5-Aza-dC suppresses melanoma progression by inhibiting GAS5 hypermethylation

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Abstract. The in-depth study of melanoma pathogenesis has revealed that epigenetic modifications, particularly DNA methylation, is a universal inherent feature of the development and progression of melanoma. In the present study, the analysis of the tumor suppressor gene growth arrest-specific transcript 5 (GAS5) demonstrated that its expression was downregulated in melanoma, and its expression level had a certain negative association with its methylation modification level. The promoter of GAS5 presented with detectable CpG islands, and methylation-specific polymerase chain reaction analysis demonstrated that GAS5 was actually modified by methylation in melanoma tissues and cells; however, no methylation modification of GAS5 was detected in normal tissues. Following the treatment of melanoma cells with 5-Aza-2'-deoxycytidine (5-Aza-dC), GAS5 methylation was significantly reversed. The analysis of melanoma cell proliferation revealed that 5-Aza-dC inhibited A375 and SK-MEL-110 cell proliferation in a time-dependent manner. Further analysis of apoptosis demonstrated that 5-Aza-dC significantly increased the apoptosis level of the two cell lines. Moreover, migration analysis of melanoma cells revealed that 5-Aza-dC significantly reduced cell migration. Furthermore, 5-Aza-dC significantly decreased the invasive ability of the two cell lines. However, when the expression of GAS5 was silenced, the effects of 5-Aza-dC on cell proliferation, apoptosis, invasion and migration were not significant. Furthermore, the subcutaneous injection of A375 cells in nude mice successfully resulted in xenograft tumor formation. However, following an intraperitoneal injection of 5-Aza-dC, the volume and weight of xenograft tumors and Ki-67 expression were significantly reduced, and caspase-3 activity and GAS5 expression were enhanced; following the silencing of GAS5, the antitumor effect of 5-Aza-dC was significantly blocked. On the whole, the present study demonstrates that 5-Aza-dC inhibits the growth of melanoma, and its function may be related to the methylation modification of GAS5.

Introduction

In recent years, the incidence of malignant melanoma has been increasing rapidly worldwide, with an annual growth rate of 3-5%, and has become one of the most rapidly growing tumors (1). Malignant melanoma is associated with a poor prognosis and a high mortality rate; there are also limited treatment options available (2). At present, although research progress has been made as regards the pathogenesis of malignant melanoma, the exact molecular mechanisms of malignant melanoma remain to be fully elucidated.

It has been recently reported that the occurrence of melanoma may not only be dependent on genetic factors, but may be also affected by epigenetic modifications, particularly DNA methylation (3). CpG island methylation of tumor suppressor gene promoters has been proven to be related to the occurrence and progression of multiple human malignant tumors, and can also be used as a biomarker to evaluate the prognosis of melanoma (4). For example, Tanemura *et al* (5) analyzed the CpG island methylation status of tumor-related gene promoter region and revealed that the hypermethylation of RASSF1A, WIF1, SOCS1 and TFPI2 was increased with the progression

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of malignant melanoma clinical stage, with MINT31 methylation in stage III melanoma being related to disease prognosis. Therefore, the further understanding of melanoma-related DNA methylation genes may prove to be of utmost importance for elucidating the regulatory mechanisms of gene expression in melanoma and melanoma occurrence and development.

In addition, long non-coding RNAs (lncRNAs) play a crucial regulatory role in cell physiological activities, and their abnormal expression has been closely related to the occurrence and development of tumors (6). Among lncRNAs, growth arrest-specific transcript 5 (GAS5) is a lncRNA with 651 nucleotides that is expressed at low levels in a variety of tumors. The GAS5 expression level has been shown to be closely related to the clinicopathological characteristics of tumors and patient prognosis, and GAS5 overexpression has been reported to inhibit the growth of metastasized tumors *in vivo* (7,8). Studies have demonstrated that lncRNAs may be also affected by DNA methylation (9,10). For example, Cheng *et al* (10) previously revealed that nine lncRNA methylated genes were significantly related to the overall survival of patients with glioma, which could be used to predict the risk of glioma patients. Although the expression of GAS5 has been reported to be downregulated in melanoma (8), it has not been specified whether this may be caused by methylation modification.

Gene silencing caused by DNA methylation has been reported to be important for tumor occurrence and development, and demethylation agents can restore the expression of silenced genes and continue to exert their antitumor biological effects (11). 5-Aza-2'-deoxycytidine (5-Aza-dC) is currently known as a markedly effective demethylating agent that can block cell cycle, induce apoptosis, promote differentiation, reduce invasion and metastasis, and inhibit tumor cell growth (12). However, there are few studies on 5-Aza-dC in melanoma (13,14). Therefore, present study aimed to investigate GAS5 methylation levels in melanoma and to confirm the role of 5-Aza-dC in melanoma, in order to provide therapeutic targets and diagnostic methods for the treatment of melanoma.

Materials and methods

Patient samples and bioinformatics analysis. Fresh-frozen and paraffin-embedded tissues from patients diagnosed with melanoma (n=8) at Yunnan Cancer Hospital were analyzed. Fresh-frozen biopsy specimens from the nevus of eight individuals were used as control samples. All skin biopsy specimens contained at least 70% of the cells as normal or melanoma cells. The present study was approved by the Ethics Committee of Yunnan Cancer Hospital, the Third Affiliated Hospital of Kunming Medical University (KY201939). Written informed consent was obtained from each patient included in this study. The analysis of GAS5 expression level was derived from The Cancer Genome Atlas (TCGA; https://portal.gdc.cancer.gov/) database, and the correlation between GAS5 methylation and expression was downloaded from the MEXPRESS (https://mexpress.be/) database.

Cells and cell culture. Human melanoma cell line A375 (SCSP-533) was purchased from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences, and SK-MEL-110 was provided by the authors' laboratory. The cells were cultured in Dulbecco's modified Eagle medium (DMEM; Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin (Gibco; Thermo Fisher Scientific, Inc.) and 100 μg/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.). All cells were maintained at 37˚C in an incubator with 5% (v/v) CO_2. Cells were incubated with or without 20 μM 5-Aza-dC (Sigma-Adrich; Merck KGaA) for 48 h as previously described (15).

