Isocitrate Dehydrogenase 2 Dysfunction Contributes to 5-hydroxymethylcytosine Depletion in Gastric Cancer Cells

NAN-HUA CHOU[1*], CHUNG-YU TSAI[1*], YA-TING TU[2], KUO-CHIANG WANG[1], CHI-HSIANG KANG[1], PO-MIN CHANG[1], GUAN-CHENG LI[2], HING-CHUNG LAM[3], SHIUH-INN LIU[1] and KUO-WANG TSAI[2,4]

[1]*Division of General Surgery, Department of Surgery, Kaohsiung Veterans General Hospital, Kaohsiung, Taiwan, R.O.C.;*
[2]*Departments of Medical Education and Research, Kaohsiung Veterans General Hospital, Kaohsiung, Taiwan, R.O.C.;*
[3]*Center for Geriatrics and Gerontology, Kaohsiung Veterans General Hospital, Kaohsiung, Taiwan, R.O.C.;*
[4]*Department of Chemical Biology, National Pingtung University of Education, Pingtung, Taiwan, R.O.C.*

Abstract. *The isocitrate dehydrogenase (IDH) family of enzymes comprises of the key functional metabolic enzymes in the Krebs cycle that catalyze the conversion of isocitrate to α-ketoglutarate (α-KG). α-KG acts as a cofactor in the conversion of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC). However, the relationship between 5hmC and IDH in gastric cancer remains unclear. Our study revealed that the 5hmC level was substantially lower and 5mC level was slightly higher in gastric cancer tissues; however, 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) levels did not change significantly in these tissues. We further examined the expression levels of IDH1 and IDH2 in gastric cancer tissues and observed that IDH2 levels were significantly lower in gastric cancer tissues than in the adjacent normal tissues. The ectopic expression of IDH2 can increase 5hmC levels in gastric cancer cells. In conclusion, our results suggested that IDH2 dysfunction is involved in 5hmC depletion during gastric cancer progression.*

Epigenetic regulation entails histone modification and DNA methylation, that are involved in the regulation of cell growth and development in mammals (1, 2). DNA methylation at the gene promoter region can silence its transcriptional activity. During carcinogenesis, the promoter region of tumor suppressor genes is gradually hypermethylated, resulting in silencing of tumor suppressor genes. A sixth base, 5-hydroxymethylcytosine (5hmC), has been recently reported to have a crucial role in the activation of gene expression through the DNA demethylation process (3, 4). Moreover, 5hmC levels have been frequently observed to be decreased in several cancers, including colon, breast, lung, kidney, liver, gastric and breast cancers (5-9). Tahiliani *et al*. (10) reported that the ten-eleven translocation (TET) enzyme can convert 5-methylcytosine (5mC) to 5hmC. Furthermore, TET proteins can oxidize 5hmC to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC), which subsequently generate cytosine through base-excision repair (11, 12).

The isocitrate dehydrogenase (IDH) family of enzymes comprises of the major functional metabolic enzymes in the Krebs cycle that catalyze the conversion of isocitrate to α-ketoglutarate (α-KG) and produce NADPH. NADPH is believed to have a critical role in the modulation of the oxidative state in a cell (13, 14). Three classes of IDH isoenzymes have been classified in mammalian cells: cytosolic *IDH1*, mitochondrial *IDH2* and *IDH3* (15). Among them, *IDH1* and *IDH2* mutations are frequently observed in acute myeloid leukemia and glioma (13, 16, 17). Studies have reported that *IDH1* (R132H) and *IDH2* (R172K) mutations exhibit a gain of function in the reduction of α-KG to a stereospecific D-stereoisomer of 2-hydroxyglutarate (13, 14, 18, 19). Moreover, α-KG acts as a cofactor in the hydroxylation of 5mC to 5hmC by TET proteins in the DNA demethylation process. Therefore, a high frequency of *IDH1* or *IDH2* mutation induces global DNA hypermethylation because of α-KG reduction (16, 20, 21). However, the actual mechanism underlying the involvement of the *IDH* gene in 5hmC depletion in gastric cancer cells remains unclear. This study evaluated the correlation between DNA modification

These Authors contributed equally to this study.

