**Supplementary Materials and Methods**

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**Supplementary Materials and Methods**

**Study subjects**

For the discovery stage, 580 bladder cancer cases were recruited from Nanjing (China) starting in May 2003 and 1,101 controls were selected from the same geographical region. The validation stage included 1,050 cases and 1,403 controls, and were mainly recruited from the First Affiliated Hospital and Huai-An Affiliated Hospital of Nanjing Medical University between January 2003 and May 2013. The demographic characteristics of all individuals were demonstrated in **Table S1** and **Table S3**.

**Screening and functional prediction for SNPs**

The scoring criteria are as follows: For HaploReg v4.1 and SNPinfo Web Server, the SNP function score is the sum of the function annotation items; for the RegulomeDB database, the SNP function score is the reverse score of the database; for the CancerSplicingQTL, only SNPs with splicing quantitative trait loci (sQTL) effects score one point; the sum of the four scores is the total score for each candidate SNP.

**SNP genotyping**

Genotyping of the discovery stage was conducted using Illumina Human Omni ZhongHua Bead chip and HumanOmniExpress chip. Genotyping for rs558814 in the validation stages was conducted using TaqMan assays (Applied Biosystems). Blinding of technicians was used to control the quality of the genotyping process. The sequence of primers and probes for the TaqMan were showed in **Table S7**.

**Patient sample collection and extraction of DNA and RNA**

A total of 51 pairs of bladder cancer tissues with 24 peripheral blood samples were collected in this study. All samples were originally collected from patients with bladder cancer undergoing surgery in Jiangsu Province Hospital of Traditional Chinese Medicine and The First Affiliated Hospital of Nanjing Medical University. Blood samples of each subject were collected to extract genomic DNA using DNA extraction kit (TIANGEN), and total RNA was collected using TRIzol Reagent (Invitrogen) from tissues according the manufacturers’ protocols.

**Cell culture, isolation of cytoplasmic and nuclear RNA**

In this study, three human bladder cancer cell lines (EJ, T24 and J82) and a human bladder epithelial cell line (SV-HUC-1) were used for experiments. All cell lines were purchased from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Among these cells, SV-HUC-1, EJ and T24 cells were cultured with 1640 medium (Biological Industries) containing 10% FBS (Gibco), 100 U/ml penicillin (Gibco) and 100 μg/ml streptomycin (Gibco), and J82 cells were cultured with MEM medium (KeyGEN). All cells were cultured at 37°C in a 5% CO2 atmosphere in a humidified incubator. Total RNA was collected from the parental and infected cells using TRIzol Reagent (Invitrogen) according the manufacturers’ protocols. In addition, cytoplasmic and nuclear RNA were extracted from cells and purified using Protein and RNA Isolation Kit (Thermo Fisher) according to the manufacturer’s protocols.

**Quantitative RT-PCR (qRT-PCR)**

After isolation of RNA, total RNA was reverse transcribed into cDNAs with a High-Capacity cDNA Reverse Transcription Kit (Invitrogen) according to the manufacturer’s protocols, and the cDNA templates were used to quantify the expression of lncRNA *BCLET* or *MSANTD2*. Quantitative RT-PCR (qPCR) was conducted with SYBR Green qPCR system (Vazyme) using LightCycler480 or LightCycler96 Real-Time PCR System (Roche). *GAPDH* or *U6* were used as endogenous controls for cytoplasmic and nuclear RNA expression, respectively. The comparative Ct method was used to calculated relative expression of RNA or transcripts. The primers were synthesized by Realgene and sequences were presented in **Table S7**. Because the specific primers of the transcript *BCLET*-long are difficult to design, the expression of *BCLET*-long was calculated by total expression of *BCLET* removing the expression of *BCLET*-short in our study.

