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Original Article

Methylation-mediated loss of SFRP2 enhances melanoma cell invasion via Wnt signaling

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Abstract: Wnt signaling plays an essential role in the initiation and progression of melanoma tumors. The Secreted Frizzled Related Proteins (SFRPs) are a family of proteins that suppress Wnt signaling. The methylation of SFRPs reduces their activity, and hence augments Wnt signaling. However, whether the methylation of SFRP2, a member of SFRPs, may be involved in the pathogenesis of melanoma is not known. Here we investigated the expression levels of SFRP2 in melanoma specimens. We found that SFRP2 mRNA wassignificantly decreased and methylation of SFRP2 gene was significantly increased in malignant melanoma tumors ascompared to the paired adjacent non-tumor tissue. Moreover, SFRP2 expression was significantly decreased in the malignant melanoma celllines, HTB63, A2058 and A375, but not in the non-transformed melanocyte cell line, Hermes 3A. The demethylation of SFRP2 gene by 5'-aza-deoxycytidine (5-aza-dCyd) in melanoma cell lines restored SFRP2 expression, at both mRNA and protein levels, and suppressed cell invasion. Furthermore, the demethylation of SFRP2 geneappeared to inhibit nuclear retention of a key Wnt signaling factor, β-catenin, in melanoma cell lines. Together, these data suggest that SFRP2may function as a melanoma invasion suppressor byinterfering with Wnt signaling, and the methylation of SFRP2 gene may promote pathogenesis of melanoma.

Keywords: Melanoma, Secreted Frizzled Related Proteins (SFRPs), methylation, 5-aza-dCyd, Wnt signaling, β-catenin, cancer invasion

Introduction

Wnt signaling plays an essential role in a variety of biological processes during development and tissue hemostasis [1, 2]. A delicate control of Wnt signaling is crucial for the proper maintenance of the organism, while aberrant Wnt signaling may lead to developmental defects and disease initiation and progression [1-7]. Wnt signaling is also involved in the progression of malignant melanoma, which comprises the majority of skin cancer deaths due to its highly metastatic behavior [8-10]. Once melanoma has migrated to distal tissue, there are currently very few effective treatments that are available [11, 12]. Hence, there is a great need for a better understanding of melanoma invasion, migration and metastases.

The Frizzled proteins are a family of G-protein coupled receptors that are negative regulators Wnt signaling [1, 2]. All Wnt-ligands and most of their cognate receptors contain a cysteine-rich domain (CRD) that mediate the molecular binding [13, 14]. Wnt signaling consists of canonical and non-canonical pathways. In the canonical Wnt signaling pathway, β-catenin is a key factor. At the resting state when no Wnt ligand binds to the frizzled/low density lipoprotein receptor related protein (LRP) receptor complexs, cytosolic β-catenin is recruited to a multi-protein "destruction complex" consisting of several proteins including adenomatous polyposis coli (APC), Axin, and glycogen synthase kinase-3β (GSK-3β), resulting in the phosphorylation of β-catenin by GSK-3β and subsequent degradation of β-catenin via the ubiquitin proteosome pathway. At the activated state, Wnt protein binds to the Frizzled/LRP receptor complex and transduces a signal to Dishevelled (Dvl) to alter the composition of the "destruction complex", resulting in suppression of β-catenin degrada-

tion to allow accumulation of β-catenin in the cytoplasm and their subsequent translocation to the nucleus. Nuclear β-catenin thus interacts with T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors to activate the TCF target genes [13, 14]. In the noncanonicalWnt pathway which is not solidly defined, β-catenin appears to be dispensable. To summarize, activation of Wnt signaling by certain ligands may activate canonical signaling through β-catenin, through suppression of β-catenin proteolytic degradation and its nuclear translocation and retention [13, 14].

The regulation Wnt signaling is executed by a variety of different modulators including the family of Secreted Frizzled-Related Proteins (SFRPs) [15-18]. Till now, five human SFRPs have been detected in that all these SFRPs contain a CRDhomologous to the Frizzled CRD for Wnt ligand binding [15-18]. SFRP proteins have been shown to inhibit activation of canonical Wnt signaling. SFRPs are found to downregulate in several cancers, which are often indicators of poorprognosis [15-18]. Indeed, in recent years, accumulating evidence has supported SFRPs as tumor suppressors, since their expression is often silenced in cancer by promoter hypermethylation [15-18]. However, whether the methylation of SFRP2, a member of SFRPs, may be involved in the pathogenesis of melanoma is not known.

