# Wnt7a activates canonical Wnt signaling, promotes bladder cancer cell invasion, and is suppressed by miR-370-3p

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## Materials and methods

**Sample preparation for mass spectrum.** Cultured 5637 HMI and NMI cells were washed thrice with ice-cold PBS and lysed in SDS lysis buffer (4% SDS, 100 mM Tris-HCl, 0.1 M DTT, pH 7.6). The lysates were sonicated, then denatured and reduced at 95°C for 5 min. The protein concentration was determined using fluorescence quantification as described (1). Peptides were prepared following the filter-aided sample preparation (FASP) procedure (2). Briefly, samples were loaded in 10 kDa centrifugal filter tubes, washed twice with 200 μl UA buffer (8 M urea in 0.1 M Tris-HCl, pH 8.5), alkylated with 50 mM iodoacetamide in UA buffer for 30 min in the dark, washed thrice with UA buffer again and finally washed thrice with 50 mM triethyl ammonium bicarbonate (TEAB). All above steps were centrifuged at 12,000 g at 20°C. Proteins were digested overnight by trypsin (Promega) at a concentration of 1:50 (w/w) in 50 mM TEAB. After digestion, peptides were eluted by centrifugation. The peptides concentration was determined by BCA protein quantification kit.

**Isobaric Labeling with Tandem Mass Tags (TMT).** The isobaric labeling experiment was conducted by reference to the TMT kit instructions. As suggested, TMT reagents (0.8 mg) were dissolved in anhydrous acetonitrile (41 μl) and added to each 100 μg peptides (dissolved in 100 μl 100 mM TEAB) to achieve a final acetonitrile concentration of approximately 30% (v/v). Following incubation for 1 hr at room temperature, 8 μl 5% hydroxylamine was added to the sample and incubated for 15 min to quench the reaction. The TMT-labeled samples were pooled at equal amounts, and then subjected to vacuum centrifugation dryness and C18 solid-phase extraction desalting (3M Empore).

**High pH Reverse Phase fractionation.** 150 μg pooled sample was fractionated by high pH reverse phase method using a Waters XBridge BEH300 C18 column (150×1.0 mm, OD 5 μm) at a flow rate of 0.2 ml/min on Agilent 1200 systems. Solvent A (10 mM $NH_4COOH$, adjusted to pH 10.0 with $NH_3 \bullet H_2O$) and a nonlinear increasing concentration of solvent B (90% ACN, 10 mM $NH_4COOH$, adjusted to pH 10.0 with $NH_3 \bullet H_2O$) were used to separate peptides. A 90 min gradient was set as follows, 1%-5% B in 2 min; 5%-25% B in 40 min; 25%-40% B in 22 min; 40%-95% B in 2 min; 95% B for 4 min; 95%-1% B in 4 min; 1% B for 16 min. Elutes of the starting 72 min were collected every 2 min into 36 fractions, which were further combined by a concatenation strategy into 18 fractions (1 & 19, 2 & 20, ..., 18 & 36). The 18 fractions of peptides were dried by vacuum centrifugation, resuspended in 0.1% FA and subjected for mass spectrometry analysis.

**Mass spectrometry analysis.** Peptides were separated using a home-made micro-tip C18 column (75 μm × 200 mm) packed with ReproSil-Pur C18-AQ, 3.0 μm resin (Dr. Maisch GmbH, Germany) on a nanoflow HPLC Easy-nLC 1000 system (Thermo Fisher Scientific),

using a 90 min linear gradient at 300 nl/min from 5% to 28% of acetonitrile with 0.1 % (v/v) formic acid. Peptides were analyzed on an Orbitrap Elite mass spectrometry. Data-dependent acquisition was performed using Xcalibur software in positive ion mode at a spray voltage of 2.30 kV. The MS1 full scan was set at a resolution of 60,000 by orbitrap mass analyzer (300-1500 m/z) following 'top 10' MS2 scans generated by HCD fragmentation at a resolution of 15,000. The normalized collision energy (NCE) was 38.0%, and the dynamic exclusion settings were: repeat count, 1; repeat duration, 30 sec; exclusion duration, 30 sec. Isolation window was set at 2.0 m/z. Other parameter settings, minimum signal required, 5,000; activation time, 100 ms; FT first mass value, 100 m/z.

**Database searching.** Mass spectrometric data were analyzed using MaxQuant 1.5.8.0 against the human Uniprot database (downloaded in July, 2016) (3). MS2 based reporter ion quantification was chosen with reporter mass tolerance set at 0.01 Da. Carbamidomethyl cysteine was searched as a fixed modification, oxidized methionine and protein N-term acetylation as variable modifications. Enzyme specificity was set to trypsin/P. Two missing cleavage site was allowed. The tolerances of first search and main search for peptides were set at 20 ppm and 4.5 ppm respectively. The peptide and protein false discovery rate (FDR) was fixed at a significant level not greater than 0.01. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier (Project accession No.: PXD007709).