Correspondence to: Kuo-Wang Tsai, Department of Medical Education and Research, Kaohsiung Veterans General Hospital, Kaohsiung 813, Taiwan, R.O.C. Tel: +886 734221211510, Fax: +886 73468056, e-mail: kwtsai@vghks.gov.tw

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and *IDH* gene expression in gastric cancer cells and provides an alternative explanation for 5hmC depletion in gastric cancer cells.

Materials and Methods

Clinical samples and DNA and RNA extraction. We obtained 58 gastric cancer and corresponding adjacent normal tissue samples from patients with gastric cancer who underwent surgery at the Department of Surgery, Kaohsiung Veterans General Hospital, Kaohsiung, Taiwan. Our study protocol was independently reviewed and approved by the Institutional Review Board of Kaohsiung Veterans General Hospital (IRB approval number: VGHKS12-CT3-10). The methods were carried out in accordance with approved guidelines and all patients provided informed consent. Total RNA and DNA from the tissues were extracted using the TRIzol reagent (Invitrogen; Thermo Fisher Scientific Inc., Waltham, MA, USA). Briefly, the tissue samples were homogenized in 1 ml of the TRIzol reagent. The protein and DNA were extracted using 0.2 ml of chloroform and RNA was precipitated using 0.6 ml of isopropanol. Genomic DNA was extracted from the remaining liquid by adding 0.5 ml of a back extraction buffer (4 M guanidine thiocyanate, 50 mM sodium citrate and 1 M Tris; pH 8.0) and DNA was precipitated using 0.4 ml of isopropanol. The concentration, purity and amount of total RNA and DNA were determined using the Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA).

DNA dot-blot assay. DNA samples (100 μg) were added to the denaturation buffer (0.4 mM NaOH and 10 mM EDTA) and denatured at 100˚C for 10 min. Furthermore, the samples were chilled on ice for 5 min and applied on a positive-charged nylon membrane (Roche, Basel, Switzerland). The membrane was UV cross-linked and dried for 1 h at 70˚C. Duplicated membranes were probed with anti-5mC (1:1,000, Cat. No.: 61255; Active Motif, Carlsbad, CA, USA), anti-5hmC (1:5000, Cat. No.: 39769; Active Motif), anti-5caC (1:1,000, Cat. No.: 61225; Active Motif) and anti-5fC antibodies (1:2,500, Cat. No.: 61223; Active Motif) overnight. Subsequently, the membranes were probed with either a rabbit or mouse IgG antibody conjugated to horseradish peroxidase (HRP) for 1 h at room temperature (RT). After the membranes were washed three times with phosphate-buffered saline (PBS)-Tween, the immunoreactive dot was detected using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Inc., Piscataway, NJ, USA). The membranes were stained with methylene blue as a loading control.

Immunohistochemical analysis and scoring. Human gastric carcinoma tissue microarrays containing adjacent normal tissues (No: IMH-341) and tumors (No: IMH-316) from 59 patients with gastric cancer were obtained from IMGENEX (Imgenex, Inc., San Diego, CA, USA). Immunohistochemical (IHC) analysis was performed using the Novolink Max Polymer Detection System (Leica, Newcastle upon Tyne, UK). The slides were deparaffinized in xylene and rehydrated through descending grades of alcohol. Antigen retrieval was performed by immersing the slides in Tris-EDTA (10 mM; pH 9.0) for 10 min at 125˚C in a pressure boiler. Endogenous peroxidase activity was blocked by incubating the slides for 30 min with 3% hydrogen peroxide in methanol at RT. Subsequently, primary antibodies were immediately applied and the slides were incubated overnight at 4˚C in a wet chamber. The primary antibodies used were rabbit polyclonal anti-IDH1 (1:400; Gene Tex, San Antonio, TX, USA) and rabbit polyclonal anti-IDH2 (1:200; Gene Tex) in a Tris-buffered saline solution with 5% bovine serum albumin. After being washed with PBS, the slides were incubated with an HRP-conjugated secondary antibody for 10 min at RT and the sections were counterstained with hematoxylin.

The relative gene expression levels were scored and calculated on the basis of staining intensity (0, no signal; 1, mild; 2, moderate; and 3, strong) and the proportion of positively stained tumor cells (scored as 0%-100%) was calculated in the whole field of each core. The score of individual samples was calculated using the following formula: intensity × percentage of positively stained tumor cells.