**Construction of luciferase plasmids and luciferase reporter assays**

For the detection of the transcriptional activity of rs558814, the lncRNA *BCLET* promoter containing rs558814 A or G allele was synthesized into the pGL3-basic vector by GENEray. Sanger sequencing was used to verify the sequence. Lipofectamine 3000 (Thermo Fisher) was used to co-transfect the constructed luciferase reporter gene plasmid and internal reference PRL-SV40 plasmid (GENEray) into the three bladder cancer cells (EJ, J82 and T24) according to manufacturer's instructions. About 24h after transfection, the luciferase activity of the cells was detected using a dual-luciferase assay system (Promega). The ratio of the fluorescence value of firefly to the fluorescence value of Renilla was calculated to compare the difference in transcription activity of plasmids carrying different alleles.

**Construction of overexpression plasmids and siRNAs**

For the overexpression of *BCLET*-long, *BCLET*-short, and *MSANTD2-004*, three transcript sequences were synthesized and subcloned into the pcDNA3.1 vector (GENEray). For the knockdown of lncRNA *BCLET*, the lncRNA Smart Silencer was constructed by RiboBio and the specific sequences were shown in **Table S7**. About 24h after cell transfection into two cancer cells (T24 and J82), the effect of overexpression or knockdown was detected using qPCR.

**Cell proliferation, colony formation, cell transwell, and apoptosis assays**

For cell proliferation assay, the transfected cells were cultured with complete medium and seeded in 96-well plate at a density of 5,000 cells/well. The cell numbers were quantified using CCK-8 Kit (Dojindo) at 8h, 24h, 48h, and 72h, respectively. For the colony formation assay, the resuspended cells were placed in 6-well plate containing complete medium at a density of 1000 cells/well. After approximately 10 days of incubation, the cells were fixed with paraformaldehyde and stained with crystal violet (Beyotime). The number of cell colonies is counted under the microscope. For cell migration and invasion analysis, cells were resuspended in serum-free medium (3.0×104 cells/well for migration and 6.0×104 cells/well for invasion) and placed in Transwell inserts (Millipore) with or without Matrigel (BD Biosciences) of 24-well plate containing complete medium in the lower chamber. After incubation for 24h or 48h, invaded or migrated cells were fixed with paraformaldehyde, stained with crystal violet (Beyotime) and counted under the microscope. For cell apoptosis, 24h after cell transfection, the cells were resuspended in 100μL Buffer and incubated with FITC Annexin V (Vazyme) and PI (Vazyme) for 15min in the dark, and the proportion of apoptotic cells was measured on a flow cytometer (Becton Dickinson). All experiences were performed in triplicate.