**Protein association network analysis.** STRING database was used to predict protein-protein interactions. The low confidence score (0.15) was chosen for searching more potential interactions. The network was reconstructed in Cytoscape 3.4.

**MTT assay.** 5637 parental cells, HMI and NMI cells were seeded at 2,500 cells/well in 96-well plates, respectively. The viable cells were measured by MTT (3-(4,5-dimethylthiazol -2-yl)-2,5-diphenyltetrazolium bromide) assay at the indicated time points, as described previously (6).

**Immunofluorescence and confocal microscopy.** 5.0 μm-thick human UBC paraffin sections were blocked by 5% bovine serum albumin (BSA)/ Phosphate Buffered Saline (PBS), followed by the incubation with the primary antibodies against with Wnt7a (Abcam, ab100792, 1:100) and β-catenin (BD Biosciences, Cat # 610153, 1:400) overnight at 4 $^{o}$C. Alexa 488 or Alexa 555-conjugated secondary antibody (Molecular Probes, Invitrogen) was added for 1 hr at room temperature. Finally, tissues were counterstained with DAPI and fluorescent images were visualized by a confocal microscope (Leica TSC SP5 II, Wetzlar, Germany).

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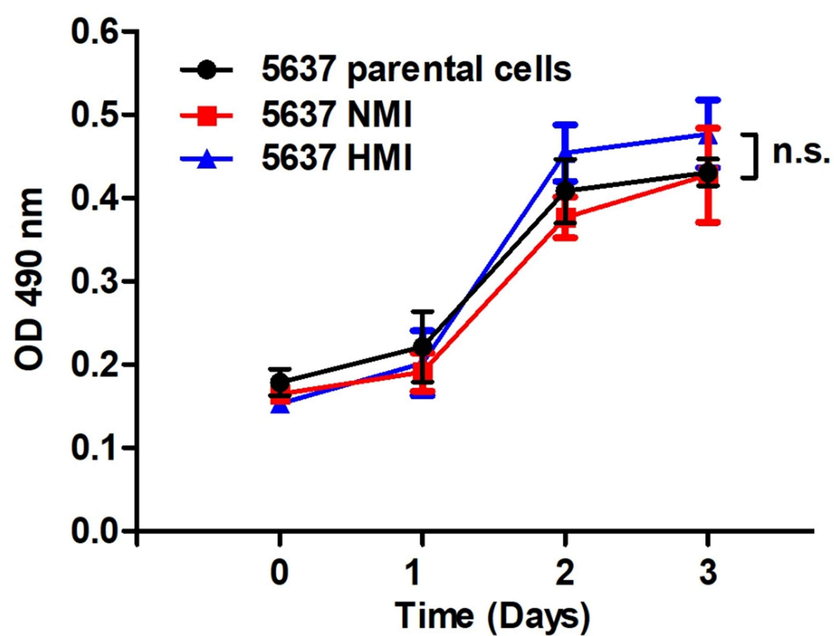
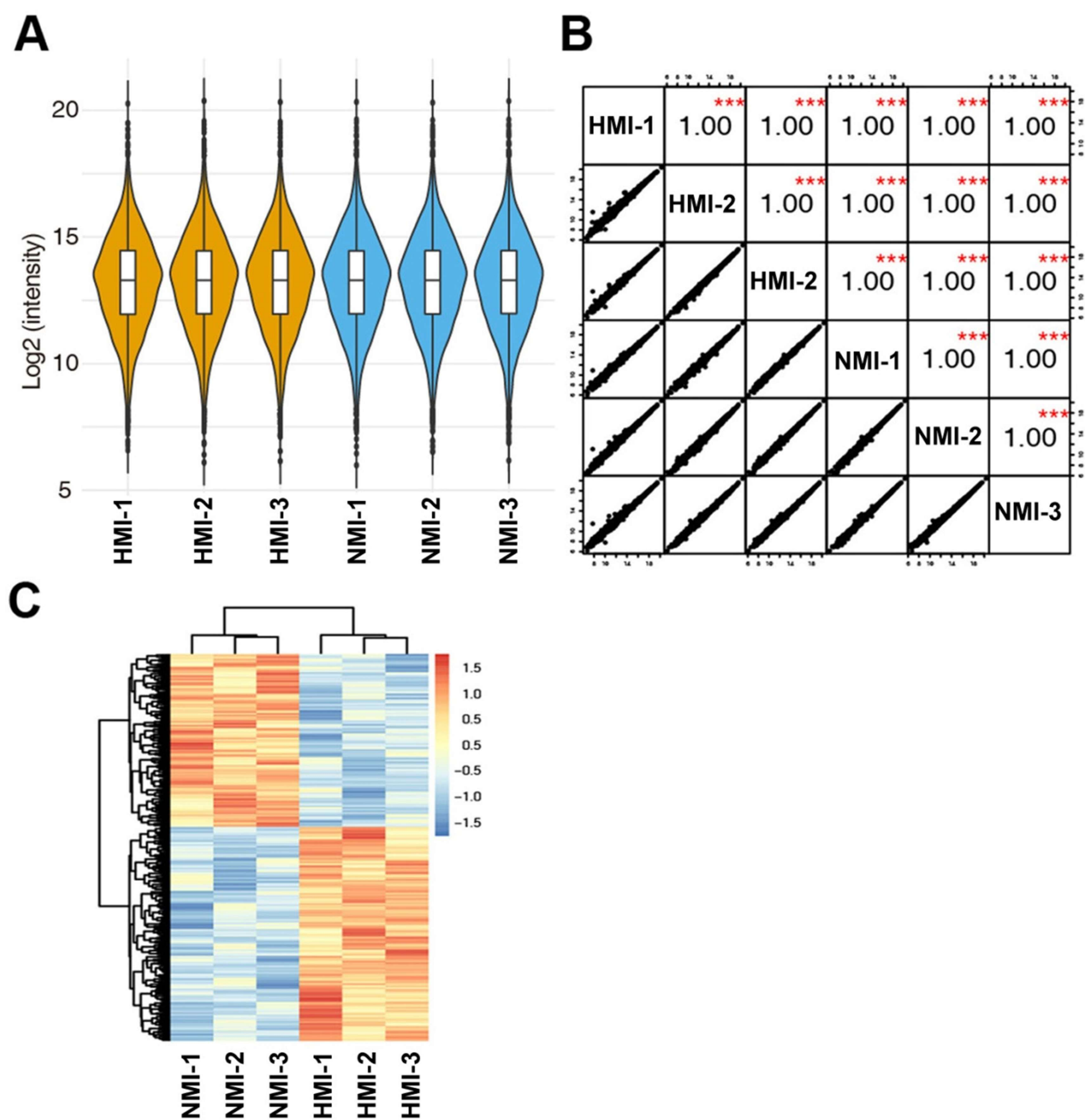
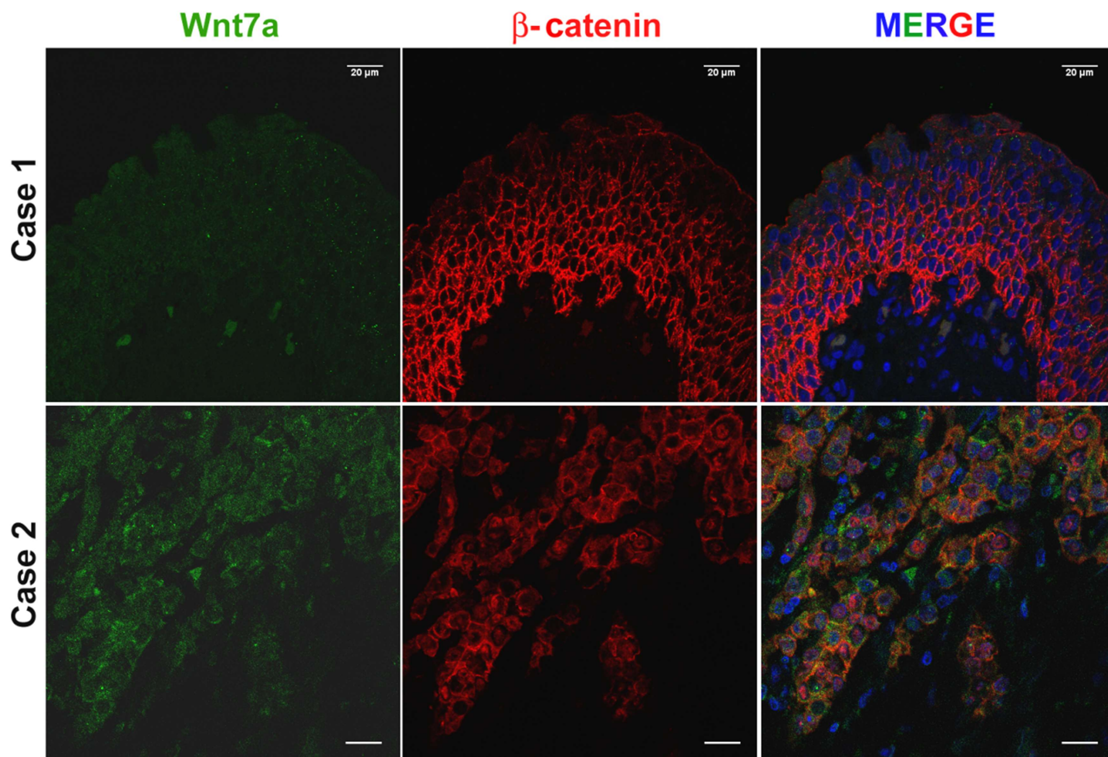


