Based on Bioinformatics Studies on the Effect of alpha1H on Key Genes, Pathways in Bladder Cancer and IncRNA on Patient Prognosis

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Abstract

Objective: To analyze the differential genes, IncRNA, signaling pathway and patient prognosis of bladder cancer after alpha1H treatment.

Methods: Sequencing data GSE172112 for patients in clinical trials with a new bladder cancer drug were downloaded from the GEO database, The bladder cancer tissues using the new drug alpha1H (alpha1- oleate) and placebo were analyzed in the R language, The differentially expressed genes were selected; Differential genes were analyzed for KEGG pathway enrichment using the DAVID database, To explore the effect of a lpha1H treatment on pathways in patients with bladder cancer; at the same time, IncRNA expression data from the Bladder Cancer (BLCA) dataset were also downloaded through the TCGA database, First, screening for differential IncRNA expression, The screening results were then analyzed by univariate Cox regression to initially screen for IncRNA associated with prognosis, The key IncRNA affecting the prognosis were further screened out, The prognostic model was also constructed using multivariate Cox regression analysis.

Results: There yielded 394 significantly upregulated genes and 385 significantly downregulated genes in bladder cancer tissue after a lpha1H treatment. Through gene signaling pathway enrichment analysis, the upregulated genes were mainly enriched in the signaling pathways regulating the pluripotent line of stem cell cells, the TGF-signaling pathways, and the cell cycle. The downregulated genes were mainly enriched in the MAPK signaling pathway, phagosomes, and the TNF signaling pathway. After a lpha1H treatment, of 119 differentially expressed IncRNA, from the lnc2cancer database, two genes were found to be potentially associated with bladder cancer prognosis, and the final analysis confirmed the key IncRNA affecting prognosis. The results of survival analysis showed that high expression of LINC00152 unfavorable unfavorable patient prognosis and the new drug alpha1H reduces LINC00152 favors patient prognosis.

Conclusion: Alpha1H can cure bladder cancer by regulating hallmark signaling, TGF-beta signaling, and LINC00152.

1. Background

Bladder cancer (Bladder cancer, BC) is a common malignancy in the male genitinary system, and a 2018 global cancer statistics reported that the incidence of BC was 4.5% in men and a mortality rate of 2.8% [1]. The main clinical presenting symptom of BC is hematuria[2]. 90% of BC occurrence is caused by the malignant proliferation of urothelial cells, most of which are located in the bladder[3]. BC can be classified into non-muscle-invasive bladder cancer and muscle-invasive bladder cancer[2]. Currently, more than 80% of bladder tumors are non-muscle-invasive papillary tumors, and although their 5-year survival rate is 90%, approximately 70% of these patients will relapse[4].
In 2002, BCG first reported the effective use of BCG in the treatment of superficial BC by Morales et al[5]. During the subsequent decades, the researchers made great clinical progress in treating BC by surgical resection, chemotherapy, and immunocheckpoint inhibitors. Transurethral bladder tumor resection is often used clinically for the treatment of non-muscle-invasive bladder cancer[6]. Platinum-based chemotherapy is often used for patients with muscle-invasive bladder cancer[7]. In 2020, the European Urology Guidelines Association reported on treatment for muscle invasive and metastatic bladder cancer, they clearly stated that cisplatin-based chemotherapy remains preferred in patients with BC metastatic. Secondly, immunotherapy is recommended for patients positive for programmed death ligand 1 (PD-L1), or with carboplatin for PD-L1-negative patients. For second-line therapy of BC metastatic disease, Pymtuzumab therapy is recommended[8]. It is well known that immunotherapy has better effects and less side effects compared with the strong toxic side effects and high recurrence rate of traditional chemotherapy therapy. In recent years, despite the success of immunosuppressive therapy in BC, the objective response to immunosuppressive monotherapy remains within the 20% range, suggesting a lower response to immunotherapy in some patients[9]. Therefore, developing a less toxic antitumor complex for BC would be very popular. Human milk albumin is flexible and able to form stable protein-fatty acid complexes with fatty acids. Among them, the alpha1-oleate generated by its binding to oleic acid has been confirmed by many researchers to have antitumor effects. It has been shown that alpha1H shows better therapeutic effects in the mouse MB49 bladder cancer model[10]. In an animal study, mice found a dose-dependent reduction in tumor size, bladder size and bladder weight in alpha1H treated mice compared to the counterfeit drug group, and increased alpha1H dose had no toxic side effects on healthy tissues[6]. In a single-center, placebo-controlled, double-blind phase I / II interventional clinical trial of nonmuscle invasive bladder cancer, the infusion of alpha1H in the bladder resulted in massive cell loss of tumor cells, smaller tumors, apoptosis after treatment, and suppressed expression of cancer-related genes[11].