**URLs**

dbSNP: http://hgdownload.soe.ucsc.edu

RegulomeDB: http://www.regulomedb.org/

HeploRegv4.1:http://pubs.broadinstitute.org/mammals/haploreg/haploreg.php

SNPinfo Web Server: http://snpinfo.niehs.nih.gov/snpinfo/snpfunc.html

CancerSpliceQTL Database: http://www.cancersplicingqtl-hust.com

Ensembl: http://grch37.ensembl.org/index.html

GTEx: https://gtexportal.org/home/

CPAT: http://lilab.research.bcm.edu/cpat/

TCGA: http://cancergenome.nih.gov/

Kaplan-Meier Plotter: http://kmplot.com/analysis/

**Supplementary Figure 1**

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**Supplementary Figure 2**

E:\4_课题\6 膀胱癌选择性剪切\Manhattan plot.tif

**Supplementary Figure 3**

**E:\4_课题\6 膀胱癌选择性剪切\膀胱癌选择性剪切\分层分析森林图_画板 1.tif**

**Supplementary Figure 4**

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**Supplementary Figure 5**

SF4_画板 1

**Supplementary Figure 6**

**E:\4_课题\6 膀胱癌选择性剪切\km_201009_160124_405100_MSANTD2.tif**

**Table S1. The demographic characteristics of case-control study in the discovery stage**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Variables** | **Cases**  **(n = 580)** | |  | **Controls**  **(n = 1,101)** | |  | ***P*a** |
| **N** | **%** |  | **N** | **%** |  |
| Age (mean±SD) | 64.7 ± 12.1 | |  | 64.5 ± 12.1 | |  | 0.689 |
| Sex |  |  |  |  |  |  |  |
| Male | 481 | 82.9 |  | 905 | 82.2 |  | 0.707 |
| Female | 99 | 17.1 |  | 196 | 17.8 |  |  |
| Smoking status |  |  |  |  |  |  |  |
| Never | 313 | 54.0 |  | 717 | 65.1 |  | < 0.001 |
| Ever | 264 | 45.5 |  | 384 | 34.9 |  |  |
| Unknown | 3 | 0.5 |  | 0 | 0 |  |  |
| Grade |  |  |  |  |  |  |  |
| Low | 287 | 49.5 |  |  |  |  |  |
| Intermediate | 174 | 30.0 |  |  |  |  |  |
| High | 86 | 14.8 |  |  |  |  |  |
| Otherb | 33 | 5.7 |  |  |  |  |  |
| Stage |  |  |  |  |  |  |  |
| Non-muscle invasive | 377 | 65.0 |  |  |  |  |  |
| Invasive | 152 | 26.2 |  |  |  |  |  |
| Other | 51 | 8.8 |  |  |  |  |  |

aTwo-sided *t* test or χ2 test.

bOther includes papilloma and missing data.

**Table S2. Association between 9 candidate AS-SNPs and bladder cancer risk in the discovery stage**

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **SNP** | **Chr** | **Position** | **Allelea** | **Gene** | **MAF** | | ***P*HWE** | **Call rate** | **OR**  **(95% CI)** | ***P*b** |
| **Cases** | **Controls** |
| rs558814 | 11 | 124675214 | A/G | *RP11-677M14.7* | 0.28 | 0.34 | 0.09 | 0.96 | 0.78 (0.67-0.91) | 1.91×10-3 |
| rs1877022 | 11 | 41842944 | G/C | *RP11-375D13.2* | 0.07 | 0.09 | 0.21 | 1.00 | 0.72 (0.55-0.95) | 1.88×10-2 |
| rs7220814 | 17 | 7290695 | A/G | *TNK1* | 0.19 | 0.22 | 1.00 | 1.00 | 0.81 (038-0397) | 2.33×10-2 |
| rs28359631 | 1 | 230898397 | A/G | *RP11-99J16\_A.2* | 0.14 | 0.12 | 0.39 | 0.99 | 1.27 (1.02-1.57) | 3.13×10-2 |
| rs2075276 | 22 | 21363744 | T/C | *THAP7-AS1* | 0.12 | 0.15 | 0.12 | 0.98 | 0.79 (0.64-0.98) | 3.27×10-2 |
| rs11176575 | 12 | 40820208 | G/A | *RP11-115F18.1* | 0.42 | 0.46 | 0.86 | 0.99 | 0.85 (0.74-0.99) | 3.37×10-2 |
| rs496797 | 11 | 94225807 | C/T | *MRE11A* | 0.47 | 0.50 | 0.72 | 1.00 | 0.86 (0.75-0.99) | 4.34×10-2 |
| rs2100431 | 15 | 60770850 | C/A | *NARG2* | 0.07 | 0.06 | 0.15 | 1.00 | 1.33 (1.01-1.77) | 4.41×10-2 |
| rs7157977 | 14 | 31858209 | C/T | *HEATR5A* | 0.33 | 0.36 | 0.60 | 1.00 | 0.85 (0.74-0.99) | 4.66×10-2 |

SNP, single nucleotide polymorphism; MAF, minor allele frequency; HWE, Hardy-Weinberg equilibrium; OR, odds ratio; CI, confidence interval.

aMajor/Minor.

b*P* values were calculated from logistic regression analysis adjusted for age and sex.