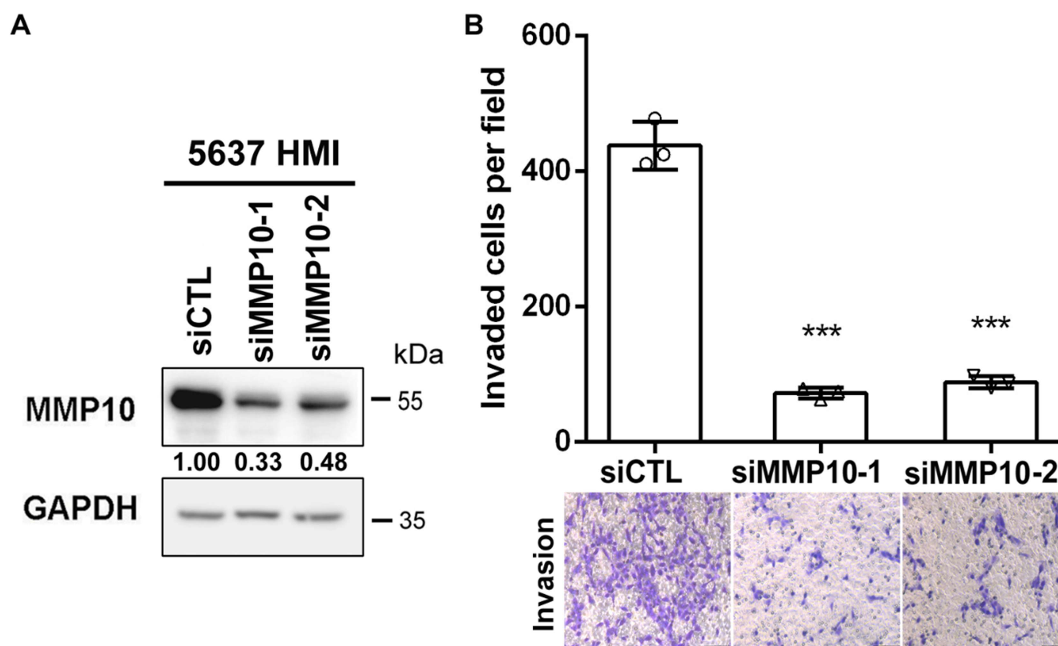

**Supplementary Figure S1. No differences of cell proliferation rates among 5637 parental cells and its derivative cells.** 5637 parental cells, 5637 HMI and 5637 NMI cells were seeded at 2,500 cells/well in 96-well plate and viable cells were measured by MTT assay at the indicated time points. n.s., not significant.