Transfection. GAS5 siRNA (5'-CUUGCCUGGACCAGC UUAATT-3') and negative control (NC) siRNA (5'-UUCUCC GAACGUGUCACGUTT-3') were synthesized by Guangzhou RiboBio Co., Ltd. A total quantity of 50 nM GAS5 siRNA or NC siRNA was transfected into melanoma cells at 37˚C using Lipofectamine 3000® (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. siRNA and transfection reagent were mixed and incubated for 20 min at room temperature, and then added to the cells. Following 4 h of culture, the cells were replaced with fresh medium to continue culture. Subsequently, the GAS5 expression level was analyzed at 24 h post-transfection.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) from melanoma cells or tissues, and mRNA expression levels were analyzed using a One Step TB Green PrimeScript RT-PCR kit (Takara Biotechnology Co., Ltd.) according to the manufacturer's instructions. The reactions were performed as followed: 5 min at 42˚C, 10 at 95˚C, and 30 cycles of 5 sec at 95˚C, 30 sec at 60˚C. The primer sequences used were as follows: GAS5 forward, 5'-GCACAC AGGCATTAGACAGA-3' and reverse, 5'-AAGCCGACTCTC CATACCTT-3'; U6 (used as the internal control) forward, 5'-CTCGCTTCGGCAGCACA-3' and reverse, 5'-AACGCT TCACGAATTTGCGT-3'. The relative quantification value of RNA was calculated by applying the $2^{-\Delta\Delta Cq}$ method (16).

Genomic DNA extraction. Melanoma cells were collected by centrifugation (8,000 x g) at room temperature for 5 min, and genomic DNA was extracted with the Wizard Genomic DNA Purification kit (Promega Corporation). Cell precipitates were then lysed with 600 μl nuclei lysis solution (included with the kit), and then incubated at 65˚C for 15 min. RNase A (3 μl) was added to the cell lysate and incubated at 37˚C for 15 min, and subsequently, 200 μl protein precipitation solution (included with the kit) were added. Following centrifugation at 14,000 x g for 4 min at room temperature, the supernatant was pipetted into an EP tube and 600 μl isopropanol (Sigma-Adrich; Merck KGaA) was added. Following centrifugation at 14,000 x g for 1 min at room temperature, 600 μl of 70% ethanol were added to wash the precipitate. Finally, following centrifugation at 14,000 x g for 2 min at room temperature for ethanol removal, 30 μl of DNA rehydration solution (included with the kit) were added, in order to dissolve DNA.

Bisulfite modification of DNA. DNA methylation levels were detected using the EpiTect Bisulfite kit (Qiagen GmbH). A total DNA quantity of 500 ng was added to the Bisulfite Mix

and DNA Protect Buffer, and then place into a PCR thermal cycler for bisulfite modification. The conversion conditions were applied as follows: 95˚C, 5 min; 60˚C, 25 min; 95˚C, 5 min; 60˚C, 85 min; 95˚C, 5 min; 60˚C, 175 min; 20˚C storage. This modification resulted in the conversion of unmethylated cytosine residues into uracil, while the methylated cytosines remained unchanged. Following bisulfite conversion, 560 μl Buffer BL were added to the above reaction solution, and all solutions were added to the EpiTect spin column. Subsequently, 500 μl washing Buffer BW was added following centrifugation at 14,000 x g for 1 min at room temperature, and then 500 μl of Buffer BD was added to incubate for 15 min. Following centrifugation at 14,000 x g for 1 min at room temperature, the EpiTect spin column was washed twice with Buffer BW and then placed into a new Eppendorf tube. Buffer EB (20 μl) was added, to dissolve the converted DNA. Following centrifugation at 14,000 x g for 2 min at room temperature, the converted DNA was stored at -20˚C for later use.

Methylation-specific polymerase chain reaction (MSP). The methylation levels of the CpG-rich region in the GAS5 promoter were analyzed using MSP. Firstly, an online program MethPrimer (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi) dedicated to methylation analysis was used to predict the CpG islands of GAS5 promoter. When the sequence of GAS5 promoter region was input, MethPrimer could automatically analyze the possible CpG islands and design usable primers. The primer pair sequences used were as follows: methylated-sense, 5'-GTTGGAATGTAGTGGTTCGATA-3', and methylated-antisense, 5'-GCCAACATAATAAAACCCCGT-3'; unmethylated-sense, 5'-AGGTTGGAATGTAGTGGTTT-3', and unmethylated-antisense, 5'-ACCAACATAATAAAACCCCATCT-3'. The methylated primers amplified methylation-specific PCR products (M), when CpG sites were methylated, whereas unmethylation-specific PCR product (U) was present when CpG sites were unmethylated. When the sites were partially methylated, M and U bands were simultaneously present. Ex Taq HS (Takara Biotechnology Co., Ltd.) was used for PCR amplification and the reactions were performed as follows: 5 min at 95˚C, and 30 cycles of 30 sec at 95˚C, 30 sec at 60˚C, 40 sec at 72˚C.

Cell counting kit-8 (CCK-8) assay. The concentration of melanoma cells was adjusted to 1x10^5 cells/ml, and 100 μl cells per well were seeded onto 96-well plates. Subsequently, 10 μl CCK-8 solution (Dojindo Laboratories, Inc.) were added at various time points (0, 24, 48 and 72 h). Following incubation for 4 h at 37˚C, data were read using a microplate reader (multiscan MK3; Thermo Fisher Scientific, Inc.) at a wavelength of 450 nm.

Apoptosis analysis. Culture supernatants of each group were collected and placed on ice. Following trypsinization for 2 min, the cells were resuspended in the culture supernatant collected above, and the cell density was then adjusted to ~1x10^6 cells/ml. Subsequently, 1.25 μl Annexin V-FITC (Nanjing KeyGen Biotech Co., Ltd.) were added into 0.5 ml of the above cell suspension, and the reaction was kept away from light at room temperature (18-24˚C) for 15 min. The supernatant was then removed by centrifugation at 1,000 x g for 5 min at room temperature. The cells were gently resuspended with 0.5 ml of pre-cooled 1X binding buffer, and then incubated with 10 μl propidium iodide (Nanjing KeyGen Biotech Co., Ltd.) in the dark at 4˚C for 15 min. Finally, a flow cytometer BD FACScalibur (BD Biosciences) was used to analyze cell apoptosis and data analysis was performed using FlowJo V10 software.