Here we investigated the expression levels of SFRP2 in melanoma specimens and malignant melanoma cell lines, HTB63, A2058 and A375, compared to the non-transformed melanocyte cell line, Hermes 3A. The demethylation of SFRP2 gene was induced by 5'-aza-deoxycytidine (5-aza-dCyd) in melanoma cell lines, and the effects on at levels of SFRP2 expression, as well as suppressed cell invasion, cellular β-catenin location, were analyzed.

Materials and methods

Patient tissue specimens

Thirty melanoma patients (male 17, female 13; aged 32 to 45) were included in the current study. The specimens from these patients were histologically diagnosed at the First Affiliated Hospital of Chongqing Medical University from 2010 to 2014. For the use of these clinical materials for research purposes, prior patient's consents and approval from the Institutional Research Ethics Committee were obtained.

Cell culture

The malignant melanoma cell lines A375, A2058 and HTB63, and a non-transformed melanocyte cell line Hermes 3A, were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and grown in 5% CO_2 at 37°C in a humidified atmosphere. The culture media were RPMI 1640 medium supplemented with 10% fetal bovine serum, 105 U/L penicillin, 100 mg/L streptomycin, 2 mmol/l glutamine, 7.5 μg/ml phenol red, 200 nmol/l TPA, 200 pmol/l Cholera toxin, 10 nmol/l Endothelin-1, and 10 ng/ml human stem cell factor (R&D systems, Minneapolis, MN, USA). The cells were regularly analyzed for the absence of mycoplasma contamination.

Transwell cell invasion assay

The transwell cell invasion assay was performed using a Fluorometric Cell Migration Assay kit with polycarbonate membrane inserts (5-μm pore size; Cell Biolabs, San Diego, CA, USA). Cells were serum-starved overnight in DMEM prior to initiation of the experiment. Cells were then incubated at 37°C for 24 hours to allow cell migration through the membrane. Migratory cells were detached from the underside of the membrane and subsequently lysed and detected by CyQuant GR dye (Invitrogen). Fluorescence measurement was performed in a FluoStar Optima fluorescence plate reader with a 485/520 nm filter set.

Quantitative PCR (RT-qPCR)

Total RNA was extracted using RNeasy kit (Qiagen, Hilden, Germany). For cDNA synthesis, complementary DNA (cDNA) was randomly primed from 2 μg of total RNA using the Omniscript reverse transcription kit (Qiagen). RT-qPCR was subsequently performed in triplicate with a 1:4 dilution of cDNA using the Quantitect SyBr green PCR system (Qiagen). All primers were purchased from Qiagen. Data were collected and analyzed using $2^{-\Delta\Delta Ct}$ method. Values of genes were first normalized against α-tubulin, and then compared to the experimental controls.