**Table S3. The demographic characteristics of the validation stage case-control study**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Variables** | **Cases**  **(n = 1,050)** | |  | **Controls**  **(n = 1,403)** | |  | ***P*a** |
| **N** | **%** |  | **N** | **%** |  |
| Age(mean±SD) | 64.8 ± 12.7 | |  | 65.2 ± 9.3 | |  | 0.320 |
| Sex |  |  |  |  |  |  |  |
| Male | 839 | 79.9 |  | 1,107 | 78.9 |  | 0.544 |
| Female | 211 | 20.1 |  | 296 | 21.1 |  |  |
| Smoking status |  |  |  |  |  |  |  |
| Never | 533 | 52.7 |  | 866 | 61.7 |  | < 0.001 |
| Ever | 497 | 47.3 |  | 537 | 38.3 |  |  |
| Former | 272 | 25.9 |  | 358 | 25.5 |  |  |
| Current | 225 | 21.4 |  | 179 | 12.8 |  |  |
| Grade |  |  |  |  |  |  |  |
| Low | 517 | 49.2 |  |  |  |  |  |
| Intermediate | 370 | 35.3 |  |  |  |  |  |
| High | 163 | 15.5 |  |  |  |  |  |
| Stage |  |  |  |  |  |  |  |
| Non-muscle invasive | 688 | 65.5 |  |  |  |  |  |
| Invasive | 362 | 34.5 |  |  |  |  |  |

aTwo-sided t test or χ2 test.

**Table S4. Association of** **rs558814 with bladder cancer risk in the validation stage**

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Genetic model** | **Genotypes** | **Cases** | |  | **Controls** | | **Adjusted OR (95% CI)** | ***P*a** |
| **N** | **%** |  | **N** | **%** |
| Additive | A | 1,440 | 69.2 |  | 1,859 | 66.3 | 1.00 |  |
|  | G | 640 | 30.8 |  | 943 | 33.7 | 0.88 (0.78-0.99) | 0.033 |
| Codominant | AA | 502 | 48.3 |  | 617 | 44.0 | 1.00 |  |
|  | AG | 436 | 41.9 |  | 625 | 44.6 | 0.86 (0.72-1.02) | 0.075 |
|  | GG | 102 | 9.8 |  | 159 | 11.4 | 0.79 (0.60-1.04) | 0.088 |
| Dominant | AA | 502 | 48.3 |  | 617 | 44.0 | 1.00 |  |
|  | AG/GG | 538 | 51.7 |  | 784 | 56.0 | 0.84 (0.72-0.99) | 0.037 |
| Recessive | AA/AG | 938 | 90.2 |  | 1,242 | 88.7 | 1.00 |  |
|  | GG | 102 | 9.8 |  | 159 | 11.4 | 0.85 (0.65-1.10) | 0.219 |

OR, odds ratio; CI, confidence interval.

a*P* values were calculated from logistic regression analysis adjusted for age and sex.

**Table S5. Stratification analyses for rs558814 and bladder cancer risk**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Variables** | **Genotypes (Cases/Controls)** | | | | | **Adjusted OR**  **(95% CI)** | ***P*a** |
| **AA** | |  | **AG/GG** | |
| **N** | **%** |  | **N** | **%** |
| Age |  |  |  |  |  |  |  |
| ≤ 65 | 234/313 | 47.3/45.2 |  | 261/380 | 52.7/54.8 | 0.91 (0.72-1.15) | 0.412 |
| > 65 | 268/304 | 49.2/42.9 |  | 277/404 | 50.8/57.1 | 0.78 (0.62-0.97) | 0.027 |
| Sex |  |  |  |  |  |  |  |
| Male | 405/469 | 48.7/42.4 |  | 426/636 | 51.3/57.6 | 0.77 (0.65-0.93) | 0.005 |
| Female | 97/148 | 46.4/50.0 |  | 112/148 | 53.6/50.0 | 1.13 (0.79-1.61) | 0.519 |
| Smoking status | |  |  |  |  |  |  |
| Never | 257/380 | 46.9/43.9 |  | 291/485 | 53.1/56.1 | 0.89 (0.72-1.10) | 0.291 |
| Ever | 245/237 | 49.8/44.2 |  | 247/299 | 50.2/55.8 | 0.80 (0.63-1.03) | 0.080 |
| Former | 131/153 | 48.7/42.9 |  | 138/204 | 51.3/57.1 | 0.79 (0.58-1.09) | 0.153 |
| Current | 114/84 | 51.1/46.9 |  | 109/95 | 48.9/53.1 | 0.83 (0.56-1.24) | 0.361 |

OR, odds ratio; CI, confidence interval.

a*P* values were calculated from logistic regression analysis adjusted for age and sex.