As bioinformatics developed, researchers found that lncRNA expression is closely related to tumor genesis and progression[12]. This suggests that lncRNA can serve as a tumor biomarker as well as a therapeutic target. In recent years, many studies have found lncRNA to be associated with the diagnosis and prognosis in BC patients. Li Gang et al[13] found that lncRNA TUC338 expression was upregulated in early BC patients and showed early diagnostic value. After surgical resection of the BC tumours, the plasma lncRNA TUC338 levels were significantly downregulated. Luo Huarong et al[14] found that plasma lncRNA CASC11 was upregulated in early BC patients as compared with healthy controls. MA ZHIPENG et al[15] found that lncRNA SNHG5 was upregulated in BC tissues and promoted BC cell proliferation by targeting p27, which subsequently had adverse effects on patient prognosis. In addition, lncrna UCA1 was found in a meta-analysis to be a diagnostic biomarker for BC[16].

In this study, we screened differential genes for BC patients after alpha1H treatment and placebo-treated BC patients, BC patients and placebo-treated BC patients, followed by KEGG signaling enrichment
analysis of the above differential genes, and confirmed prognostic key lncRNA for alpha1H treatment of bladder cancer.

2. Results

2.1 alpha1H treatment affects protein-coding gene expression in patients with bladder cancer

We collected gene expression sequencing data from clinical experimental patients with alpha1H, a new bladder cancer drug, GSE172112, with simple preliminary preprocessing and statistics, in which 39 patients using the new drug and 42 patients using placebo were tested, and gene expression data from a total of 81 samples were tested. Subsequently, using the R package "DESeq2", we transformed the gene read count data detected by the samples into the corresponding expression values, combined with the RNA classification information in the R package "biomaRt" package, yielding a total of 20,356 PCG and 12,382 lncRNA (including 7,109 lincRNA and 5,273 antisense).

First, for 20,356 protein-coding genes, using the patient drug profile, we performed differential expression analysis between patients using the new drug alpha1H versus patients using placebo. We counted the mean expression of each protein-coding gene in both patient groups, calculated the corresponding FC values, and recorded the Wilcoxon rank-sum test p-values, finally obtaining 781 differentially expressed genes with a p-value <0.01, containing 396 significantly up-regulated genes and 385 significantly downregulated genes (Table 1, Figure 1).

Table 1

| Term                                              | Count | PValue       |
|---------------------------------------------------|-------|--------------|
| hsa 04550:Signaling pathways regulating pluripotency of stem cells | 10    | 3.52E-04     |
| hsa 04350:TGF-beta signaling pathway              | 6     | 0.010437931  |
| hsa 04110:Cell cycle                              | 7     | 0.013608859  |
| hsa 03018:RNA degradation                         | 5     | 0.03338814   |
| hsa 03420:Nucleotide excision repair              | 4     | 0.037922436  |
| hsa 00280:Valine, leucine and isoleucine degradation | 4   | 0.037922436  |
| hsa 04330:Notch signaling pathway                 | 4     | 0.039997665  |

Furthermore, we distributed differentially expressed protein-coding genes to functionally annotated significantly up-and significantly down-regulated genes among. For the 394 genes that were significantly upregulated, We found that their function is mainly associated with the following KEGG pathway:
"hsa04550: Signaling pathways regulating pluripotency of stem cells" regulates signaling in stem cells, "hsa04350: TGF-beta signaling pathway" TGF-beta signaling pathway, "hsa04110: Cell cycle" cell cycle, et al. (Table 1, Figure 2).