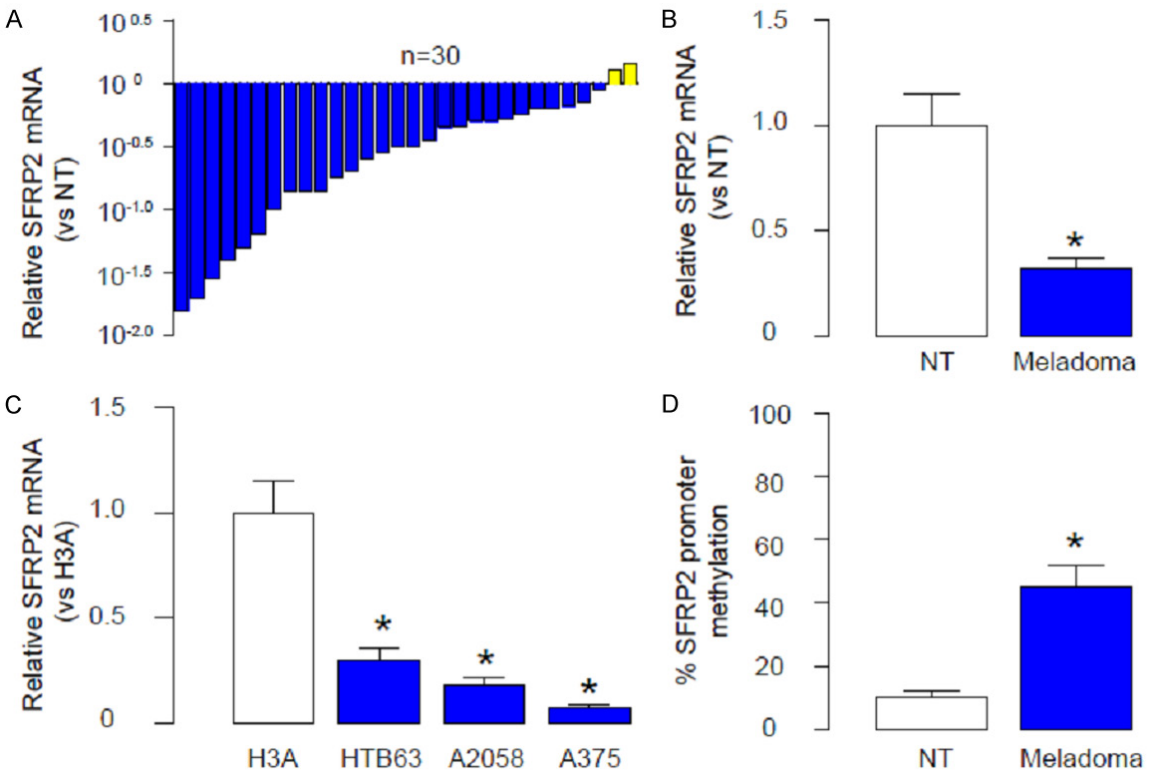

Figure 1. Decreased SFRP2 expression and increased SFRP2 gene methylation are detected in melanoma specimens. (A, B) SFRP2 mRNA levels were analyzed in malignant melanoma tumors as compared to the paired adjacent non-tumor tissue (NT), shown by individual levels (A), and by mean ± SD (B). (C) SFRP2 mRNA levels were analyzed in the malignant melanoma cell lines, HTB63, A2058 and A375, and a non-transformed melanocyte cell line, Hermes 3A (H3A). (D) The quantification of SFRP2 gene methylation by BS in specimens. *p<0.05, N=30.

Western blot

Total protein was extracted and homogenized in RIPA lysis buffer (1% NP40, 0.1% SDS, 100 μg/ml phenylmethylsulfonyl fluoride, 0.5% sodium deoxycholate, in PBS) on ice. The supernatants were collected after centrifugation at 12000× g at 4°C for 20 min. Protein concentration was determined using a BCA protein assay kit (Bio-rad, China), and whole lysates were mixed with 4×SDS loading buffer (125 mmol/l Tris-HCl, 4% SDS, 20% glycerol, 100 mmol/l DTT, and 0.2% bromophenol blue) at a ratio of 1:3. Protein samples were heated at 100°C for 5 min and were separated on SDS-polyacrylamide gels. The separated proteins were then transferred to a PVDF membrane. The membrane blots were first probed with a primary antibody. After incubation with horseradish peroxidase-conjugated second antibody, autoradiograms were prepared using the enhanced chemiluminescent system to visualize the protein antigen. The signals were recorded using X-ray film. Nuclear and cytosol proteins were isolated with Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific, Rockford, IL, USA). Primary antibodies were rabbit anti-SFRP2 (Abcam, Cambridge, MA, USA), anti-β-catenin (Sigma-aldrich), and anti-α-tubulin (Cell Signaling, San Jose, CA, USA). α-tubulin was used as protein loading controls. Secondary antibody is HRP-conjugated anti-rabbit (Jackson ImmunoResearch Labs, West Grove, PA, USA). Images shown in the figures were representative from 5 individuals. Densitometry of Western blots was quantified with NIH ImageJ software (Bethesda, MA, USA). The protein levels were first normalized to α-tubulin, and then normalized to experimental controls.