Cell migration assay. A total quantity of 1x10^5 cells were resuspended in 100 μl serum-free DMEM, and then added to the upper Transwell chamber (BD Biosciences) of Transwell inserts (8-μm pore size, BD Biosciences), while 600 μl complete DMEM were added to the lower chamber. After the cells were cultured for 12-48 h, the cells in the upper chamber were removed by using cotton swabs. The migratory cells were fixed with 4% paraformaldehyde for 20 min, washed with PBS, then stained with crystal violet (Sigma-Adrich; Merck KGaA) for 10 min at room temperature, and photographed using a Leica DM4000B microscope (Leica Microsystems GmbH), for subsequent experimental result statistical analysis.

Cell invasion. For cell invasion assay, 40 μl Matrigel (BD Biosciences) were added into the pre-cooled upper Transwell chamber of Transwell inserts (8-μm pore size), and following coagulation, serum-free DMEM was added to balance overnight. Afterwards, 100 μl serum-free DMEM containing 1x10^5 cells were added to the upper chamber, and 600 μl complete DMEM was added to the lower chamber. The following experimental steps were carried out according to the detection method previously described for cell migration evaluation.

Nude mouse tumorigenicity assay and immunohistochemistry. A total of 16 male BALB/c nude mice (SPF grade, 4-5 weeks old, weighing 18-20 g) were purchased from the Experimental Animal Center of Guangzhou University of Chinese Medicine. All mice were housed (21-25˚C with 45-55% humidity) at a 12-h light-dark cycle and provided with food and water *ad libitum*. A A375 cell suspension (0.1 ml/mouse, containing 5x10^6 cells) was injected subcutaneously into nude mice. After 1 week, a subcutaneous tumor mass appeared. Subsequently, 5-Aza-dC was injected intraperitoneally at a dose of 3 mg/kg for 3 consecutive weeks as previously described (17). Tumor size was measured twice a week, and the tumor size and weights were calculated and recorded. When the tumor volume reached ~500 mm^3, the mice were anesthetized using 3% sevoflurane, then euthanized by using CO_2 at flow rate of 30%/min for 7 min, followed by cervical dislocation, and tumors were excised on day 31. Xenograft tumors were fixed in formalin (10%) for 24 h at room temperature and then immunohistochemical evaluation was performed, using an antibody against Ki-67 (1:200; cat. no. ab15580, Abcam). Dense tumor segments (~4 μm) were blocked with 3% bovine serum albumin (BSA; Beyotime Institute of Biotechnology) for 30 min at room temperature, and incubated with Ki-67 antibody (0.5 μg/ml) overnight at 4˚C. Subsequently, 4-μm-thick slices were incubated with goat anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody (1:100; cat. no. ab6789; Abcam) for

