Appendix S1

SUPPLEMENTARY MATERIALS AND METHODS

Data collection

Our cohort was composed of 237 samples of NS (n=28), AK (n=42), BD (n=35) and invasive SCC (n=132) from the Affiliated Hospital of Guizhou Medical University. Detailed information on the cases is shown in Table 1. Hematoxylin and eosin (H&E) stained sections were reviewed and evaluated, and samples fulfilling the criteria for the appropriate diagnoses of AK, BD and SCC were selected for study. Archived formalin-fixed paraffin-embedded (FFPE) blocks were cut to make 4 μm sections for immunohistochemistry (IHC), RNAscope and multiple immunofluorescence staining. The study was approved by the Ethics Committees of Affiliated Hospital of Guizhou Medical University (Approval Number: 2021541).

IHC analyses of RGR expression

Details about the methods of IHC and further semiquantitative assessment followed previous reports.[1] Briefly, 4 μm sections with different types of skin tissues were dewaxed and rehydrated according to standard methods. Antigen retrieval was conducted with retrieval solution (ethylenediaminetetraacetic acid [EDTA], pH 9.0, ZLI-9069 from ZSGB-BIO) for 4 min using a pressure cooker. H_2O_2 (3%; PV-9000; ZSGB-BIO) was applied to block endogenous enzyme activity and subsequently incubated in a serum-free blocking solution (ZLI-9056; ZSGB-BIO). Then, the primary rabbit anti-human antibody of RGR (Abbkine, Cat# ABP56042) with dilution 1:300 , involucrin (Affinity Biosciences, Cat# AF0186) with dilution 1:200, or CEA (ZSGB-Bio, Cat# ZM-0062) with dilution 1:200 at 4 °C overnight, followed by treatment with horseradish peroxidase-conjugated (HRP) IgG goat anti-rabbit secondary antibody (DAKO, Glostrup, Denmark) for 30 min, and finally the UltraView Polymer DAB Detection Kit (Ventana/Roche) according to the recommended manufacturing protocol.

RGR expression on all stained slides was scored by two independent investigators. The semiquantitative assessment method was carried out by using percentages of 3+ (strong), 2+ (moderate), 1+ (weak), and 0 (negative) staining of tumor cells for each sample. The overall score was calculated by the percentage of positive tumor cells (3×x %+2× x %+1×x %=total score) to equal a range of 0-300.[2]

RNAScope RGR mRNA in situ hybridization assay

The mRNA expression level of RGR in SCC tissues was evaluated by an RNAscope kit (Advanced Cell Diagnostics (ACD), Newark, CA, USA) according to the manufacturer's instructions.[3] Briefly, the slides were deparaffinized and then treated with hydrogen peroxide for 10 minutes at room temperature. These sections were incubated with RNA scope target retrieval reagent (RNAscope kit, REF 322000) for 15 minutes at 100 °C

and digested by protease (RNAscope kit, REF 322330) for 30 minutes in a HybEZ oven (ACD) at 40 °C. Then, the sections were hybridized with the probe Hs-RGR-C1 (ACD, ref. 1178911-C1), positive control probe 3-plex Positive Control Probe-Hs (ACD, ref. 320861), and negative control probe: DapB (ACD, ref. 310043) in a HybEZ oven (ACD) at 40 °C for two hours. Finally, slides were treated with the HD 2.5 detection kit-brown (ACD, ref. 322300) and a mixture of DAB solutions A and B (1:1), followed by counterstaining with hematoxylin (ACD, ref. ASGH1-20). The slides were dried at 20 °C for 15 minutes and mounted with Vector Labs Vectamount (ACD, ref.321584). All the sections were scanned at the maximum available magnification (40×) by light microscopy (LEICA DM6). The images of RGR RNA expression were analyzed by QuPath software (version 0.2.3).

Multiple immunofluorescence staining

Based on the previous method,[4] costaining of RGR and involucrin, CD31, PCNA or Ki67 in NS and cSCC tissues was performed. The steps from paraffin dewaxing and hydration to primary antibody incubation were the same as the above IHC staining method. After the first primary antibody of anti-RGR (1:300; Abbkine, Cat# ABP56042) overnight at 4 °C, secondary antibody marked with HRP (abs50012; Absin, China) incubate at room temperature (RT) for 30 min in the dark condition. Next, the sections were stained with monochromatic fluorescent dyes 570-tyramide signal amplification (TSA) solution (abs50012; Absin) at a 1:200 dilution for 30 min at RT in the dark. The above steps were repeated, and the slides were again immersed in antigen and then stained with the secondary antibody anti-involucrin (1:200; Affinity Biosciences, Cat# AF0186), anti-CD31(1:100; ZSGB-Bio, TA500121), anti-PCNA (1:1200; Cell Signaling Technology, Inc) or anti-Ki67 (1:200; Abcam) and the corresponding secondary antibody and monochromatic fluorescent dyes 520-TSA (abs50012; Absin) or 650-TSA (abs50012; Absin). DAPI (abs50012; Absin) at a 1:100dilution at RT for 5 min was used for nuclear staining.

Cell culture

The A431 cell line (human cSCC cell) was purchased from ATCC and maintained in DMEM (Wako Pure Chemical Industries, Ltd.) supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma-Aldrich; Merck KGaA) in a 37 °C humidified incubator containing 5% CO2.