Methylation-specific polymerase chain reaction (MSP) analysis

Methylation-specific primers were designed based on the promoter sequence of SFRP2, with 5'-GGGTCGGAGTTTTTCGGAGTTGCGC-3' as the forward primer and 5'-CCGCTCTCTTCGCTAAATAGACTCG-3' as the reverse primer, with a PCR

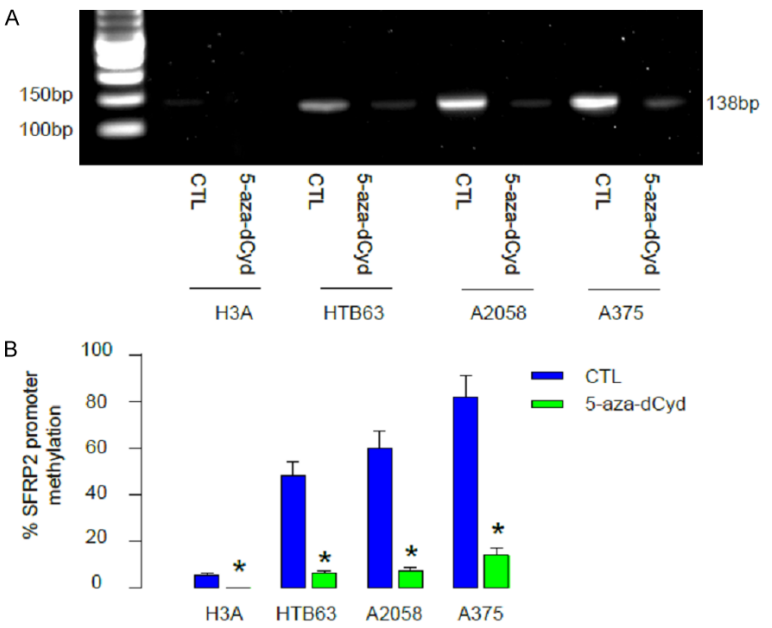

Figure 2. Demethylation of SFRP2 gene is induced by 5-aza-dCyd in melanoma cells. We used 5'-aza-deoxycytidine (5-aza-dCyd) to treat malignant melanoma cell lines, HTB63, A2058 and A375, and the non-transformed melanocyte cell line, Hermes 3A (H3A). (A, B) The methylation-specific polymerase chain reaction (MSP) was done, shown by representative MSP gels (A), and by quantification (B). *p<0.05, N=5.

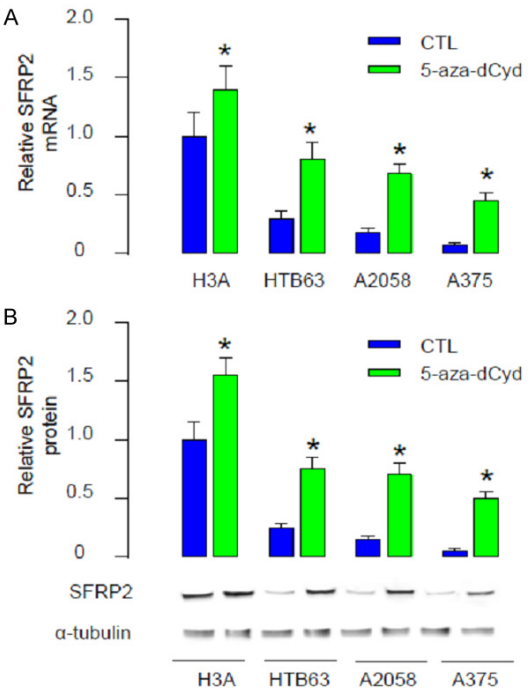

Figure 3. Demethylation of SFRP2 gene restores SFRP2 expression in melanoma cells. We used 5'-aza-deoxycytidine (5-aza-dCyd) to treat malignant melanoma cell lines, HTB63, A2058 and A375, and the non-transformed melanocyte cell line, Hermes 3A (H3A). (A, B) The SFRP2 expression was analyzed, at mRNA (A) and protein (B) levels. *p<0.05. N=5.

product of 138 bp. The sequences 5'-TTTTGGGTTGGAGTTTTTTGGAGTTGTGT-3' and 5'-AACCCACTCTCTTCACTAAATACAACTCA-3' were used for the forward and reverse non-methylation-specific primers, respectively, with a PCR product of 145 bp. The following thermal cycling conditions were used: initial denaturation at 95°C for 5 min; 35 cycles of denaturation at 94°C for 45 sec, annealing at 50°C for 30 sec and extension at 72°C for 45 sec; final extension at 72°C for 10 min. The 138-bp MSP product was isolated using electrophoresis in a 1.5% agarose gel and analyzed using an ultraviolet (UV) gel imaging system (Image-Quant 350; GE Healthcare Co., Little Chalfont, UK).