**Table S6. Association analyses for rs558814 and bladder cancer risk in clinicopathological subgroup**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Variables** | **Genotypes** | | | | | **Adjusted OR (95% CI)** | ***P*a** |
| **AA** | |  | **AG/GG** | |
| **N** | **%** |  | **N** | **%** |
| Controls | 617 | 44.0 |  | 784 | 56.0 |  |  |
| Grade |  |  |  |  |  |  |  |
| Low | 249 | 48.9 |  | 260 | 51.1 | 0.83 (0.67-1.01) | 0.067 |
| Intermediate | 176 | 47.7 |  | 193 | 52.3 | 0.86 (0.68-1.08) | 0.181 |
| High | 77 | 47.5 |  | 85 | 52.5 | 0.85 (0.62-1.19) | 0.343 |
| Stage |  |  |  |  |  |  |  |
| Non-muscle invasive | 316 | 46.5 |  | 364 | 53.5 | 0.91 (0.76-1.10) | 0.324 |
| Invasive | 186 | 51.7 |  | 174 | 48.3 | 0.73 (0.58-0.92) | 0.008 |

OR, odds ratio; CI, confidence interval.

a*P* values were calculated from logistic regression analysis adjusted for age and sex.

**Table S7. Sequences of primers and probes used in this study**

|  |  |  |  |
| --- | --- | --- | --- |
| **Experiments** | **SNP/Gene** | **Description** | **Sequences (5'-3')** |
| TaqMan | rs558814 | Forward | TTTGAAGCCGAGGACTTTGTC |
|  |  | Reverse | GCCTGCCTGGCATTCTTCTA |
|  |  | P-G | FAM-TTCACTCCCAATGGA-MGB |
|  |  | P-A | HEX-ACTCCCAATAGATTCA-MGB |
| RT-PCR | *BCLET* | Forward | GGTAGGTGTGGCCGTTTGTA |
|  | Reverse | GCAACTTCCAAAGCACGGAG |
|  | *BCLET*-short | Forward | AGTAGCACAGCACATCCAGT |
|  | Reverse | TTGCATTCATTGGTCAGCATCC |
|  | *MSANTD2* | Forward | ATTCACAGGAGGACTGGGGAA |
|  | Reverse | TGCATGATGTCTCTCTTCTGTG |
|  | *MSANTD2*-*004* | Forward | ATCTGTGGTCCGTACCTGGA |
|  | Reverse | CCCATCCTCCCTGACCAAAC |
|  | *GAPDH* | Forward | CCGGGAAACTGTGGCGTGATGG |
|  | Reverse | AGGTGGAGGAGTGGGTGTCGCTGTT |
|  | *U6* | Forward | CTCGCTTCGGCAGCACA  AACGCTTCACGAATTTGCGT |
|  | Reverse | AACGCTTCACGAATTTGCGT |
| RIP | *MSANTD2-RIP* | Forward | GCTGGACAGACTTTTCAAGGC |
|  |  | Reverse | AGTACTCCCGAAGTCGCTTG |
| LncRNA  Smart  Silencer | *BCLET* |  | CAATCAAGGACAGTAAACA |
|  |  | CAACCAAGATGGAATAACA |
|  |  | CCAGCAGAAAAGTTACTTT |
|  |  | GAGAATTAATACAGGCTCCA |
|  |  | ACCAGCTGACGACAACCATA |
|  |  | TCCAATACCAGTCTCCATCC |