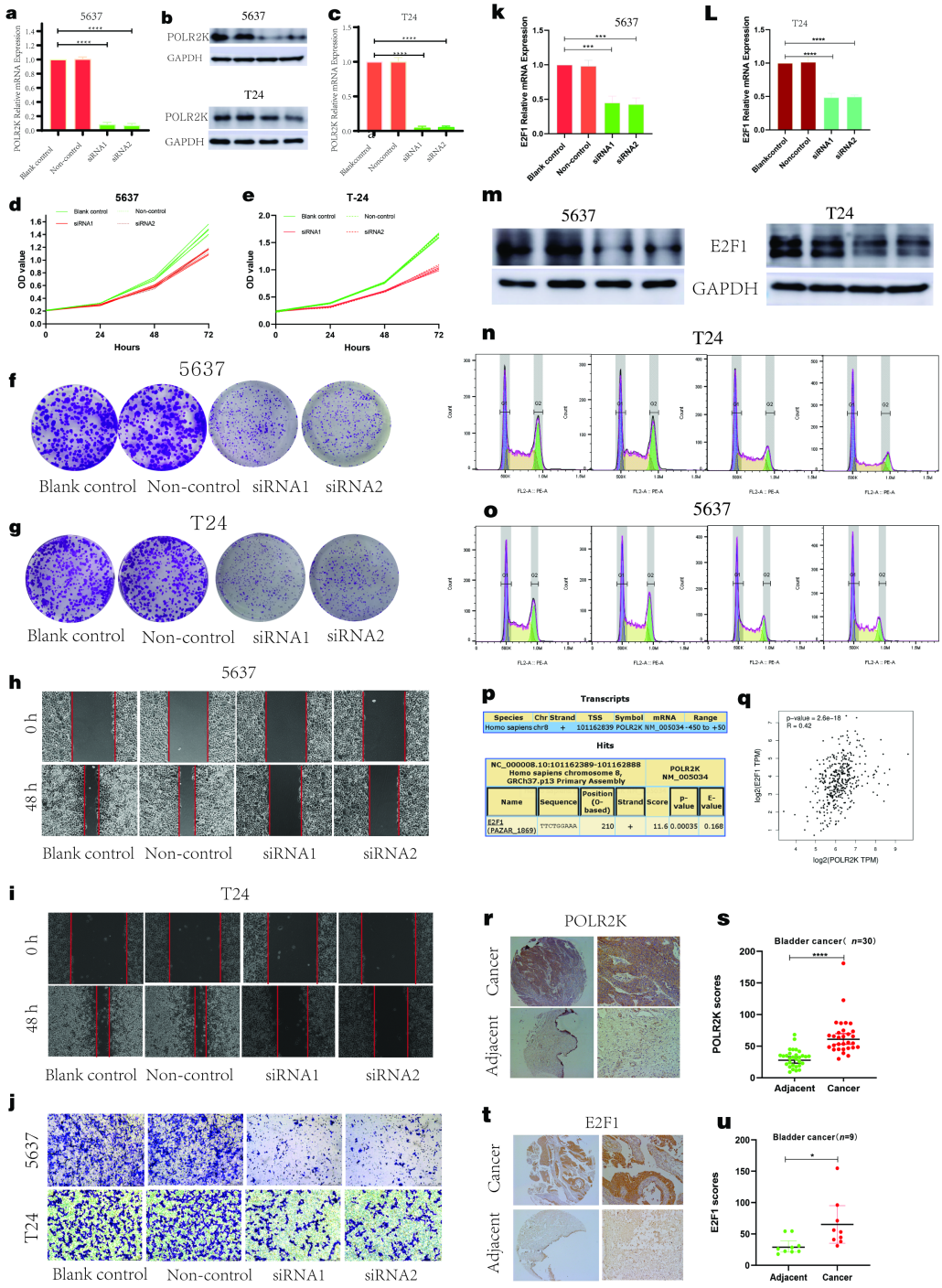

Figure 8

Cell function experiments validate POLR2K-E2F1 pathway and POLR2K as a positive indicator of BLCA. (a-c) Knockdown efficiency of siRNA was verified at mRNA and protein level at 48 h.(d-e) MTT was used to evaluate cell viability of 5637 and T24cells after POLR2K knockdown. (f-g) Colony formation was deployed to assess cell growth ability after knocking down POLR2K. (h-j)Wound healing and Transwell were performed to determine the migration and invasion ability of 5637 and T24cells after POLR2K knockdown. (k-m) The effects of POLR2K knockdown on E2F1 both at mRNA and protein level.(N-O) POLR2K knockdown could induce cell cycle G1arrest. (p) The binding sequence of E2F1 to POLR2K promoter. The predication was based on JASPAR database. (q) The correlation between POLR2K and E2F1 at mRNA level. Data were retrieved from TCGA database. (r-s) POLR2K immunohistochemical staining from bladder cancer tissues and matched adjacent bladder tissues from each

patient. (t-u) E2F1 immunohistochemical staining from bladder cancer tissues and matched adjacent bladder tissues from each patient.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryMaterialRevisedversion.docx