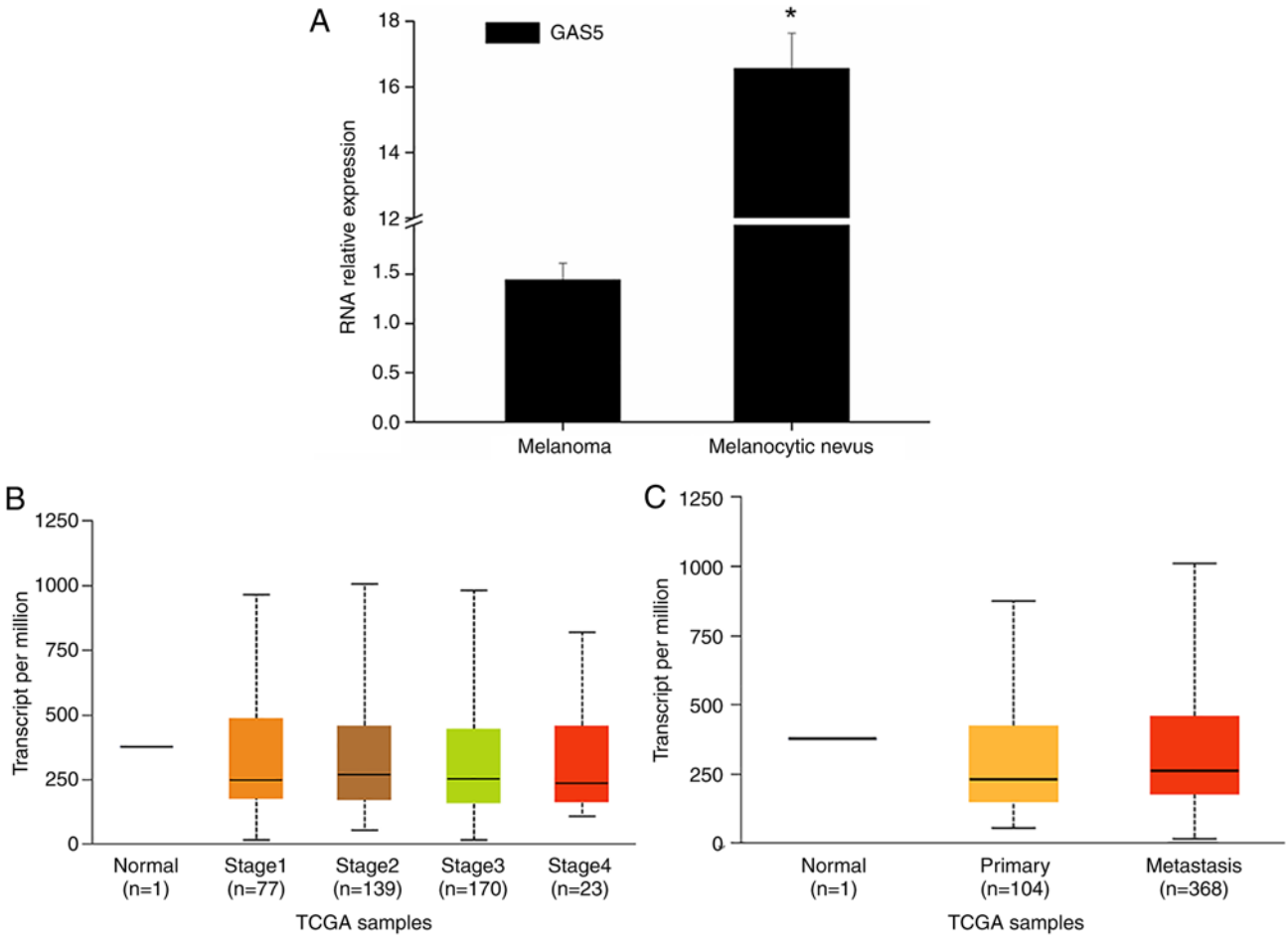

Figure 1. GAS5 expression levels in melanoma tissues. (A) The expression of GAS5 in melanoma and normal tissues was detected using reverse transcription-quantitative PCR. *P<0.05 vs. melanoma. (B and C) Data from (TCGA, https://portal.gdc.cancer.gov/) database demonstrated that GAS5 expression was significantly downregulated in melanoma. GAS5, growth arrest-specific transcript 5; TCGA, The Cancer Genome Atlas.

30 min at 37˚C. All sections were photographed and observed under an Eclipse TS100 microscope (Nikon Corporation). Caspase-3 activity was detected using a Caspase-3 Assay kit (cat. no. ab39401, Abcam) according to the manufacturer's instructions. The present study was approved by the Ethics Committee of Animal Research Institute of Yunnan Cancer Hospital.

Statistical analysis. The data are presented as the mean ± standard deviation (SD), and all the experiments were repeated at least three times. Statistical analysis was performed using SPSS 20.0 software (IBM Corp.) by applying an unpaired Student's t-test for comparisons between 2 groups, a two-way analysis of variance (ANOVA) to compare between groups or one-way ANOVA followed by Tukey's post-hoc test for mean separation including correction for multiple comparisons. P<0.05 was considered to indicate a statistically significant difference.

Results

GAS5 is hypermethylated in melanoma tissues and cells. GAS5 has been reported to be an important tumor suppressor gene in a variety of tumors (7); however, the association between GAS5 expression and methylation levels in melanoma has not been reported, at least to the best of our knowledge. In the present study, it was revealed that GAS5 expression in melanoma tissues was downregulated in general (Fig. 1A). TCGA data analysis also revealed that the expression of GAS5 was significantly downregulated in melanoma (Fig. 1B and C). At the same time, MEXPRESS data evaluation revealed that GAS5 expression and methylation levels in melanoma had a certain negative correlation (r=-0.094~-0.308; Fig. 2), indicating that the downregulation of GAS5 expression in melanoma may be related to the modification of methylation. The GAS5 promoter region exhibited evident CpG islands (Fig. 3A). The methylation level of GAS5 promoter detected using MSP revealed that GAS5 did have methylation modifications in melanoma tissues, since the methylated primers amplified methylation-specific PCR products (M); however, no GAS5 methylation was detected in normal tissues, as the band of unmethylation-specific PCR product (U) was present (Fig. 3B). The simultaneous detection of GAS5 methylation level in A375 and SK-MEL-110 melanoma cells also revealed that GAS5 was methylated, and the methylation of GAS5 was significantly reduced after 5-Aza-dC treatment (Fig. 3C). At the same time, 5-Aza-dC significantly upregulated the expression of GAS5, while GAS5 siRNA significantly inhibited the expression of GAS5 and blocked the expression-promoting effect of 5-Aza-dC (Fig. 4A and B). Therefore, the

Figure 2. Correlations between the GAS5 methylation and expression level in melanoma. The data from MEXPRESS (https://mexpress.be/) database demonstrated that the methylation of GAS5 had a certain negative correlation with its expression level (correlation coefficient r=-0.094~-0.308). The statistical analysis indicators (r and P-value) on the right demonstrate that the expression of GAS5 negatively correlated with the promoter methylation level of GAS5. *P<0.05, **P<0.01 and ***P<0.001 vs. normal control. GAS5, growth arrest-specific transcript 5.

downregulation of GAS5 expression in melanoma may be related to its methylation modification, and 5-Aza-dC may be able to reverse GAS5 methylation modification.

5-Aza-dC inhibits melanoma cell proliferation and promotes apoptosis. The evaluation of the effects of 5-Aza-dC on the proliferation of melanoma cells demonstrated that 5-Aza-dC inhibited the proliferation of A375 cells in a time-dependent manner (Fig. 4C) and similar results were also observed in another melanoma cell line, SK-MEL-110 (Fig. 4D). However, when the expression of GAS5 was silenced, the effect of 5-Aza-dC on cell proliferation was not significant compared to the control (Fig. 4C and D). The continued detection of cell apoptosis demonstrated that 5-Aza-dC significantly increased the apoptotic level of A375 cells (Fig. 4E and F), and also significantly enhanced the apoptosis of SK-MEL-110 cells (Fig. 4G and H). However, GAS5 siRNA attenuated the pro-apoptotic effects of 5-Aza-dC (Fig. 4G and H). Therefore, 5-Aza-dC significantly inhibited the proliferation of melanoma cells and promoted apoptosis by upregulating the expression of GAS5, indicating that 5-Aza-dC may present with an evident tumor suppressor function.