Lentiviral infection

According to the standard lentiviral production protocol (GeneChem, Shanghai, China), lentiviral particles (LV-RGR-RNAi [103556-1] and LV-control-RNAi [CON313]) were obtained via GV493 vectors in A431 cells. Lentiviral particles (LV-RGR [KL29757-4] and LV-control [CON335]) were also obtained via GV358 vectors in A431 cells. The supernatant of the virus was harvested for the following studies. Cells were cultured on 12-well plates at a density of 3×10^5 cells/well at 37 °C with 5% CO2 for 24 hours. Lentiviral particles at an MOI =10 were added to the medium to infect A431 cells grown to 30-40% confluence in 12-well plates. After 72 hours, the medium was replaced with fresh medium to continue the culture. When 80% of the cells showed

green fluorescence, selection pressure medium containing 5 μg/mL puromycin was added. Within 3-4 weeks, puromycin-resistant cell colonies were collected to measure the transfection efficiency of lentiviral infection by western blot and RT–qPCR.

RNA isolation and sequencing

Total RNA was extracted using TriPure (Roche), and the three groups of cell samples (LV-RGR-RNAi, LV-RGR, control) were sequenced by Illumina (NEB, USA, Catalog #: E7370L). Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in NEBNext First Strand Synthesis Reaction Buffer (5X). First strand cDNA was synthesized using random hexamer primers and M-MuLV Reverse Transcriptase (RNase H). Second strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities. After adenylation of the 3' ends of DNA fragments, NEBNext adaptors with hairpin loop structures were ligated to prepare for hybridization. To preferentially select cDNA fragments 370~420 bp in length, the library fragments were purified with the AMPure XP system (Beverly, USA). Then, 3 μL USER Enzyme (NEB, USA) was used with size-selected, adaptor-ligated cDNA at 37 °C for 15 min followed by 5 min at 95 °C before PCR. Then, PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer. Finally, PCR products were purified (AMPure XP system), and library quality was assessed on the Agilent 5400 system (Agilent, USA) and quantified by QPCR (1.5 nM). The qualified libraries were pooled and sequenced on Illumina platforms with the PE150 strategy at Novogene Bioinformatics Technology Co., Ltd. (Beijing, China). According to the effective library concentration and data amount needed, quality control and bioinformatics analysis were performed. QPCR and western blotting were used to validate the differential expression of candidate genes with cell differentiation.

Real-time quantitative PCR (RT–qPCR**)**

Total RNA was isolated by TRIzol (Invitrogen, Carlsbad, CA, USA) and reverse transcribed with a Fasting cDNA Dispelling RT SuperMix reverse transcriptase kit (KR118, TIANGEN) according to the manufacturer's instructions. Quantitative real-time reverse transcriptase PCR was performed using the Real-time PCR System (Bio-Rad, San Francisco, USA) with SYBR Green PCR Master Mix (Tiangen Biotech, Beijing, China). The expression of target gene RNA was calculated by the $2^{-\Delta\Delta Ct}$ method, and human GAPDH was used as an internal control. The primers (Shanghai Generay Bioteach, China) used were as follows: RGR F, 5'-TCGCAGACGTGACTTCCATC-3', RGR R, 5'-CTTGGTTCGGTCCTTCTCCC-3'; AKT F, 5'-CGTTCCGTCCTCTGGTGTCTG-3', AKT R, 5'-GTGTTGTTGTTGCTGCTTGCTATTG-3'; Involucrin F, 5'-TCCTCCTCCAGTCAATACCCATCAG-3', Involucrin R, 5'-GGCTCCTTCTGCTGTTGCTCAC-3';

Western blot analysis

Cells were lysed in RIPA lysis buffer (Solarbio, Beijing, China, R0010) containing 1 mM PMSF (Solarbio,

R0010) to obtain the protein extract. The total extracted proteins (20 μg) were separated by 10% SDS–PAGE and then transferred to polyvinylidene difluoride (PVDF) membranes (IPVH00010, Merck KGaA, Darmstadt, Germany). The PVDF membranes were blocked with 5% nonfat milk in TBST (T1085, Solarbio, Beijing, China) for two hours at RT and then incubated with the primary antibodies at 4 °C overnight. The primary antibodies used were as follows: anti-RGR (1:1000, Invitrogen, PA5-10993) and anti-phospho-Akt (1:1000, Abbkine, ABP0059). Anti-involucrin (1:1000; Affinity Biosciences LTD, AF7021) and anti-GAPDH (1:5000; Affinity Biosciences LTD, AF7021) were used as internal controls. After rinsing with TSBT, the membranes were incubated with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibody (goat anti-rabbit IgG H&L [ab97051] or goat anti-mouse IgG H&L [ab6789, Abcam, Cambridge, UK]) at RT for one hour. The protein bands were visualized with an enhanced chemiluminescence (ECL) kit (KF001; Affinity Biosciences Ltd., Beijing, China) with a Bio-Imaging system (Bio-Rad Laboratories, Inc., California, USA). The band intensities were quantified using Quantity One software (Bio-Rad, Hercules, CA, USA).

Statistical Analyses

GraphPad Prism (version 8.0) software was used for statistical analysis. Continuous variables are presented as the mean ± SD or median with interquartile range (IQR) when the distribution was skewed. The analysis of variance to compare means of two or more than two groups was performed by *t* tests or one-way. Both Mann–Whitney (two groups) and the Kruskal–Wallis (more than two groups) tests were used to compare the nonparametric distributions. Statistically significant differences were considered when $P < 0.05$ (***$P < 0.001$, **$P < 0.01$, *$P < 0.05$).

References

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