But for the 385 significantly downregulated genes, we found that their function was mainly with (Table 2, Figure 3). like the following KEGG pathway-associated: "hsa 04010:MAPK signaling pathway" MAPK signaling pathway, "hsa04145:Phagosome" phagosomal, "hsa04668:TNF signaling pathway" TNF signaling pathway

Table 2

| Term                              | Count | PValue        |
|-----------------------------------|-------|---------------|
| hsa05132: Salmonella infection    | 10    | 1.45E-04      |
| hsa04010: MAPK signaling pathway  | 15    | 0.002761576   |
| hsa05152: Tuberculosis            | 12    | 0.00324906    |
| hsa04145: Phagosome              | 10    | 0.009423262   |
| hsa04060: Cytokine-cytokine receptor interaction | 13 | 0.013038165 |
| hsa04668: TNF signaling pathway  | 8     | 0.013680707   |
| hsa04910: Insulin signaling pathway | 9     | 0.017016212   |
| hsa04064: NF-kappa B signaling pathway | 7     | 0.017281951   |
| hsa04062: Chemokine signaling pathway | 10    | 0.033343318   |
| hsa05146: Amoebiasis             | 7     | 0.040529418   |
| hsa04621: NOD-like receptor signaling pathway | 5     | 0.043877413   |
| hsa05020: Prion diseases         | 4     | 0.046418919   |

2.2 alpha1H treatment affects IncRNA expression in patients with bladder cancer

In addition, for the non-coding RNA molecule IncRNA, the protein detected in the GSE172112 dataset, we also performed a differential expression analysis. As with the analysis of protein-coding genes, for 12,382 IncRNA, we finally obtained 119 significantly differentially expressed IncRNA, including 53 significantly upregulated IncRNA and 66 significantly downregulated IncRNA (Table 2, Figure 4).
Further, we obtained from the lnc2cancer database (http://bio-bigdata.hrbmu.edu.cn/lnc2cancer/index). The 369 bladder cancer-related IncRNA entries were downloaded from html) and two of these IncRNA were closely associated with bladder cancer and LINC00152 with TUG1 (Table 3). We found that both IncRNA were upregulated in bladder cancer patients in most literature reports, and in the GSE172112 dataset, we also compared the expression of patients using new drugs and placebo.

Table 3

Related information of LINC00152 and TUG1 recorded in lnc2Cancer database

| IncRNA      | Cancer.name    | Methods                                                                 | Expression.pattern | Pubmed.ID   |
|-------------|----------------|-------------------------------------------------------------------------|--------------------|-------------|
| LINC00152   | gallbladder cancer | qPCR, RNAi, Western blot, RIP, Luciferase reporter assay etc.          | up-regulated       | 28077595    |
| LINC00152   | gallbladder cancer | qPCR, RNAi, Western blot, ChiP, Luciferase reporter assay, Cell proliferation assay etc. | up-regulated       | 27829993    |
| LINC00152   | bladder cancer   | qRT-PCR, ect.                                                           | down-regulated     | 29988223    |
| TUG1        | bladder cancer   | qPCR, Luciferase reporter assay, Western blot                          | up-regulated       | 30925453    |
| TUG1        | bladder cancer   | qPCR, Western blot, in vitro knockdown                                 | up-regulated       | 31308746    |
| TUG1        | gallbladder cancer | qPCR, RNAi, Western blot, Luciferase reporter assay, Cell proliferation assay etc. | up-regulated       | 28178615    |
| TUG1        | bladder cancer   | qPCR, RNAi, Western blot, RIP, Luciferase reporter assay etc.          | up-regulated       | 26318860    |
| TUG1        | bladder cancer   | qPCR etc.                                                               | up-regulated       | 29029461    |
| TUG1        | bladder cancer   | qRT-PCR, Luciferase reporter assay, in vitro knockdown                 | up-regulated       | 29321088    |
| TUG1        | bladder cancer   | qPCR, Western blot, MIT                                                | up-regulated       | 28503069    |
| TUG1        | bladder cancer   | qPCR, Western blot etc.                                                | up-regulated       | 28376901    |
| TUG1        | bladder cancer   | qPCR, RNAi, MTT assay etc.                                             | up-regulated       | 27460088    |

For LINC00152, we found that the mean expression was 5.8159 in patients using new drugs, compared to 6.5003 in placebo patients and a Wilcoxon test p-value of 0.0038, showing that LINC00152 expression
was significantly reduced by using new drugs.