Bisulfite sequencing (BS) analysis of CTGF promoter methylation

BS primers for the CTGF promoter region were designed to avoid methylated CpGs. Once the DNA sample treated with sodium bisulfite was fully sulfonated, the BS product was amplified with forward: 5'-TTGTTTGTAAGGTAATTATTAG-3' and outside reverse 5'-ATTTTCTTAACCTTTTTT-ATAC-3' and inside reverse: 5'-AAACAAAAAAA-AAACCAAAC-3', with a product size of about 200 bp. The polymerase chain reaction (PCR) thermal cycling conditions were: initial denaturation at 95°C for 5 min; 35 cycles of denaturation at 94°C for 45 sec, annealing at 52°C for 30 sec and extension at 72°C for 30 sec; final extension at 72°C for 10 min. The 200-bp amplification product was isolated using electrophoresis in a 1.5% agarose gel and visualized under UV light. A 10 μl aliquot of the PCR product was subjected to further sequencing by the Beijing Genomics Institute (Beijing, China). The BS primer amplification products from the samples of the three groups were compared with completely sulfonated promoter target sequences using the JellyFish 1.3 data application software (Field Scientific, LCC, Lewisburg, PA, USA). The methylation level was calculated as: (mC/C-G) x 100%.

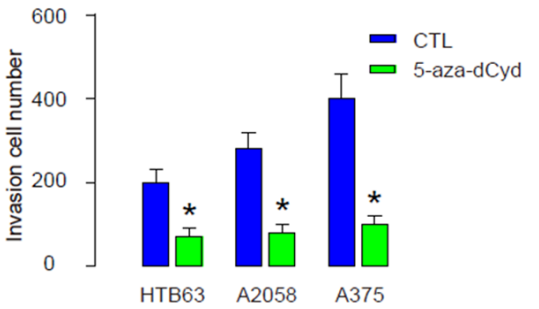

Figure 4. Demethylation of SFRP2 gene suppresses melanoma cell invasion. We used 5'-aza-deoxycytidine (5-aza-dCyd) to treat malignant melanoma cell lines, HTB63, A2058 and A375. The cell invasion was analyzed in a transwell cell invasion assay. *p<0.05, N=5.

Statistics

All statistical analyses were carried out using the GraphPad Prism 6.0 statistical software (GraphPad Software, Inc. La Jolla, CA, USA). All values in cell and animal studies are depicted as mean ± standard deviation and are considered significant if $p < 0.05$. All data were statistically analyzed using one-way ANOVA with a Bonferroni correction, followed by Fisher' Exact Test for comparison of two groups. Bivariate correlations were calculated by Spearman's Rank Correlation Coefficients.

Results

Decreased SFRP2 expression and increased SFRP2 gene methylation are detected in melanoma specimens

Wee investigated the expression levels of SFRP2 in melanoma specimens. We found that SFRP2 mRNA was significantly decreased in malignant melanoma tumors as compared to the paired adjacent non-tumor tissue (NT), shown by individual levels (**Figure 1A**), and by mean ± SD (**Figure 1B**). Moreover, SFRP2 expression was significantly decreased in the malignant melanoma cell lines, HTB63, A2058 and A375, but not in the non-transformed melanocyte cell line, Hermes 3A (**Figure 1C**). Next, we checked methylation of SFRP2 gene in malignant melanoma specimens, and found that SFRP2 gene methylation was significantly increased in malignant melanoma tumors as compared to NT (**Figure 1D**).

Demethylation of SFRP2 gene is induced by 5-aza-dCyd in melanoma cells

Then, we used 5'-aza-deoxycytidine (5-aza-dCyd) to treat malignant melanoma cell lines, HTB63, A2058 and A375, and the non-transformed melanocyte cell line, Hermes 3A. We found that demethylation of SFRP2 gene is efficiently induced by 5-aza-dCyd in melanoma cells, shown by representative methylation-specific polymerase chain reaction (MSP) gels (**Figure 2A**), and by quantification (**Figure 2B**).