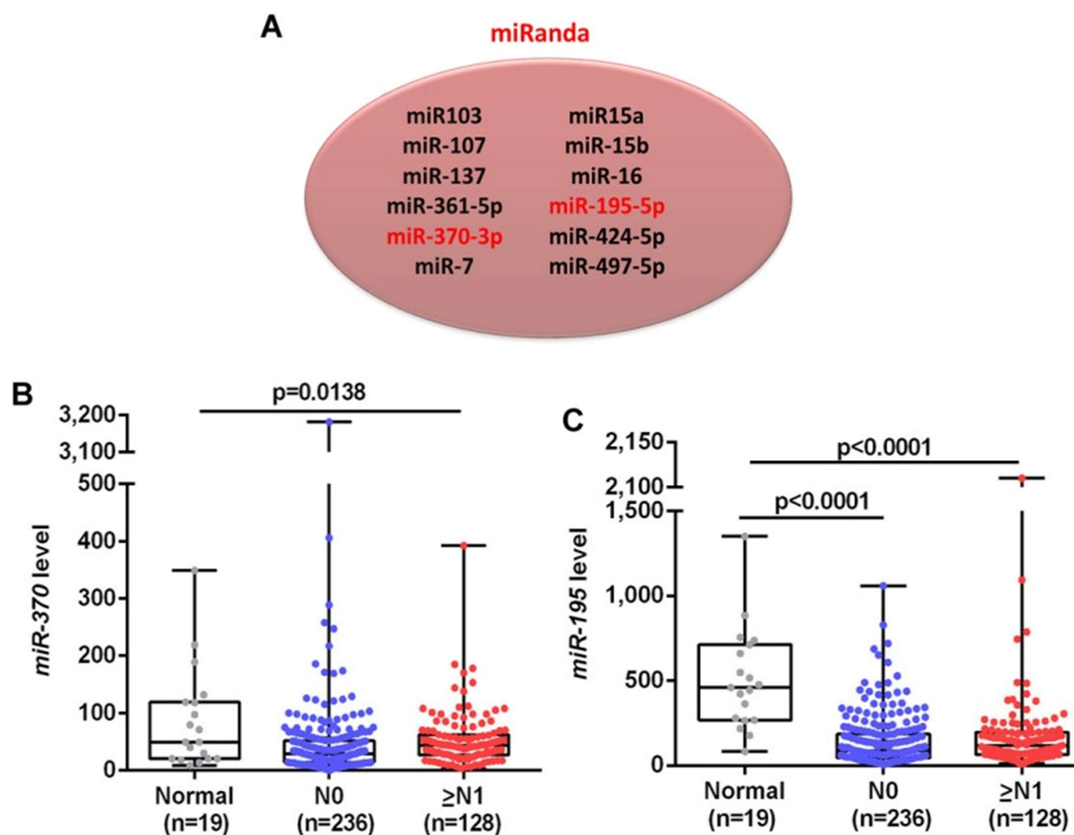

**Supplementary Figure S2. Quality control of mass spectrum data.** (A) The boxplot shows distributions of protein reporter intensities adjusted by median normalization in each channel. (B) The quantification correlation plot. Pearson coefficients were calculated using all quantified proteins for each pair of samples. (C) Hieratical clustering analysis using proteins with significant p<0.05.




**Supplementary Figure S3. Expressions and localizations of Wnt7a and β-catenin in human UBC samples.** The human UBC sections were stained with Wnt7a (Green) and β-catenin (Red) antibodies, followed by counterstaining with DAPI (Blue). Scale bar, 20 μm.




**Supplementary Figure S4. Depletion of MMP10 in 5637 HMI cells suppressed the invasion capacity by transwell assay.** (A) Knockdown of MMP10 in 5637 HMI cells by two independent siRNAs was detected by western blotting. GAPDH served as an internal loading control. (B) Transwell invasion assay. After transfected with siRNAs to MMP10 and control siRNA (siCTL), $1\times10^5$ cells were trypsinized and seeded in each transwell with 10% FBS medium as chemoattractant. After 15 hr, cells were fixed with formaldehyde and stained with crystal violet. Cell number was counted under microscope. Representative transwell images were shown below the graph. Scale bar, 100 μm. ***, $p<0.001$.




**Supplementary Figure S5. The expression level of putative Wnt7a targeting miRNAs in UBC samples.** (A) Bioinformatic analysis of Wnt7a targeting miRNAs by miRanda algorithm. (B,C) The plots of two underexpressed miRNAs, miR-370 (B) and miR-195 (C), in either UBC samples without lymph node metastatic lesion (N0, n=236) or UBC samples and UBC samples with lymph node metastatic lesions (≥ N1, n=128), compared with normal bladder tissues (n=19) by using TCGA provisional database.