But for TUG1, we found that the mean expression was 12.3502 in patients with new drugs, but 12.0904 in placebo, and a Wilcoxon test p-value of 0.001, showing that using new drugs could not reduce TUG1 expression.

Combined with the two IncRNA analysis described above, we suggest that the new drug alpha1H most likely caused a series of gene expression changes by reducing LINC00152 expression and ultimately played a good role in killing tumor cells.

### 2.3 alpha1H treatment altered part of the hallmark pathway activation in patients with bladder cancer

Using differential expression analysis between the two classes of patients in GSE172112, we found that many differentially expressed genes were generated in alpha1H-treated patients, and that treatment of alpha1H at a single gene level had a huge effect on patient gene expression. Further, we evaluated whether both types of patients have a huge impact on the activation of certain pathways from the perspective of the overall pathways.

Therefore, we collected and collated gene members of the 50 hallmark pathways from the MsigDB database, rated each sample in GSE172112 for pathway activation using the "ssgsea" algorithm in the R package "GSVA", and assessed score differences between samples using the Wilcoxon rank-sum test. Finally, we found that in alpha1H treatment compared with placebo, Activation of the following pathways in patients: where "REACTIVE_OXYGEN_SPECIES_PATHWAY", "P53_PATHWAY", "UV_RESPONSE_UV", "HYPOXIA", "APOPTOSIS", "TNFA_SIGNALING_VIA_NFKB", "COMPLEMENT", "IL6_JAK_STAT3_SIGNALING", "INFLAMMATORY_RESPONSE" is all downregulated (Table 4).

| Table 4 |
| --- |

Differences in ssGSEA scores of 50 hallmark pathway activation in patients in the GSE172112 dataset
| Hallmark pathway                     | mean ssGSEA in alpha1H treated | mean ssGSEA in placebo treated | wilcox.test pValue |
|-------------------------------------|---------------------------------|--------------------------------|-------------------|
| REACTIVE_OXYGEN_SPECIES_PATHWAY     | 1.569619674                    | 1.582734513                    | 0.004120032       |
| P53_PATHWAY                         | 1.507696636                    | 1.521012228                    | 0.007985337       |
| UV_RESPONSE_UP                      | 1.424304396                    | 1.434798911                    | 0.012893519       |
| HYPOXIA                             | 1.42652941                     | 1.439254409                    | 0.021327705       |
| APOPTOSIS                           | 1.531946053                    | 1.544429318                    | 0.02244453        |
| TNFA_SIGNALING_VIA_NFKB            | 1.428124292                    | 1.465793485                    | 0.023021538       |
| COMPLEMENT                          | 1.374210535                    | 1.397835555                    | 0.033326204       |
| IL6_JAK_STAT3_SIGNALING            | 1.33525268                     | 1.368464526                    | 0.036656563       |
| INFLAMMATORY_RESPONSE              | 1.281653791                    | 1.317457585                    | 0.04728863        |
| MTORC1_SIGNALING                   | 1.607157413                    | 1.616700174                    | 0.050597275       |
| HEME_METABOLISM                    | 1.405351602                    | 1.409403022                    | 0.063037602       |
| KRAS_SIGNALING_UP                  | 1.341771721                    | 1.361403026                    | 0.067223154       |
| IL2_STAT5_SIGNALING                | 1.40733034                     | 1.423663491                    | 0.076272281       |
| PI3K_AKT_MTOR_SIGNALING            | 1.536935306                    | 1.542465415                    | 0.103223941       |
| ALLOGRAFT_REJECTION                | 1.30101474                     | 1.328268003                    | 0.115903328       |
| COAGULATION                         | 1.247726476                    | 1.260215792                    | 0.122681092       |
| CHOLESTEROL_HOMEOSTASIS            | 1.492168982                    | 1.49907447                     | 0.158421731       |
| ANDROGEN_RESPONSE                  | 1.610609182                    | 1.615768853                    | 0.172916472       |
| UNFOLDED_PROTEIN_RESPONSE          | 1.587649281                    | 1.593578296                    | 0.178980994       |
| NOTCH_SIGNALING                    | 1.483836676                    | 1.479281759                    | 0.185200472       |
| INTERFERON_GAMMA_RESPONSE          | 1.49277404                     | 1.513236111                    | 0.198110796       |
| ESTROGEN_RESPONSE_LATE             | 1.413869042                    | 1.418128337                    | 0.201437702       |
| PROTEIN_SECRETION                  | 1.708610457                    | 1.70515225                     | 0.211659698       |
| EPITHELIAL_MESENCHYMAL_TRANSITION  | 1.409575339                    | 1.432730605                    | 0.211659698       |
| GLYCOLYSIS                          | 1.437199485                    | 1.443256898                    | 0.222247114       |
| APICAL_SURFACE                     | 1.298814008                    | 1.308220393                    | 0.22951047        |
| ESTROGEN_RESPONSE_EARLY            | 1.455088631                    | 1.457645328                    | 0.244534355       |
| APICAL_JUNCTION                    | 1.368342672                    | 1.377967747                    | 0.260227498       |
Based on the above results, we found that the downregulation of "P53_PATHWAY" indicates the decreased proliferation capacity of tumor cells; while "TNFA_SIGNALING_VIA_NFKB", "IL6_JAK_STAT3_SIGNALING" and "INFLAMMATORY_RESPONSE" pathways indicate the alleviated immunoinflammatory response in patients after using the new drug alpha1H, indicating the removal of tumor cells (Figure 5).

