Appendix S1

MATERIALS AND METHODS

Clinical protocol and patient information

For real-time PCR and immunostaining, tissue samples of melanoma, naevus and normal skin were collected from patients during routine diagnostic procedures. Relevant information was collected from the medical records of melanoma patients to determine their clinical stage according to the staging system for melanoma of the skin from the American Joint Committee on Cancer Staging Manual, 7th edition (11). Seventy-three primary cutaneous melanomas were obtained from 73 patients (31 men and 42 women) whose ages ranged from 27 to 93 years (mean 67 years). Primary cutaneous melanomas had Clark's levels ranging from I to V and Breslow depths ranged from in situ to 21 mm. Of the primary cutaneous melanomas, there were 35 cases of acral melanoma, 22 cases of CSD melanoma (melanoma arising from chronic sun-damaged skin), 14 cases of non-CSD melanoma and 2 cases of mucous melanoma (12). The 6 benign naevi were from 6 patients (3 males and 3 females). Their ages ranged from 9 to 74 years (mean 38 years). Histologically, they included junctional, compound and intradermal variants. The 11 normal skin tissue samples from 11 patients were used as age- and sex-matched controls. The samples were preserved at −80°C prior to use. For the FCM, melanoma cells were obtained from surgically resected primary tumours and lymph node metastases. Institutional review board approval and written informed consent were obtained before the patients were enrolled, in accordance with the Declaration of Helsinki.

Western blotting

The human melanoma cell lines were obtained from ATCC (Manassas, VA, USA), the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan) and Dr Y. Kawakami, Keio University (Japan). Normal human epithelial melanocyte (NHEM) cell lines were obtained from DS Pharma Biomedical (Osaka, Japan). The proteins of these cell lines were extracted using radioimmunoprecipitation assay (RIPA) buffer (Wako, Japan). For the western blot, proteins were separated using 10% sodium dodecyl sulphate-polyacrylamide gels. The proteins were then transferred to membranes using the Trans-Blot Turbo Transfer System (Bio-Rad Laboratories, Hercules, CA, USA) and the membranes were blocked with 1% skimmed milk/Tris-buffered saline, 0.2% Tween-20 (TBST) for 1 h. The membranes were then incubated with anti-ARID3B antibody at a dilution of 1:1000 (ab32481, rabbit polyclonal, Abcam, Cambridge, UK) at 4ºC overnight. The membranes were then incubated with antirabbit immunoglobulin G–horse radish peroxidase (IgG-HRP) (Bio-Rad Laboratories) for 1 h. After washing the membranes with TBST, bands of β-actin and ARID3B were detected using the Enhanced Chemiluminescence system (Amersham Bioscience, Piscataway, NJ, USA).

PCR of melanoma/normal skin tissue mRNA

Real-time PCR was performed using mRNA from melanoma and normal skin tissue. Twenty μg frozen tissue samples were macroscopically dissected and homogenized and then treated with NucleoSpin RNA (Takara Bio Inc., Shiga, Japan) to extract the mRNA, which was used to prepare the cDNA. The ARID3B expression was determined using real-time PCR using the Thermal Cycler Dice TP800 (Takara Bio Inc., Shiga, Japan) and specific primers (F:GGCTCGCATTCAGTCCTGTG, R:CAGCTTACCGGGCTGAGGTATC). Primers specific for GAPDH (QIAGEN PPH00150F-200) were used as the control. The relative mRNA expression levels of each sample were calculated using the calibration line method.

Tissue immunostaining analysis

The expression of ARID3B was investigated through immunohistochemical studies. Four μm sections of formalin-fixed, paraffin-embedded melanoma/naevus tissues were prepared, deparaffinized in xylene and dehydrated in a graded series of ethanol. The antigen was activated by heating the solution containing citrate buffer (pH 6.0) in a microwave for approximately 15 min and then cooling at room temperature. The intrinsic peroxidase activity was blocked with 0.3% hydrogen peroxide, diluted in methanol, for 30 min, followed by blocking of non-specific staining with 5% goat serum for 20 min. The same anti-ARID3B antibody used for Western blotting was used at a dilution of 1:100 and the samples were incubated at 4ºC overnight. The following day, the samples were incubated with rabbit MAX-PO (anti-rabbit IgG+HRP, Nichirei, Tokyo, Japan) for 45 min. Samples were then stained with 3,3'-diaminobenzidine tetrahydrochloride (Dojindo, Kumamoto, Japan) for less than 3 min to avoid non-specific staining. After rinsing with distilled water, the samples were counterstained with Giemsa stain. Furthermore, analyses were performed to determine whether ARID3B expression had a significant correlation with overall survival in melanoma patients.

Flow cytometry

FCM was performed with fresh melanoma tissue excised during surgery. Raw melanoma tissue samples were homogenized using a biomasher (Nippi Inc., Tokyo, Japan). Homogenized cells underwent antigen-antibody reactions involving ARID3B and CD271. Because ARID3B antigens were thought to localize to the nuclei of melanoma cells, the cells were initially perforated using IntraPrep Permeabilization reagent (Beckman Coulter Inc., Brea, CA, USA).