5-Aza-dC inhibits the migration and invasion of melanoma cells. Migration and invasion are the main biological characteristics of tumor cells. The evaluation of the effects of 5-Aza-dC on melanoma cell migration revealed that 5-Aza-dC significantly inhibited A375 cell migration (Fig. 5A and B), and similar results were also observed in the SK-MEL-110 cells (Fig. 5A and B). Following the addition of 5-Aza-dC, the migration of the cells was markedly inhibited (Fig. 5A and B). At the same time, the evaluation of cell invasion level revealed that 5-Aza-dC significantly inhibited A375 and SK-MEL-110 cell invasion levels (Fig. 5C and D). However, when the expression of GAS5 was silenced, the inhibitory effects of 5-Aza-dC on cell migration and invasion were significantly

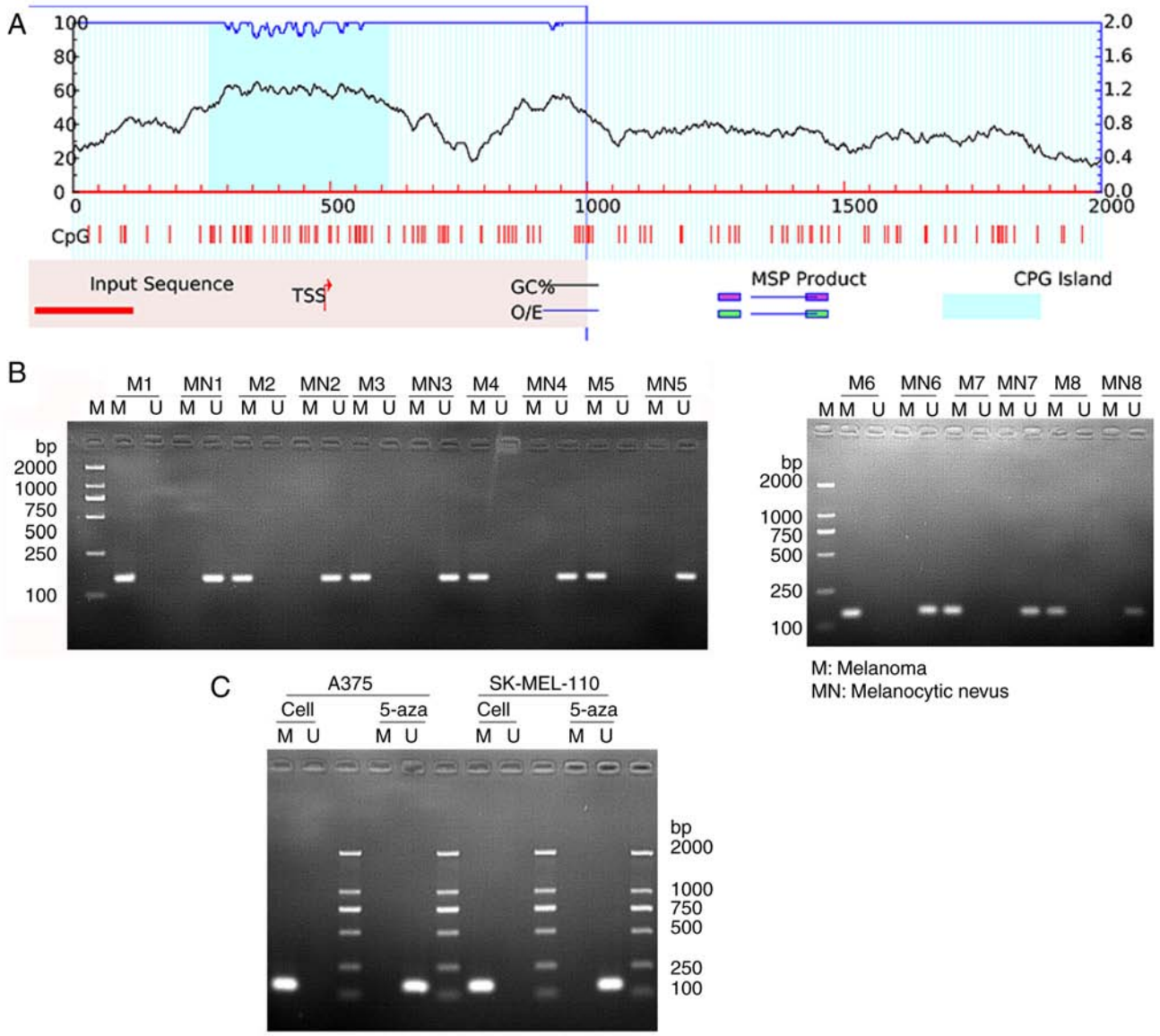

Figure 3. Detection of GAS5 promoter methylation levels in melanoma. (A) GAS5 promoter region had obvious CpG islands. (B) GAS5 promoter methylation levels in melanoma and normal tissues was detected by MSP. M, methylation-specific PCR products; U, unmethylation-specific PCR products. (C) Following melanoma cell treatment with 5-Aza-dC, the methylation level of GAS5 was detected using MSP. GAS5, growth arrest-specific transcript 5; MSP, methylation-specific polymerase chain reaction; 5-Aza-dC, 5-Aza-2'-deoxycytidine.

impeded (Fig. 5). Therefore, 5-Aza-dC may inhibit the migration and invasion of melanoma cells by upregulating the expression of GAS5.

5-Aza-dC inhibits the growth of melanoma xenografts. Although there have been several studies on the role of 5-Aza-dC in tumors (12-15), there are few *in vivo* studies on 5-Aza-dC and melanoma. Thus, the role of 5-Aza-dC *in vivo* remains to be elucidated. In the present study, the subcutaneous injection of A375 cells in nude mice successfully led to the formation of xenograft tumors. Following the intraperitoneal injection of 5-Aza-dC, although 5-Aza-dC had a certain effect on the body weight of nude mice (Fig. 6A), it significantly inhibited the growth of tumors, demonstrating that the volume and weight of xenograft tumors were significantly reduced (Fig. 6B-D). In addition, the expression of Ki-67 was significantly decreased (Fig. 6E and F), while caspase-3 activity and GAS5 expression were significantly increased (Fig. 6G and H) in the mice treated with 5-Aza-dC. However, when the expression of GAS5 was silenced, the antitumor effect of 5-Aza-dC was markedly inhibited (Fig. 6B-D) and the expression level of Ki-67 and caspase-3 activity were markedly restored to the control levels (Fig. 6E-G). Therefore, 5-Aza-dC may possibly inhibit the growth of melanoma xenografts *in vivo*, and its effect is closely related to GAS5 levels.

Discussion

Malignant melanoma is highly invasive and highly metastatic, and its morbidity and mortality have been continuously increasing (2). Therefore, the further exploration of malignant melanoma pathogenesis and the designation of novel therapeutic targets for the treatment of malignant melanoma is of utmost urgency.