## Supplementary Table S1. The list of altered protein levels in 5637 HMI *vs* NMI cells.

| Uniprot | Gene Symbol | HMI-1 | HMI-2 | HMI-3 | NMI-1 | NMI-2 | NMI-3 | FC* (HMI/NMI) | p value (*t* test) |
|---|---|---|---|---|---|---|---|---|---|
| P50453 | SERPINB9 | 9880.66 | 10303.4 | 10759.7 | 5568.77 | 6686.2 | 6241.98 | 1.65 | 0.00054817 |
| P05109 | S100A8 | 83007 | 86329 | 86749.3 | 56637.8 | 62371.7 | 56887.4 | 1.52 | 0.00026996 |
| P30740 | SERPINB1 | 32539.7 | 32070.7 | 32682.6 | 22861.3 | 24972.1 | 22878.8 | 1.42 | 0.00025689 |
| P09238 | MMP10 | 19810.6 | 19781.8 | 19520.6 | 14724.5 | 14432.5 | 14838.7 | 1.34 | 4.9525E-06 |
| Q5JV61 | FRMD3 | 5509.86 | 6596.16 | 6148.69 | 4606.46 | 5314.54 | 4596.5 | 1.33 | 0.03433336 |
| O00755 | WNT7A | 30150.1 | 29650.4 | 29410.3 | 21235 | 24782.3 | 22238.9 | 1.33 | 0.0029236 |
| O60732 | MAGEC1 | 5546.02 | 6533.82 | 6921.93 | 5045.25 | 4762.77 | 5010.49 | 1.30 | 0.02918655 |
| P06702 | S100A9 | 37241.7 | 37522.4 | 38437.9 | 30741 | 29138.8 | 28976.1 | 1.29 | 0.00026501 |
| O60635 | TSPAN1 | 573.167 | 583.635 | 685.016 | 506.403 | 412.804 | 455.631 | 1.28 | 0.02541519 |
| P03973 | SLPI | 11267.7 | 11094.4 | 11357.6 | 8778.38 | 8814.51 | 9105.8 | 1.28 | 5.4729E-05 |
| P03956 | MMP1 | 78326.4 | 83122.7 | 81224.2 | 60939.5 | 65519.1 | 64028.2 | 1.27 | 0.00085581 |
| P00813 | ADA | 9602.69 | 9558.03 | 10039.3 | 7621.04 | 7811.34 | 7610.92 | 1.26 | 0.00025058 |
| P05120 | SERPINB2 | 76875.8 | 80050.1 | 75563.7 | 59045.8 | 61164 | 62334.5 | 1.26 | 0.00053414 |
| H7C1W6 | CBS | 2127.2 | 1947.12 | 2121.94 | 1854.03 | 1640.52 | 1741.04 | 1.22 | 0.02000178 |
| Q96HD1 | CRELD1 | 9631.99 | 9710.55 | 9479.31 | 7972.41 | 8401.4 | 7908.96 | 1.21 | 0.00085914 |
| A0A0C4DFU2 | SOD2 | 34989.3 | 36288.3 | 37177.2 | 30096.9 | 31792.4 | 29924.1 | 1.21 | 0.00311866 |
| H7C471 | TBC1D10A | 941.475 | 1042.47 | 990.047 | 1195.41 | 1116.67 | 1283.53 | 0.83 | 0.02123622 |
| Q02388 | COL7A1 | 12155.2 | 11879.8 | 11833.6 | 14359.4 | 13816.2 | 14428.3 | 0.83 | 0.00050204 |
| Q12962 | TAF10 | 5102.47 | 4572.89 | 4664.7 | 5639.35 | 5250.76 | 6110.24 | 0.83 | 0.04066769 |
| P46736 | BRCC3 | 5465.34 | 5387.42 | 5228.79 | 5765.73 | 6542.42 | 6701.96 | 0.82 | 0.03044322 |
| P18827 | SDC1 | 2019.22 | 1803.28 | 2095.61 | 2392.2 | 2564.49 | 2469.37 | 0.82 | 0.00754082 |
| K7EPQ7 | YIF1B | 1849.94 | 1693.74 | 1703.23 | 2478.08 | 2067.01 | 2097.7 | 0.81 | 0.03029218 |
| O75487 | GPC4 | 4948.48 | 4106.87 | 4342.88 | 5159.29 | 5552.45 | 5353.5 | 0.81 | 0.0319417 |
| Q15542 | TAF5 | 590.939 | 566.991 | 585.321 | 730.94 | 798.358 | 646.527 | 0.80 | 0.03169325 |
| P0DMQ5 | INAFM2 | 504.659 | 497.512 | 548.636 | 608.521 | 683.442 | 630.538 | 0.80 | 0.01061864 |
| P31431 | SDC4 | 860.512 | 887.893 | 857.81 | 1281.79 | 1077.44 | 987.884 | 0.80 | 0.04771194 |
| E9PR30 | FAU | 562.913 | 542.004 | 548.934 | 692.718 | 592.828 | 715.119 | 0.79 | 0.03858998 |
| P32455 | GBP1 | 12659 | 13005.3 | 12900 | 16412.5 | 15390.6 | 16317.4 | 0.79 | 0.00073695 |
| Q15464 | SHB | 756.753 | 872.085 | 746.767 | 960.505 | 965.148 | 943.003 | 0.79 | 0.01572224 |
| I3L3M4 | CLDN7 | 205.259 | 232.062 | 193.202 | 264.714 | 284.532 | 254.65 | 0.78 | 0.01615584 |
| Q9BWT7 | CARD10 | 387.473 | 450.159 | 423.962 | 554.768 | 522.041 | 552.756 | 0.77 | 0.00431099 |
| H0YD96 | SDHD | 673.603 | 748.233 | 640.551 | 952.688 | 889.322 | 838.345 | 0.76 | 0.01093581 |
| Q9P0H9 | RER1 | 6071.32 | 5666.57 | 5555.22 | 7036.72 | 7509.25 | 8042.06 | 0.75 | 0.00589341 |
| Q9BQ69 | MACROD1 | 703.173 | 767.993 | 803.101 | 866.73 | 1022.61 | 1065.7 | 0.75 | 0.02780179 |
| A0A075B730 | EPPK1 | 52124.5 | 49762.9 | 50379.8 | 67987.5 | 64511.2 | 67333.6 | 0.75 | 0.00024398 |
| P43365 | MAGEA12 | 6012.08 | 5140.44 | 5329.27 | 7187.14 | 6977.73 | 7702.39 | 0.74 | 0.00625207 |
| Q13641 | TPBG | 6667.79 | 6710.83 | 6472.54 | 7376.39 | 9329.21 | 9439.42 | 0.71 | 0.03578893 |
| Q99880 | HIST1H2BL | 2453.27 | 1990.15 | 2060.6 | 3280.84 | 2690.1 | 2996.84 | 0.69 | 0.02123717 |
| P04035 | HMGCR | 3843.72 | 3667.69 | 3806.49 | 4371.58 | 5809.26 | 5592.41 | 0.68 | 0.03006608 |

| Uniprot | Gene Symbol | HM-1 | HM-2 | HM-3 | NM-1 | NM-2 | NM-3 | FC* (HMI/NMI) | p value (*t* test) |
|---|---|---|---|---|---|---|---|---|---|
| **Q9NY87** | **SPANXC** | 2014.15 | 1949.18 | 1975.96 | 2710.14 | 3161.88 | 3000 | **0.66** | 0.00184943 |
| **E9PF05** | **SUMF1** | 1946.62 | 1695.57 | 1733.77 | 2179.42 | 2644.08 | 2947.7 | **0.66** | 0.02794741 |
| **Q9NV35** | **NUDT15** | 1048.45 | 893.905 | 965.536 | 1158.71 | 1611.52 | 1513.14 | **0.64** | 0.03381291 |