After blocking the samples with FcR Blocking Reagent (Miltenyi Biotec Inc., Auburn, CA, USA), the same anti-ARID3B antibody used for Western blotting was used at a dilution of 1:200 and the samples were incubated for 20 min at room temperature. The Rb Primary Antibody Isotype Control (rabbit primary antibody isotype control, Invitrogen) was used with the same conditions that were used for ARID3B. The CD271-FITC (clone ME20.4-1.H4, Miltenyi Biotec Inc.) was used at a 1:11 dilution for 20 min at room temperature along with mouse IgG1 isotype control-FITC (Miltenyi Biotec Inc.). Samples were labelled with goat anti-rabbit immunoglobulin G–phycoerythrin (IgG-PE) (Santa Cruz Biotechnology Inc., Dallas, TX, USA).

Immunofluorescence analysis

To detect ARID3B and CD271 expression in melanoma cells, an immunofluorescence analysis was performed using the melanoma cell line MeWo. The cells, fixed with 4% paraformaldehyde for 30 min, were permeabilized with 0.2% Triton- X. The cells were then blocked in 2% bovine serum albumin/ phosphate-buffered saline (BSA/PBS) for 1 h and incubated with the same antibodies used previously (anti-ARID3B at a 1:100 dilution; CD271-FITC: clone ME20.4-1.H4 at a 1:11 dilution) overnight at 4ºC. The cells were then incubated with goat anti-rabbit IgG-PE at a 1:100 dilution for 1 h at room temperature, washed with distilled water and analysed. Each antibody reaction was compared with isotype controls, which were the same as those used during FCM.

Evaluation of ARID3B positivity, invasive ability and proliferation of CD271$^+$ cells

Prior to the following 3 experiments, MeWo cells were sorted using the FACSAriaII system (Becton Dickinson, Franklin Lakes, NJ, USA) to obtain CD271$^+$ and CD271$^-$ melanoma cell populations. ARID3B mRNA expression was then analysed in these cell populations. mRNA was extracted from the cells and reverse transcribed into cDNA, and the ARID3B expression was analysed by real-time PCR using the Thermal Cycler Dice TP800 and specific primers (F:GGCTCGCATTCAGTCCTGTG, R:CAGCTTACCGGGCTGAGGTATC). Primers specific for GAPDH (QIAGEN PPH00150F-200) were used as the control. An invasion assay was performed to evaluate the invasive ability of CD271$^+$ cells compared with CD271$^-$ cells. A 24-well plate containing 8 μm pore size transwell inserts pre-coated with Matrigel was prepared (Corning Inc., Corning, NY, USA). After serum starved incubation with serum-free Dulbecco's Modified Eagle Medium (DMEM) in 5% CO_2 atmosphere at 37ºC for 24 h, the cells were seeded into the upper chamber of the transwell at 1×10^5/well in 500 μl serum-free DMEM. The lower chamber was filled with 750 μl DMEM supplemented with 20% foetal bovine serum as a chemoattractant. After incubating the cells at 37ºC for 24 h, the cells on the upper chamber of the transwell were removed with a cotton swab. Subsequently, the cells on the bottom of the transwell were fixed with methanol for 10 min and stained with haematoxylin and eosin. The number of cells was counted microscopically (at 200× magnification). Three independent experiments were performed. We also investigated the proliferation of CD271$^+$ cells using the Cell Proliferation enzyme-linked immunosorbent assay (ELISA) BrdU chemiluminescent assay (Roche, Basel, Switzerland). The cells were seeded at 1×10^4 cells/well in a 96-well plate and incubated in 5% CO_2 atmosphere at 37ºC for 96 h. Then cells were labelled with BrdU, which reflected the cell proliferation by being incorporated into DNA and taking the place of [^{3}H]-thymidine. BrdU-labelled cells were incubated for 2 h at 37ºC. Subsequently, after cell fixation, cells were incubated with anti-BrdU-peroxidase for 90 min at room temperature. After washing cells, 100 μl/well of substrate solution was added, the cells were incubated for 10 min at room temperature, and then light emission was measured using the FilterMax F5 microplate reader (Molecular Devices LLC, Sunnyvale, CA, USA).

Statistical analysis

The differences between 2 groups were compared using the Mann–Whitney *U* test. The Kruskal-Wallis test was used to analyse the differences among several groups. The relationship between ARID3B positivity in immunostained melanoma tissue and the overall survival was investigated using the Kaplan–Meier survival analysis, multivariate logistic regression analysis. Values of $p<0.05$ were considered to be significant. All statistical analyses were conducted using the Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA, USA), Statcel 3 (OMS, Saitama, Japan) and SAS software (SAS Institute Inc., Cary, NC, USA).