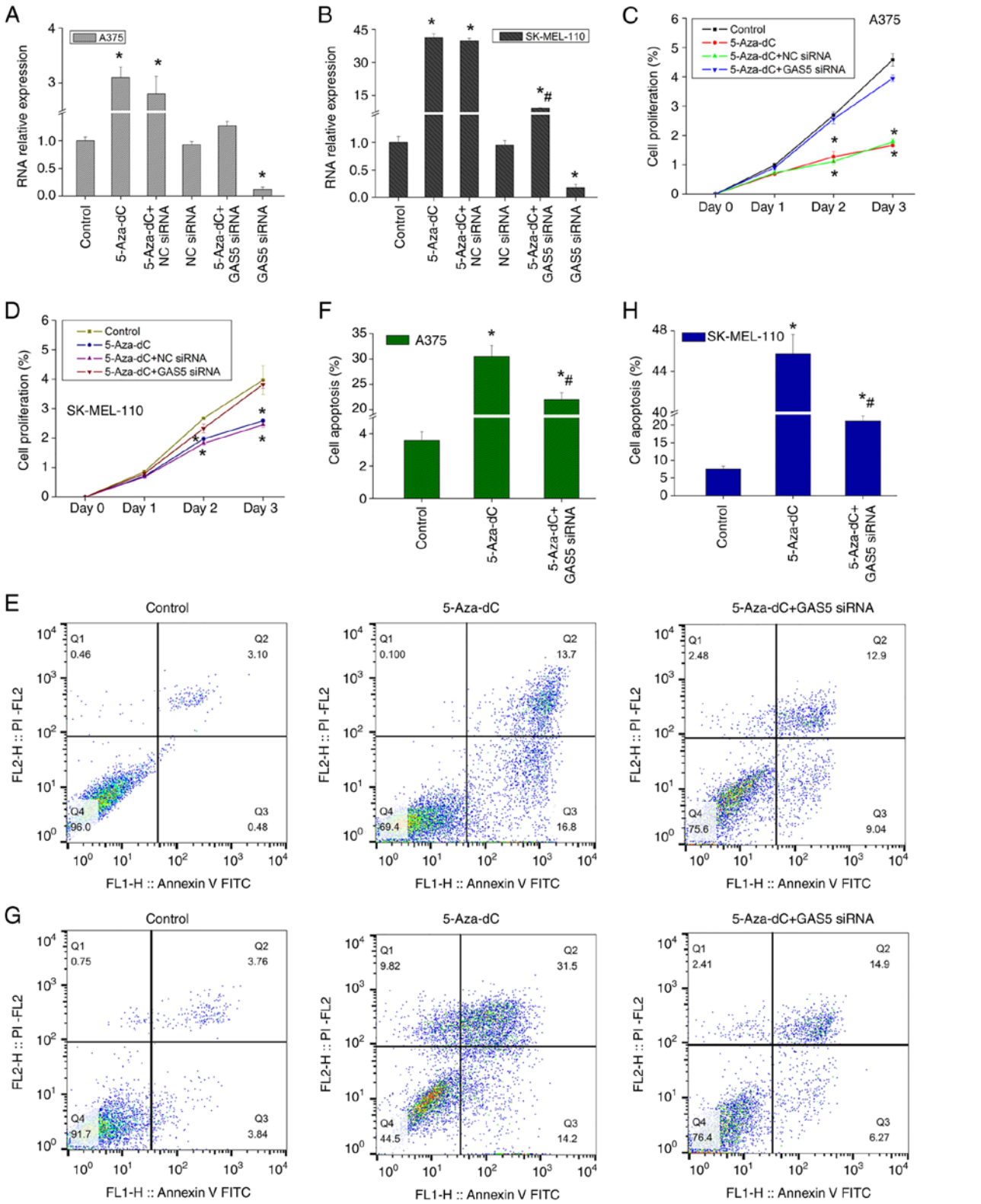

Figure 4. Regulatory effect of 5-Aza-dC on melanoma cell proliferation and apoptosis. Melanoma cells were transfected with NC or GAS5 siRNA and then treated with 5-Aza-dC. (A and B) GAS5 expression in A375 and SK-MEL-110 cells was detected using reverse transcription-quantitative PCR. (C and D) Cell proliferation was detected using CCK-8 assay. (E and F) Detection of apoptosis using flow cytometry in A375 cell. (G and H) Detection of apoptosis using flow cytometry in SK-MEL-110 cell. *P<0.05 vs. control, #P<0.05 vs. 5-Aza-dC. 5-Aza-dC, 5-Aza-2'-deoxycytidine; NC, negative control; GAS5, growth arrest-specific transcript 5.

GAS5 is an important tumor suppressor gene, playing a crucial role by regulating a variety of intracellular biological processes, including growth arrest, apoptosis, proliferation, metastasis, and DNA damage repair (7). For example, Chen *et al* (18) revealed that the downregulation of GAS5 may increase cyclin D1, CDK4, p27, NADPH