***, FC, fold changes. Only the proteins with statistical significance were listed in the table.**

## Supplementary Table S2. The putative TCF/LEF sites in human *MMP10* promoter.

| Putative TCF/LEF site | From | To | Wild-type Sequence | Mutated sequence |
|---|---|---|---|---|
| Site 1 | -756 | -739 | 5'-taaggTACAAAGgagga-3'(-) | 5'-taaggCATGAGAgagga-3' |
| Site 2 | -622 | -605 | 5'-ttatgTTCAAAGtaact-3'(-) | 5'-ttatgCTTCAGAtaact-3' |

**Notes:** The core sequences of the TCF/LEF site are in upper cases, which match the 7-base consensus TCF/LEF binding motif (5'-WWCAAWG-3') (4,5). The position is the distance from the transcription start site using human Wnt7a sequence (NM_004625.3). (-), antisense strand.

**Supplementary Table S3. List for qRT-PCR primers, siRNAs and subcloning sequences.**

| | Direction | Sequences (5'-3') |
|---|---|---|
| ***qRT-PCR primers*** | | |
| *β-actin* | Forward | TCTGGCTGAGGCTGGTTGAC |
| | Reverse | CTCCTTAATGTCACGCACGAT |
| *MMP1* | Forward | CCTGGAAAAATACTACAACCTGAAG |
| | Reverse | TTCAATCCTGTAGGTCAGATGTGT |
| *MMP10* | Forward | TGCTCTGCCTATCCTCTGAGT |
| | Reverse | TCACATCCTTTTCGAGGTTGTAG |
| *S100A8* | Forward | ATGCCGTCTACAGGGATGAC |
| | Reverse | ACTGAGGACACTCGGTCTCTA |
| *Wnt7a* | Forward | CTCCGGATCGGTGGCT |
| | Reverse | CCCATTTGTGAGCCTTCTCCT |
| *miR370-3p* | RT-primer | CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGACCAGGTT |
| | qRT-primer | ACACTCCAGCTGGGGCCTGCTGGGGTGGAA |
| *miR424-5p* | RT-primer | CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGTTCAAAAC |
| | qRT-primer | ACACTCCAGCTGGGCAGCAGCAATTCAGTG |
| *miR497-5p* | RT-primer | CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGACAAACCA |
| | qRT-primer | ACACTCCAGCTGGGCAGCAGCACACTGTGG |
| *miR195-5p* | RT-primer | CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGCCAATATT |
| | qRT-primer | ACACTCCAGCTGGGTAGCAGCACAGAAATA |
| *RNU6* | RT-primer | CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAACGCTTC |
| | qRT-primer | ACACTCCAGCTGGGACGCAAATTCGTGAAG |
| ***siRNA sequences*** | | |
| siWnt7a-1 | | GCUACGUGCUCAAGGACAAtt |
| siWnt7a-2 | | GCUGCUAUGUCAAGUGCAAtt |
| siMMP10-1 | | GGACAGUAAUCUCAUUGUUtt |
| siMMP10-2 | | CAUACAGGAUUGUGAAUUAtt |
| siNC | | UUCUCCGAACGUGUCACGUtt |
| **PCR primers for subcloning** | | |
| MMP10 WT 1F | | ATTTCTCTATCGTAGGTACCGTAGAGCAAACTCTTTGGCAAC |
| MMP10 WT 1R | | CAGTACCGGAATGCCAAGCTTCTCACTGCCCTTACCTTCT |
| MMP10 mutant 1 2F | | AATCCATCTCCTCTCTCATGCCTTAAGA |
| MMP10 mutant 1 2R | | TCTTAAGGCATGAGAGAGGAGATGGATT |
| MMP10 mutant 2 3F | | ATGGAGCAGTTATCTGAAGCATAATTATAC |
| MMP10 mutant 2 3R | | GTATAATTATGCTTCAGATAACTGCTCCAT |
| pWnt7a subcloning F | | gatgacaagcttgcggccgcgAACCGGAAAGCGCGGCGCTGCC |
| pWnt7a subcloning R | | tctgccctcgatatcgaattcCTTGCACGTGTACATCTCCGTG |

**Notes**：The nucleotides with underlines in the table were mutant nucleotides sequences to replace putative wild-type TCF/LEF binding sites.