Demethylation of SFRP2 gene restores SFRP2 expression in melanoma cells

We found that demethylation of SFRP2 gene by 5-aza-dCyd in melanoma cell lines HTB63, A2058 and A375, and in non-transformed melanocyte cell line Hermes 3A, restored SFRP2 expression, at both mRNA (**Figure 3A**) and protein (**Figure 3B**) levels. Thus, demethylation of SFRP2 gene restores SFRP2 expression in melanoma cells.

Demethylation of SFRP2 gene suppresses melanoma cell invasion

Moreover, in a transwell cell invasion assay, we found that demethylation of SFRP2 gene by 5-aza-dCyd in melanoma cell lines HTB63, A2058 and A375 significantly suppressed cell invasion (**Figure 4**). Thus, demethylation of SFRP2 gene suppresses melanoma cell invasion.

Demethylation of SFRP2 gene suppresses nuclear retention of β-catenin in melanoma cells

Finally, we aimed to analyze the effects of SFRP2 gene demethylation on the activation of canonical Wnt signaling. Then we isolated nuclear protein vs cytosol protein from the 5-aza-dCyd-treated melanoma cells. We found that demethylation of SFRP2 gene suppressed nuclear retention of β-catenin in melanoma cells, shown by representative blots (**Figure 5A**), and by quantification of the nuclear vs cytosol β-catenin ratio (**Figure 5B**). Together, based on our data in the current study, we proposed that the demethylation of SFRP2 gene may inhibit nuclear retention of the key Wnt signaling factor, β-catenin, in melanoma cell lines, to facilitate cell invasion. SFRP2 may thus func-

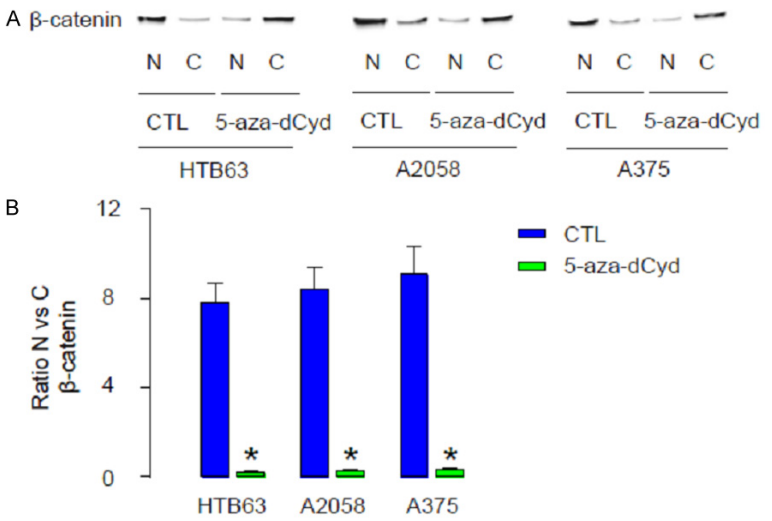

Figure 5. Demethylation of SFRP2 gene suppresses nuclear retention of β-catenin in melanoma cells. We analyze the effects of SFRP2 gene demethylation on the activation of canonical Wnt signaling. (A, B) The nuclear protein vs cytosol protein from the 5-aza-dCyd-treated melanoma cells were analyzed for β-catenin in melanoma cells HTB63, A2058 and A375, shown by representative blots (A), and by quantification of the nuclear vs cytosol β-catenin ratio (B). *p<0.05, N=5.