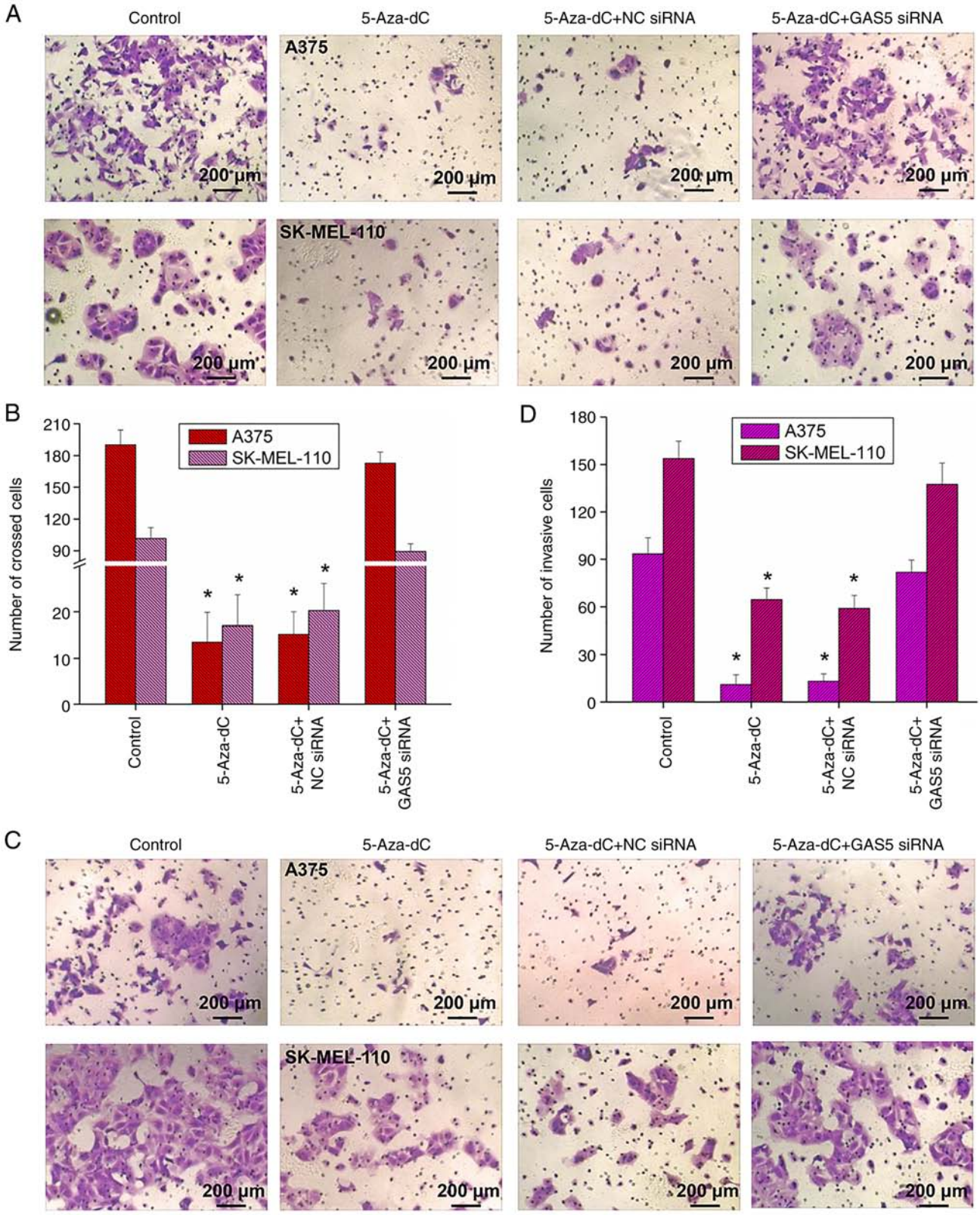

Figure 5. 5-Aza-dC inhibits the migration and invasion of melanoma cells. Melanoma cells were transfected with NC or GAS5 siRNA and then treated with 5-Aza-dC. (A and B) Detection of 5-Aza-dC regulating the migration of melanoma cells. (C and D) Effect of 5-Aza-dC on the invasion of melanoma cells. *P<0.05 vs. control. 5-Aza-dC, 5-Aza-2'-deoxycytidine; NC, negative control; GAS5, growth arrest-specific transcript 5. Scale bar, 200 μm.

oxidase 2 (NOX4), glucose-6-phosphate dehydrogenase (G6PD) and Bcl-2 expression, thereby inducing G1/S cell cycle progression and redox imbalance, inhibiting apoptosis and then promoting the growth of melanoma cells. More and more studies have shown that GAS5 is low expressed in a variety of tumors (7). The analysis of clinical samples of patients with malignant melanoma has also revealed that the expression level of GAS5 in patients with advanced tumors is