2.4 alpha1H and the TGF-beta signaling pathway
Combined with single gene-level differential analysis of overall pathway activation, we found that alpha1H affected some of gene members in TGF-beta signaling (including SMAD4, ACVR1C, ID4, SMAD9, SMAD6 and ACVR2B), but the activation of overall TGF-beta signaling was not significantly changed with Wilcoxon rank sum test p-value of 0.7528. However, SMAD4, SMAD9, SMAD6 Three SMAD family members also have important effects on TGF-beta signaling. Therefore, we focused on the expression of these three genes and found that all three SMAD family members were relatively highly expressed (Table 5) in patients treated with alpha1H.

Table 5

| gene  | mean expression in alpha1H treated | mean expression in placebo treated | FC       | wilcox.test pValue |
|-------|-----------------------------------|------------------------------------|----------|--------------------|
| SMAD9 | 8.654020345                       | 7.850127802                        | 1.102405026 | 0.003750499       |
| SMAD6 | 7.889000669                       | 7.319214986                        | 1.077847923 | 0.006899118       |
| SMAD4 | 11.30312093                       | 11.10285469                        | 1.018037366 | 0.006899118       |

To further validate the role of these three genes in patients with bladder cancer, we performed a prognostic analysis of these three genes separately, combined with the expression of BLCA patients in the TCGA program and the recorded clinical prognostic information. Among them, a total of 426 patients with detected expression and clinical prognostic information were analyzed.

For SMAD4, we divided patients into high expression groups (expression above 85.0865) and low expression group (expression below 85.0865), and the HR [95%CI] was 1.818[1.221-2.706] and the log-rank test p-value of 0.0029, indicating that the worse the prognosis of SMAD4 expression (Figure 6).

For SMAD6, we divided patients into high expression groups (expression above 34.2596) and low expression groups (expression below 34.2596), and the HR of Cox proportional hazards [95%CI] of the final constructed proportional hazards model was 0.5052[0.3783-0.6747], and the log-rank test p-value <0.0001, indicating lower SMAD6 expression (Figure 7).

For SMAD9, we divided the patients into high expression groups (expression above 11.3209) and low expression group (expression below 11.3209) according to their expression value, and the HR [95%CI] of the final constructed Cox proportional-risk model was 1.521[1.064-2.174], and the log-rank test p-value was 0.021, indicating that the worse the prognosis of patients with higher SMAD9 expression (Figure 8).

The above results show that the upregulation of SMAD6 gene expression after alpha1H treatment contributes to patient prognosis, but the upregulation of SMAD4 and SMAD9 genes is instead unfavorable to patient prognosis.
2.5 LINC00152 and patients with bladder cancer

In the analysis of differentially expressed IncRNA in the GSE172112 dataset, we found significantly downregulated LINC00152 expression in patients treated with alpha1H, and the results collected in the lnc2cancer database also showed generally high expression of this IncRNA in bladder cancer patients, we used this IncRNA to assess the prognostic value using the TCGA BLCA dataset, A total of 252 patients who examined IncRNA expression and recorded clinical prognostic information were analyzed.