**Supplementary Table S4. Relationship between Wnt7a mRNA expression and clinical pathological characteristics of 41 UBC samples for qPCR assay.**

| Characteristics | Number | Expression of *Wnt7a* | | p value |
|---|---|---|---|---|
| | | Low (n, %) | High (n, %) | |
| Age | | | | 0.0953 |
| <60 | 11 | 3 (27.2%) | 8 (72.7%) | |
| ≥60 | 30 | 17 (56.7%) | 13 (43.3%) | |
| Gender | | | | 0.7059 |
| Male | 35 | 18 (51.4%) | 17 (48.6%) | |
| Female | 6 | 2 (33.3%) | 4 (66.7%) | |
| Tumor number | | | | 0.6232 |
| Single | 18 | 8 (44.4%) | 10 (55.6%) | |
| Multiple | 23 | 12 (52.2%) | 11 (47.8%) | |
| Tumor grade | | | | 0.0500 |
| Low | 12 | 3 (25.0%) | 9 (75.0%) | |
| High | 29 | 17 (58.6%) | 12 (41.1%) | |
| T stage | | | | 0.4697 |
| < T2 | 11 | 5 (45.5%) | 6 (54.5%) | |
| ≥ T2 | 23 | 15 (65.2%) | 8 (34.8%) | |
| Tx | 7 | 0 (0.0%) | 7(100%) | |
| N stage | | | | 0.9198 |
| N0 | 33 | 16 (48.5%) | 17 (51.5) | |
| ≥ N1 | 3 | 1 (33.3%) | 2 (66.7%) | |
| Nx | 5 | 3 (60.0%) | 2 (40.0%) | |

**Notes:** Mean was used for cutoff point.

**Supplementary Table S5. Relationship between Wnt7a protein level and clinical pathological characteristics of 45 UBC samples for IHC staining.**

| Characteristics | Number | Wnt7a protein level | | p value |
|---|---|---|---|---|
| | | Low (n, %) | High (n, %) | |
| Age | | | | 0.286 |
| ≤65 | 20 | 12 (60.0%) | 8 (40.0%) | |
| >65 | 25 | 11 (44.0%) | 14 (56.0%) | |
| Gender | | | | 0.953 |
| Male | 39 | 20 (51.3%) | 19 (48.7%) | |
| Female | 6 | 3 (50.0%) | 3 (50.0%) | |
| T stage | | | | 0.626 |
| < T2 | 18 | 10 (55.6%) | 8 (44.4%) | |
| ≥ T2 | 27 | 13 (48.1%) | 14 (51.9%) | |
| N stage | | | | 0.838 |
| N0 | 19 | 10 (52.6%) | 9 (47.4%) | |
| ≥ N1 | 7 | 4 (57.1%) | 3 (42.9%) | |
| Nx | 19 | 9 (47.4%) | 10(52.6%) | |

**Notes:** Mean value was used for cutoff point.

## Supplementary Table S6. List of antibodies.

| Antibody | Company | Cat. No. | Dilution (Application) |
|---|---|---|---|
| Active β-catenin | Cell Signaling | 8814s | 1:1,000 (WB) |
| β-catenin | BD Biosciences | 610153 | 1:500 (IHC)<br>1:400 (IF) |
| E-Cadherin | Bioworld | BS1098 | 1:1,000 (WB) |
| N-Cadherin | Proteintech | 22018-1-AP | 1:1,000 (WB) |
| GAPDH | Santa Cruz | sc-32233 | 1:1,000 (WB) |
| MMP1 | Proteintech | 10371-2-AP | 1:1,000 (WB) |
| MMP9 | Cell Signaling | #13667s | 1:1,000 (WB) |
| MMP10 | R&D Systems | MAB9101 | 3.5:3,000 (WB) |
| Twist | GeneTex | GTX127310 | 1:1,000 (WB) |
| Vimentin | Proteintech | 0366-1-AP | 1:1,000 (WB) |
| Wnt7a | Abcam | ab100792 | 1:1,000 (WB);<br>1:150 (IHC)<br>1:100 (IF) |
| Zeb1 | Proteintech | 21544-1-AP | 1:1,000 (WB) |
| $2^{nd}$ antibody mouse IgG | Cell Signaling | 7076s | 1:1,000 (WB) |
| $2^{nd}$ antibody rabbit IgG | Cell Signaling | 7074s | 1:1,000 (WB) |
| $2^{nd}$ antibody mouse IgG, Alexa Flour 488 | Molecular Probes | A11008 | 1:1,000 (IF) |
| $2^{nd}$ antibody rabbit IgG, Alexa Flour 555 | Molecular Probes | A21422 | 1:1,000 (IF) |

**Notes:** WB, western blotting; IHC, immunohistochemical staining; IF, immunofluorescence.