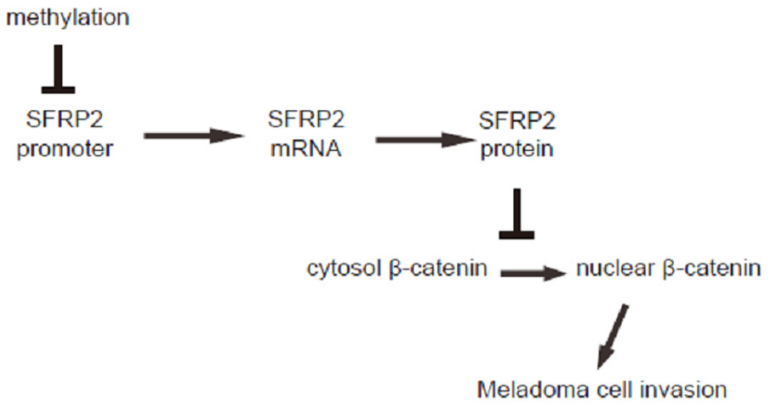

Figure 6. Schematic of the model. The demethylation of SFRP2 gene may inhibit nuclear retention of the key Wnt signaling factor, β-catenin, in melanoma cell lines, to facilitate cell invasion. SFRP2 may thus function as a melanoma invasion suppressor by interfering with Wnt signaling, and the methylation of SFRP2 gene may promote pathogenesis of melanoma.

tion as a melanoma invasion suppressor by interfering with Wnt signaling, and the methylation of SFRP2 gene may promote pathogenesis of melanoma (**Figure 6**).

Discussion

The SFRP2 plays a pivotal role in the Wnt pathway, and mainly functions as an antagonist of activation of Wnt signaling [19, 20]. Previous studies have shown that SFRP2 is a Wnt inhibitor whose promoter CpGs were hypermethylated at high frequency in colorectal cancers (CRCs) [21-27]. Indeed, the methylation of SFRP2 has been mainly investigated in CRCs, and the pattern of SFRP2 methylation appears to differ throughout the promoter during progressive tumorigenesis, showing that extensive methylation of the SFRP2 promoter was present primarily in CRCs [21-27]. However, a role of SFRP2 gene methylation in the tumorigenesis of melanoma is ill-defined.

In the current study, we investigated the expression levels of SFRP2 in melanoma specimens. We found that SFRP2 mRNA was significantly decreased, and this downregulation of SFRP2 may result from the several possible reasons, including promoter methylation. Hence, we analyzed promoter methylation of SFRP2 in patients' specimens, and the results confirmed our hypothesis. Since the SFRP2 expression was also significantly decreased in the malignant melanoma cell lines, HTB63, A2058 and A375, but not in the non-transformed melanocyte cell line, Hermes 3A, these data suggest that SFRP2 gene promoter may exist in a relative low methylation state in normal skin cells, while significantly methylated when the cells undergo phenotypic changes towards malignant melanoma. Hence, DNA methylation appears to be a regulatory mechanism of SFRP2 expression, possibly contributing to the regulation of Wnt signaling, and the pathogenesis of melanoma. Understanding the role of the demethylation of the SFRP2 promoter in the development of melanoma may lead to the identification of novel strategies and/or additional therapeutic tar-

gets for the prevention and treatment of malignant melanoma.

Based on these clinical findings, we were prompted to analyze the effects of demethylation of SFRP2 on the tumor cell biology. The demethylation of SFRP2 gene by 5-aza-dCyd in melanoma cell lines restored SFRP2 expression, at both mRNA and protein levels, resulting in a suppression of cell invasion. We also examined cell survival and growth, using an MTT and CCK-8 assay, but we did not find any alterations of these parameters by SFRP2 gene demethylation. These negative data were not shown in the result part. Thus, our data suggest that the effects of SFRP2 on melanoma cells may be mainly on cell invasion, rather than cell proliferation and survival.

Furthermore, the demethylation of SFRP2 gene appeared to inhibit nuclear retention of a key Wnt signaling factor, β-catenin, in melanoma cell lines. Hence, the regulation of SFRP2 gene methylation directly regulated canonical pathway of Wnt signaling. Together with a pivotal role of Wnt signaling in the tumorigenesis of melanoma, our study suggests that SFRP2 may function as a melanoma invasion suppressor by interfering with Wnt signaling, and the methylation of SFRP2 gene may promote pathogenesis of melanoma.

The present study had several limitations. The current molecular mechanism analyses were performed only in vitro. The analyses on an animal model may further provide evidence of a role of SFRP2 promoter methylation in the carcinogenesis of melanoma. In addition, despite the fact that previous studies and the current work have indicated that methylation is involved in the regulation of SFRP2 expression in malignant melanoma cells, the precise mechanisms underlying the control of SFRP2 gene promoter methylation still requires further investigation.

Acknowledgements

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Disclosure of conflict of interest

None.

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