We found that, given as the expression values of the LINC00152, Patients were divided into high expression groups (expression above 1.130234) and low expression groups (expression below 1.130234), The HR [95%CI] of the final constructed model of the Cox scale analysis was 3.681[1.354-10.01], The log-rank test p-value was 0.0062, It shows that the high expression of LINC00152 is unfavorable to the patient prognosis, The new drug alpha1H reduces LINC00152 and has a positive effect on curing cancer (Figure 9).

3. Discussion

In this study, by comparing the BC tissues after alpha1H treatment and after placebo treatment, a total of 781 differential genes were selected, including 396 upregulated genes and 385 downregulated genes. Upregulated genes are mainly involved in seven pathways, including the signaling pathway regulating stem cell pluripotency, TGF-signaling pathway, cell cycle, RNA degradation, nucleotide excision repair, valine, leucine, and isoleucine degradation, and Notch signaling pathway. The downregulated genes are mainly involved in 12 pathways, including Salmonella infection, MAPK signaling, pulmonary tuberculosis, phagosomes, cytokine receptor interactions, tumor necrosis factor signaling, insulin signaling, NF- B signaling, chemokine signaling, amoebosis, NOD-like receptor signaling, and prion disease. Signaling pathways associated with bladder cancer (BC) progression may be important targets for systemic therapy. Manal Elmasry et al[17] found that mitogen-activated protein kinase (MAPK / ERK) signaling is associated with BC progression, and the results showed that MAPK signaling can control BC cell activity, and that inhibition of M A P C signaling in BC xenografts would inhibit BC cell growth and reduce BC cell subsets. In this study, the genes significantly downregulated after treatment with the new drug alpha1H were associated with MAPK signaling.

In this study, we found that alpha1H can have a positive effect on the prognosis of BC through intervention in related signaling pathways, mainly including P53 signaling, IL-6 / Stat3 signaling, TNF- / NF-B signaling, etc.YE GUOMEI et al 26 found that puerurin inhibited bladder cancer T24 cell proliferation and induced apoptosis by inhibiting the SIRT1 / p53 signaling pathway. Jelena Korac-Prlic et al[18] found that blocking the IL-6 / Stat3 axis alone inhibits bladder cancer progression.Chen Jia-Liang et al[19] found that mechanical ectopic pain, depression-like behavior in cyp-induced in a mouse model of TNF- / NF-induced cystitis by inhibition of P- κB signaling. This study found that P53 pathway downregulation after alpha1H treatment indicates the decreased proliferative capacity of tumor cells and the IL-6 / Stat3 axis
and TNF- / NF-κB signaling pathway, indicating that the immunoinflammatory response was alleviated in vivo in patients after the use of the new drug alpha1H.

This study found that some of the genes in the TGF-signaling pathway changed treatment with the new drug alpha1H. Hung Tzung-Tyng et al\cite{20} found that the TGF-pathway is involved in BC progression. BIAN JING et al\cite{24} showed that BC cells utilize mutant TGF-receptors for TGF-signaling, leading to their enhanced migration and invasion, and avoiding growth arrest. Shi Heng et al\cite{21} found that LINC01451 promotes epithelial-mesenchymal transformation through the activation of the LIN28 / TGF- / Smad signaling pathway, thereby aggravating BC progression. Zhu Feng et al\cite{22} found that LncRNA AWPPH can inhibit SMAD4 through EZH2, promote BC cell proliferation, migration, and inhibit apoptosis. SMAD4 levels were downregulated in BC tissues as compared to normal bladder tissues. The results of this study show that some of the genes in the TGF-signaling pathway have changed after the new drug alpha1H treatment, and that the upregulation of SMAD6 gene expression contributes to patient prognosis, but the upregulation of SMAD4 and SMAD9 genes is instead unfavorable to patient prognosis.