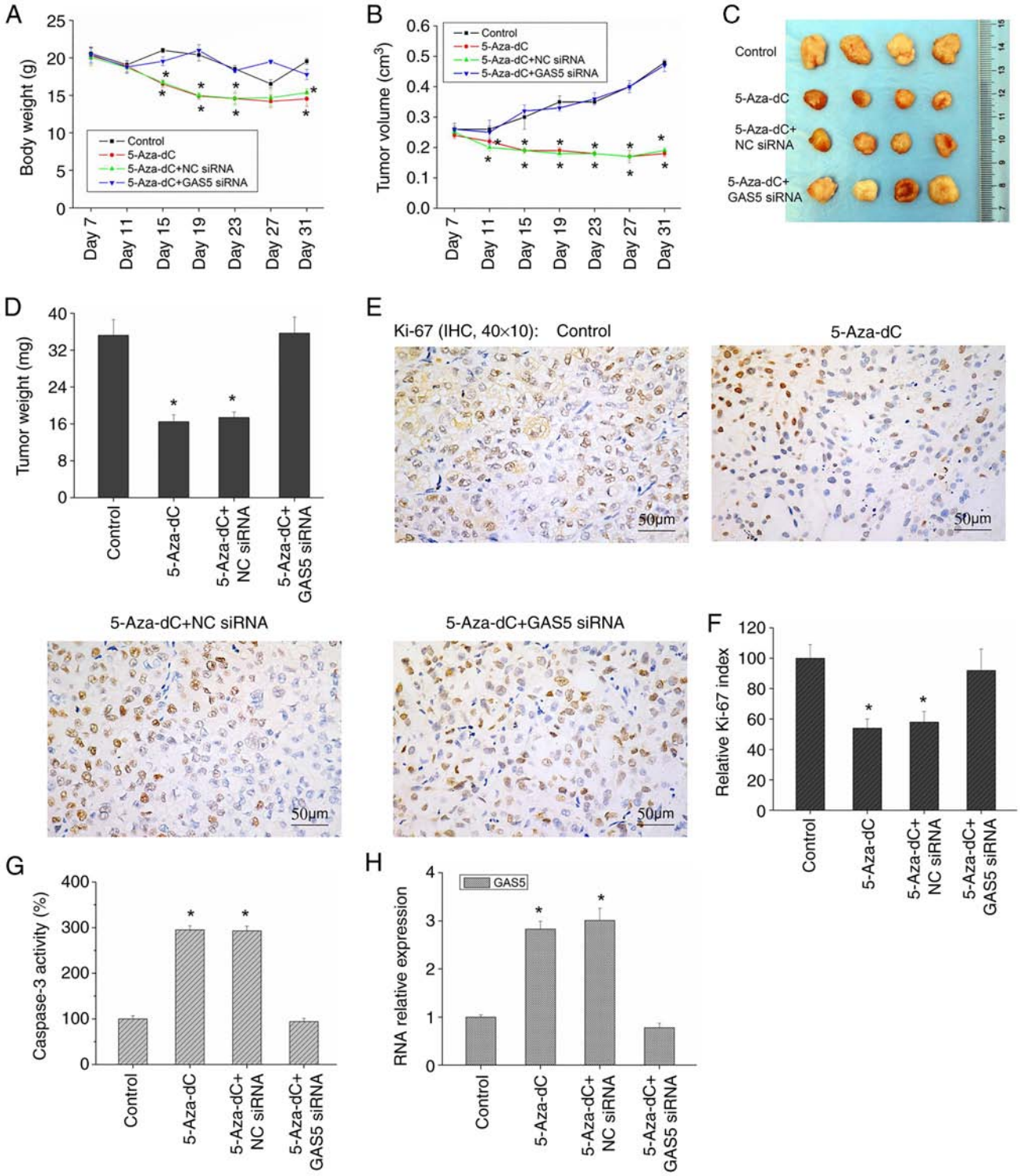

Figure 6. 5-Aza-dC inhibits the growth of melanoma xenografts. (A) Effect of 5-Aza-dC on total body weight of nude mice. (B) 5-Aza-dC significantly reduced the volume of melanoma xenografts. (C) Melanoma xenograft tumor volume. (D) 5-Aza-dC significantly reduced the tumor weight of melanoma xenografts. (E and F) Formalin-fixed xenograft tumors were immunostained with Ki-67 antibody. (G) Caspase-3 activity. (H) GAS5 expression was detected using reverse transcription-quantitative PCR. *P<0.05 vs. control. 5-Aza-dC, 5-Aza-2'-deoxycytidine; GAS5, growth arrest-specific transcript 5. Scale bar, 50 μm.

significantly lower than that of adjacent non-cancerous tissues and is also significantly associated with the TNM stage and distal metastasis of melanoma (18-21). Therefore, increasing the expression of GAS5 in tumor tissues may be a possible tumor treatment strategy.

DNA methylation, a more widely studied and a main modification process in epigenetics, is crucial for the study of tumor pathogenesis. A large amount of research data have confirmed that DNA methylation is significantly associated with tumorigenesis, particularly the abnormal hypermethylation of CpG islands in the promoter region of tumor suppressor genes, which often leads to the inactivation and silencing of the transcription level of relevant tumor suppressor genes, resulting in unrestricted cancer cell growth,

thus promoting cancer development (22,23). In the present study, the analysis of GAS5 revealed that the promoter region of GAS5 presented with apparent CpG islands, and also presented with methylation modification in melanoma tissues and cells. However, no GAS5 promoter methylation modification was found in normal tissues. The data in the MEXPRESS database demonstrated that the GAS5 expression levels in melanoma had a certain negative correlation with the level of methylation modification. Therefore, the downregulation of GAS5 expression in melanoma may be likely attributed to methylation modification. Studies on triple-negative breast cancer and cervical cancer have also revealed that GAS5 methylation levels in cancer tissues are significantly increased, culminating in the downregulation of GAS5 expression (24,25). However, Wang *et al* (26) demonstrated that although GAS5 was downregulated in gliomas, its methylation levels was not significantly altered. Another study [Wang *et al* (27)] indicated that a decreased GAS5 expression in esophageal squamous cell carcinoma may be related to the increased expression of miR-196a, and may possibly not be related to DNA methylation. Therefore, the downregulation of GAS5 expression in tumor cells may be related to a variety of regulatory mechanisms, and methylation modification may be one of the important regulatory mechanisms.

Demethylation drugs have been reported to inhibit tumor suppressor gene methylation levels in most tumor cells, thereby restoring gene expression and inhibiting tumor cell growth (11). Following the treatment of melanoma cells with 5-Aza-dC, the methylation of GAS5 was significantly reversed in the present study, and 5-Aza-dC significantly inhibited cell proliferation, migration and invasion, also inducing cell apoptosis through the upregulation of GAS5 expression, also indicating that 5-Aza-dC possibly also exerts a tumor-suppressive effect in melanoma cells. Rajaii *et al* (13) also reported that treating melanoma cells with 5-Aza-dC significantly inhibited cell invasion, growth and colony formation, and reduced tumor metastasis in mouse skin melanoma xenograft models. In the present study, further *in vivo* experiments once again demonstrated that the intraperitoneal injection of 5-Aza-dC significantly reduced the volume and weight of xenograft tumors, also inhibiting tumor growth. After implanting 5-Aza-dC-treated A375 melanoma cells into mice, it has been also revealed that 5-Aza-dC might significantly reduce tumor volumes, and may play a role by reducing the methylation level of Thrombospondin-1 (TSP1) and then restoring the expression level of TSP1 (14). In another study, decitabine (5-Aza-dC), followed by carboplatin treatment, significantly attenuated cell proliferation, resulting in a greater apoptotic response in melanoma cells, as compared to carboplatin alone (28). Moreover, clinical trials have demonstrated that 5-Aza-dC exerts a positive antitumor effect and is safe for use (29,30).

The aforementioned studies have demonstrated that the important tumor suppressor gene GAS5 is significantly methylated in melanoma, leading to the downregulation of GAS5; 5-Aza-dC may effectively inhibit the methylation of GAS5, decrease cell proliferation, migration and invasion, promote apoptosis and reduce xenograft tumor growth. This finding may provide suitable therapeutic targets and approaches for the diagnosis and treatment of melanoma.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HTN and LC made substantial contributions to the conception and design of the study. YJZ, RX and JJ conducted most of the experiments, confirmed the authenticity of all the raw data and drafted the manuscript. LZ, CHY and JZ made substantial contributions to data analysis and data interpretation. XW and DXC were involved in performing some of the experiments and data analysis. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethical Committee of Yunnan Cancer Hospital of the Third Affiliated Hospital of Kunming Medical University (KY201939). Informed consent was obtained from each patient included in this study. The use of animals was approved by the Ethics Committee of Animal Research Institute of Yunnan Cancer Hospital.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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