Expression data from Gene Expression across Normal and Tumor Tissue (GENT) database. The microarray data of *IDH1* and *IDH2* in 311 gastric cancer tissues and 57 adjacent normal tissues were obtained from the GENT database (http://medicalgenome.kribb.re.kr/GENT/search/search.php).

Real-time polymerase chain reaction (RT-PCR). Total RNA of the gastric cancer tissues was extracted using the TRIzol reagent (Invitrogen; Thermo Fisher Scientific Inc.) according to the manufacturer's protocol. Furthermore, cDNAs were obtained from a reverse transcription reaction by using oligo-dT primers and SuperScript III Reverse Transcriptase according to the manufacturer's instructions (Invitrogen; Thermo Fisher Scientific Inc.). In subsequent PCR, cDNA was used at a dilution of 1:20 in water. PCR was performed using a gene-specific forward primer and a reverse primer. The PCR protocol used was incubation at 94˚C for 5 min, followed by 35 cycles of 94˚C for 15 s, 60˚C for 30 s and 72˚C for 1 min. The gene expression levels were normalized using *GAPDH*. The primers used are listed as follows:

IDH1-F: TGCAAAAATATCCCCCGGCT
IDH1-R: TACATCCCCATGGCAACACC
IDH2-F: GCCGGCACTTTCAAAATGGT
IDH2-R: GATGGACTCGTCGGTGTTGT
GAPDH-F: TGCACCACCAACTGCTTAGC
GAPDH-R: GGCATGGACTGTGGTCATGAG

Cell invasion and colony formation assays. The gastric cancer cells were transfected with either of the RNAi oligonucleotides directed against *IDH2* (*IDH2*, sense: 5'-CAAGAACACCAUACUGAAATT-3' and antisense: 5'-UUUCAGUAUGGUGUUCUUGGT-3'), whereas random sequence siRNA oligonucleotides (*NC*, sense: 5'-GCGACGAUCUGCCUAAGAUdTdT-3' and antisense: 5'-AUCUUAGGCAGAUCGUCGCdTdT-3') were used as a negative control. *IDH2* knockdown and control cells were used to perform invasion and colony formation assays. The invasion ability of the cells was examined *in vitro* in a transwell. Briefly, the cells were re-suspended at a density of 4.5×10^5 in 2% fetal bovine serum and then added to the upper chamber of the transwells (Corning Incorporated, New York, NY, USA) with a matrigel coating for the invasion assay. The chambers were incubated in a CO_2 incubator at 37˚C for 12 or 24 h; the remaining cells in the upper chamber were removed using cotton swabs; the cells on the undersurface of the transwells were fixed in a 10% formaldehyde solution. The cells