LINC00152 may be involved in cell cycle arrest, apoptosis, epithelial to mesenchymal transformation, cell migration, and may serve as reliable biomarkers for the diagnosis of some cancer types. Tang Xian-li et al\cite{23} found that Linc00152 is highly expressed in bladder cancer patients, and that the possible oncogenic role of Linc00152 in bladder cancer is achieved through the activation of Wnt / b-Catenin signaling, suggesting that Linc00152 may be a novel biomarker for the diagnosis and prevention of bladder cancer. In this study, the constructed Cox proportional analysis model found that high LINC00152 expression unfavorable patient prognosis, while the new drug alpha1H reduced LINC00152 has a positive effect on the cancer cure.

In this comparative study, differentially expressed genes between BC tissues and BC tissues after placebo treatment. Functional and pathway enrichment analyses of differential genes revealed potential molecular pathways underlying alpha1H in multiple signaling for BC. Further combined with analyses, obtaining key LncRNA closely related to the prognosis of BC patients provide potential targets for subsequent studies on BC treatment, while elucidating the molecular mechanisms of cancer suppressor in BC patients after alpha1H treatment.

4. Conclusions

In Conclusion, based on the GEO and lnc2cancer databases, we used bioinformatics methods to screen the differentially expressed genes of bladder cancer after alpha1H treatment, which are mainly enriched in the TGF-signaling pathways, the MAPK signaling pathway, and the TNF signaling pathway and other pathways, and verified The high expression of SMAD4, SMAD9 and LINC00152 is not conducive to the prognosis of patients, and the low expression of SMAD6 is not conducive to the prognosis of patients. It points out the diagnostic and therapeutic value of LINC00152 in bladder cancer. However, the molecular mechanism of LINC00152 affecting prognosis needs further study.
5. Methods

5.1 Data collection and preprocessing

We are working from the GEO (https://www.ncbi.nlm.nih.gov) database, GSE172112, in which some patients used the new drug alpha1H (alpha1-oleate), some patients used placebo (placebo), including several tissues using the new drug alpha1H, and several tissues using placebo (placebo). We are also quoted from the UCSC Xena (https://xena.ucsc.edu) database, including the gene RNA-seq sequencing results (after quantile standardization) of the sample, and the clinical information of the patient. In addition, we also from TANRIC (https://www.tanric.org) database, with total case samples, where several were organized after the new drug alpha1H (alpha1-oleate) and several after placebo (placebo).

The TCGA database provides the raw counts and Entrez ID for the RNA-seq Level 2 mRNA. The annotation of the most complete human IncRNA was included and analyzed in the GENCODE v7 database, and the IncRNA dataset is more comprehensive than other databases. According to the chromosomal location of the exon provided by the TCGA database, the raw counts, and the reads number per thousand base from the map to the exon in the reads per 1 million map (reads per kilobase of exon per million mapped reads, RPKM) Information, Alignment to the chromosomal locations of the IncRNA in the GENCODE v7 database, if the chromosome position, the information is consistent, The exon is defined as a IncRNA. Using the R package "DESeq2", we transformed the read count values of the raw sequencing data into the gene expression values fit by the "DESeq2" package, and used the R package "biomaRt" to classify the detected RNA molecules into PCG (Protein Coding Gene) as well as IncRNA classes.

5.2 Gene differential expression analysis

For the gene expression dataset for new drug treatment, GSE172112, we divided the patient’s medication profile into two groups, one using the new drug alpha1H and the other using a placebo. The data chips in the gene expression data profiling were normalized in R 3.6.1 using the limma software package, Screening for differentially expressed genes, The Fold Change values were calculated, follow, Differential expression analysis was performed using the Wilcoxon rank-sum test, Record the test p-value, |FC (fold change)| 2 and P < 0.01 were used as screening criteria for differential genes.

5.3 KEGG pathway enrichment analysis
DAVID 6.8 database (https://david.ncifcrf.gov/) is a commonly used database for gene enrichment and functional annotation analysis. The KEGG pathway was analyzed for the differentially expressed genes using DAVID. The selection conditions were: human gene, P <0.05.

5.4 GSVA pathway difference analysis

To assess the impact of new drug use on overall pathway activation in patients, we obtained from the MsigDB database (http://www.gsea-msigdb.org/gsea/index). Gene sets for downloading 50 hallmark signaling pathways were collected in jsp). Using the "ssgsea algorithm" in the R package "GSVA", combined with a gene set of 50 hallmark signaling pathways, we scored pathway activation for each sample and subsequently scored difference comparison using Wilcoxon's rank-sum test.

