2. Materials and Methods

*2.1 Tissue samples*

Samples of RCC for immunohistochemistry were collected from 121 patients. The patients were diagnosed as having RCC who underwent nephrectomy at Hiroshima University Hospital (Supplementary Table 1). Tumor staging was performed according to the TNM stage grouping system [[1](#_ENREF_1)]. Among 121 patients with RCC, 86 patients were used for immunohistochemistry of CD44, CD133, ALDH1, p53, and PD-L1.

*2.2 Cell lines*

Human RCC-derived cell lines Caki-1, ACTH, and 786-O cells were purchased from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). All cell lines were maintained in RPMI 1640 (Nissui Pharmaceutical, Tokyo, Japan) containing 10% fetal bovine serum (Whittaker, Walkersville, MD, USA) in a humidified atmosphere of 5% CO2 and 95% air at 37°C.

*2.3 western blotting,*

Cells were lysed, as described previously [[2](#_ENREF_2)]. The lysates (40 µg) were solubilized in Laemmli sample buffer by boiling and then subjected to 10% SDS-polyacrylamide gel electrophoresis. The membrane was incubated with a primary antibody for BUB1B (1:500; OriGene Tech, Rockville, CA, USA), p53 (1:500; Cell Signaling Technology, Inc., Danvers, MA), Phospho-p53 (Ser15) (1:500; Cell Signaling Technology, Inc., Danvers, MA), CD44 (1:500; Cell Signaling Technology, Inc., Danvers, MA) (Supplementary Table 2). Peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG was used in the secondary reaction. Immunocomplexes were visualized with an ECL Western Blot Detection System (Amersham Biosciences, Piscataway, NJ, USA). β-actin (Sigma-Aldrich, St. Louis, MO, USA) was stained as a loading control.

*2.4 RNA interference,*

We used validated siRNA oligonucleotides targeting *BUB1B (sense: GCACACUAGCUGAACUAAA, antisense: UUUAGUUCAGCUAGUGUGC)* and negative control (non-targeted siRNA) (Thermo Fisher Scientific, Waltham, MA, USA). Transfection was performed using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) as described previously [[3](#_ENREF_3)].

*2.5 qRT-PCR analysis,*

Extraction of total RNA, synthesis of cDNA, and qRT-PCR were performed as described previously [[4](#_ENREF_4)]. *ACTB*-specific PCR products, which were amplified from the same RNA samples, served as internal controls. The primer sequences of BUB1B (upper: TTAGGGTGCAGCTGGATGTTT, lower: ACCCATCCCAGAAGACCTGTA)

*2.6 cell growth and invasion assays,*

We performed Water Soluble Tetrazolium Salts (WST-1) assay to examine cell viability as described previously [[5](#_ENREF_5)]. The cells were seeded at a density of 2000 cells per well in 96-well plates. Cell growth was monitored after 1, 2, and 4 days. Three independent experiments were performed.

Modified Boyden chamber assays were performed to examine cell invasion, as described previously [[6](#_ENREF_6)]. Cells were plated at 10,000 cells per well in RPMI 1640 medium in the upper chamber of an 8-µm pore diameter Transwell Insert (Corning, NY, USA) coated with Matrigel. Medium containing 10% serum was added in the bottom chamber. After two days, cells in the upper chamber were removed by scraping, and the cells remaining on the lower surface of the insert were stained with CyQuant GR dye to assess the number of cells.

*2.7 Spheroid Formation Assay*

For the generation of spheres, 2×103 cells (transfected with *BUB1B* siRNA, or negative control siRNA) were plated on 24-well ultra-low attachment plates (Corning, New York, NY, USA). Cells were grown in mTeSR medium (STEMCELL Technologies Inc., Cambridge, MA, USA). The plates were incubated at 37°C in a 5% CO2 incubator for 15 days. Sphere size was then determined using a microscope [[3](#_ENREF_3)].

*2.8 statistical analysis*

All experiments were repeated at least three times with each sample in triplicate. The results are expressed as the mean ± S.D. of the triplicate measurements. Sample sizes for relevant experiments were determined by power analysis. The two-tailed Student *t*-test or Fisher’s exact test was used to analyze the statistical significance. A paired T-test was used to compare the statistical differences between RCC tissues and their corresponding non-neoplastic kidney tissues. After Kaplan-Meier analysis was performed, any statistical difference between the survival curves of the cohorts was determined with the log-rank Mantel-Cox test. A one-way ANOVA and posthoc analysis (Tukey-Kramer) were used to compare the four immune-based subtype data. A *P*-value of <0.05 was considered statistically significant. Statistical analyses were conducted primarily using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA) and JMP 14.0.0 software (SAS Institute, Cary, NC, USA).