5.5 Screening of the LncRNA

Transcriptomic sequencing data of BC and detailed clinical data of BC patients were downloaded from the TCGA database, Expression matrix of the long-chain non-coding RNA data was extracted from the transcriptome sequencing data; The threshold was set as a corrected P <0.05 with a differential expression multiple > 4 (FDR < 0.05 and | log FC | > 2), Differentially expressed lncRNA were screened using the R software edgeR package, The expression data of the lncRNA were merged with the downloaded survival data, The lncRNA related to patient outcome after alpha1H treatment was screened by univariate Cox regression analysis; LASSO (Least Absolute Shrinkage and Selection Operator through the glmnet package and the survival package in R, LASSO) Regression analysis screening for lncRNA with more critical prognosis with alpha1H treatment. Finally, the lncRNA model associated with BC prognosis after alpha1H treatment was established by multivariate Cox regression analysis. K-M survival analysis was performed on lncRNA with statistically differences in Cox multivariate regression analysis to determine prognostic biomarkers.

5.6 survival analysis

To assess the impact of gene expression on patient prognosis, we combined the cox proportional hazards survival model in TCGA BLCA patients using the R package "survival" and performed log-rank test, record HR [95%CI] of survival model and test p-values to assess the prognostic value of genes. Kaplan-Meier curves were drawn for the genes, lncRNA using Graphpad Prim 7 statistical software and whether statistical differences between the two groups were determined by Log-rank test, considered by P <0.05 as statistically significant.

Abbreviations
| Abbreviations | English Full Name |
|---------------|------------------|
| BC            | Bladder cancer   |
| BCG           | Bacillus Calmette-Guérin |
| GEO           | Gene Expression Omnibus |
| KEGG          | Kyoto Encyclopedia of Genes and Genomes |
| TCGA          | The Cancer Genome Atlas |
| MSigDB        | The Molecular Signatures Database |
| DAVID         | Database for Annotation, Visualisation and Integrated Discovery |
| GSVA          | Gene Set Variation Analysis |
| GSE           | Gene Set Enrichment |
| FDR           | False Discovery Rate |
| LASSO         | Least Absolute Shrinkage and Selection Operator through the glmnet package and the survival package in R |
| HR            | Hazard Ratio |

**Declarations**

**Ethics approval and consent to participate**

Not applicable

**Consent for publication**

Not applicable

**Availability of data and materials**

The datasets used and analyzed during the current study are presented in the main body of this manuscript.

**Competing interests**

The author declares no conflict of interest.
Fundings
Not applicable

Authors' contributions
Zhou HY designed the study. Wang X, Yu JD, Wen HL, Wang RJ and Peng K collected and analyzed the data. Wang X wrote the manuscript.

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Figure 1

heatmap plot of the overall expression distribution of the 781 differentially expressed genes

Figure 2

396 KEGG pathway associated with significantly upregulated genes
Figure 3

KEGG pathway associated with 385 significantly downregulated genes
Figure 4

heatmap plot of the overall expression distribution of the 119 differentially expressed lncRNA
Figure 5

Differential comparison of ssGSEA activation scores for the four important hallmark signaling pathways.
Figure 6

Overall survival K-M curves of SMAD4 in the TCGA BLCA dataset

\[ p = 0.0029 \]
**Overall survival**

![Graph showing overall survival K-M curves of SMAD6 in the TCGA BLCA dataset.](image)

- Expression: SMAD6 low (red) and SMAD6 high (blue)

**Number at risk**

|          | SMAD6 low | SMAD6 high |
|----------|-----------|------------|
| Deaths   | 37        | 14         |
| Survivors| 188       | 237        |
| Total    | 225       | 251        |

*p < 0.0001*

**Figure 7**

Overall survival K-M curves of SMAD6 in the TCGA BLCA dataset
Figure 8

Overall survival K-M curves of SMAD9 in the TCGA BLCA dataset
Figure 9

Overall survival K-M curves of LINC00152 in the TCGA BLCA dataset