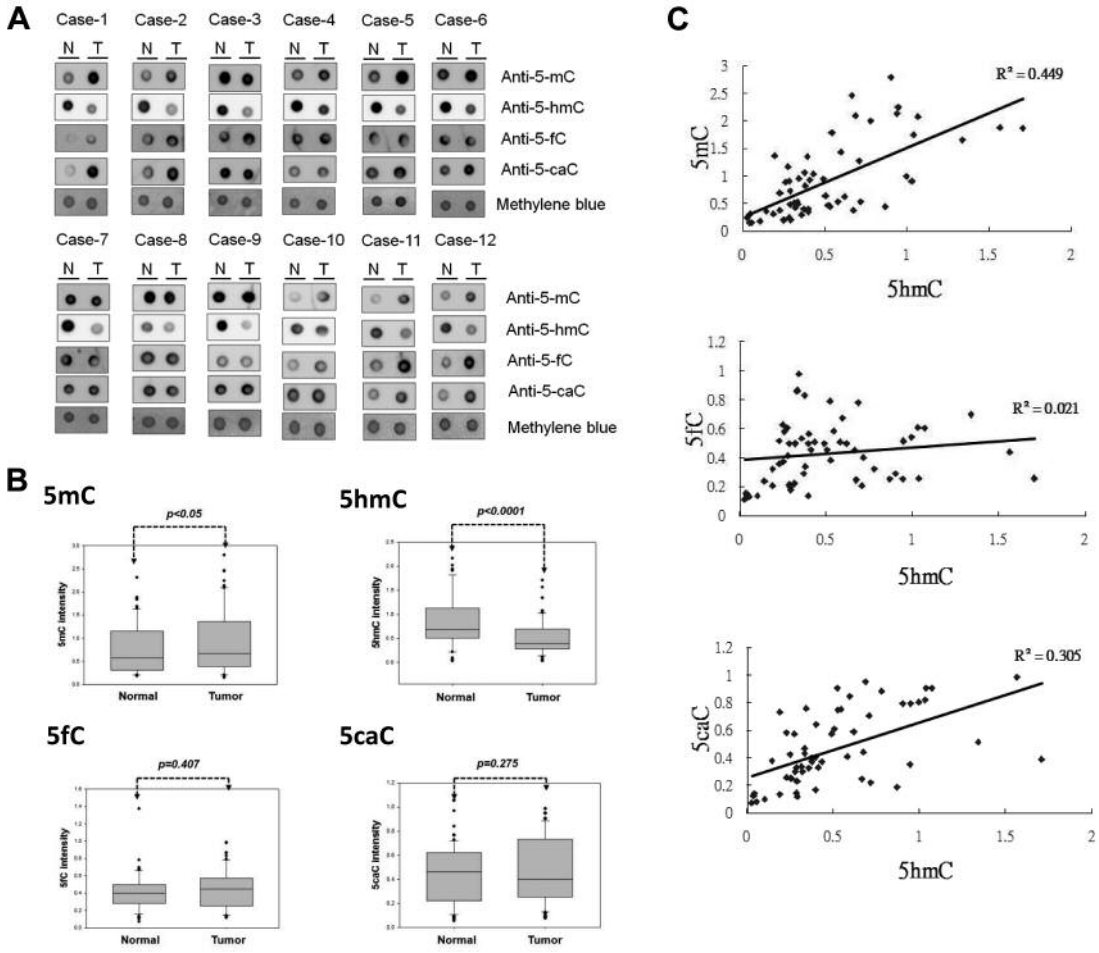

Figure 1. *Epigenetic modification status of 5mC, 5hmC, 5fC and 5Cac in human gastric cancer. (A) Dot-blot analysis was performed using anti-5hmC, anti-5mC, anti-5fC and anti-5Cac antibodies in 58 pairs of clinical gastric cancer and adjacent normal tissues; twelve representative cases are shown. Methylene blue was used as the loading control. (B) The intensity of 5mC, 5hmC, 5Cac and 5fC was quantified using the Image J software. (C) Correlations of 5hmc levels with 5mC, 5Cac and 5fC modifications were analyzed in 58 gastric cancer samples.*

were stained with a crystal violet solution and the number of gastric cancer cells in three fields was counted under a phase-contrast microscope. All the experiments were repeated three times. In a clonogenic assay, gastric cancer cells were seeded in a six-well plate at a density of 2×10^3 and transfected with 5 nM si-*IDH2* or a control. The cells were incubated in a CO_2 incubator at 37˚C for 2 weeks until the formation of colonies with substantial sizes. The medium was removed, cells were fixed in 1 ml of a 10% formaldehyde solution and the plates were incubated at RT for 2 min. After removing the fixation solution, 1 ml of a crystal violet solution was added and the plates were incubated at RT for 2 h. Subsequently, the crystal violet solution was removed and the plates were rinsed. The plates were air-dried at RT. Furthermore, 1 ml of 10% acetic acid was added to each well to dissolve the crystal violet. The absorbance of individual wells was determined at 595 nm by using the Multiskan FC spectrophotometer (Thermo Scientific).

Results

DNA modification status in gastric cancer cells. The role of 5hmC in gene regulation is extremely crucial. During gastric cancer progression, 5hmC might regulate gene expression through activation of the DNA demethylation process. However, the biological function of 5hmC in gastric cancer remains largely unknown. Therefore, we comprehensively assessed the levels of 5mC, 5hmC, 5caC and 5fC in gastric cancer genomic DNA (n=58 cases) by using the dot-blot assay. As presented in Figure 1A and B, the 5mC level was slightly higher and the 5hmC level was significantly lower in gastric cancer tissues than in the adjacent normal tissues. Moreover, no significant change was observed in 5fC and 5CaC levels between the adjacent normal and gastric cancer

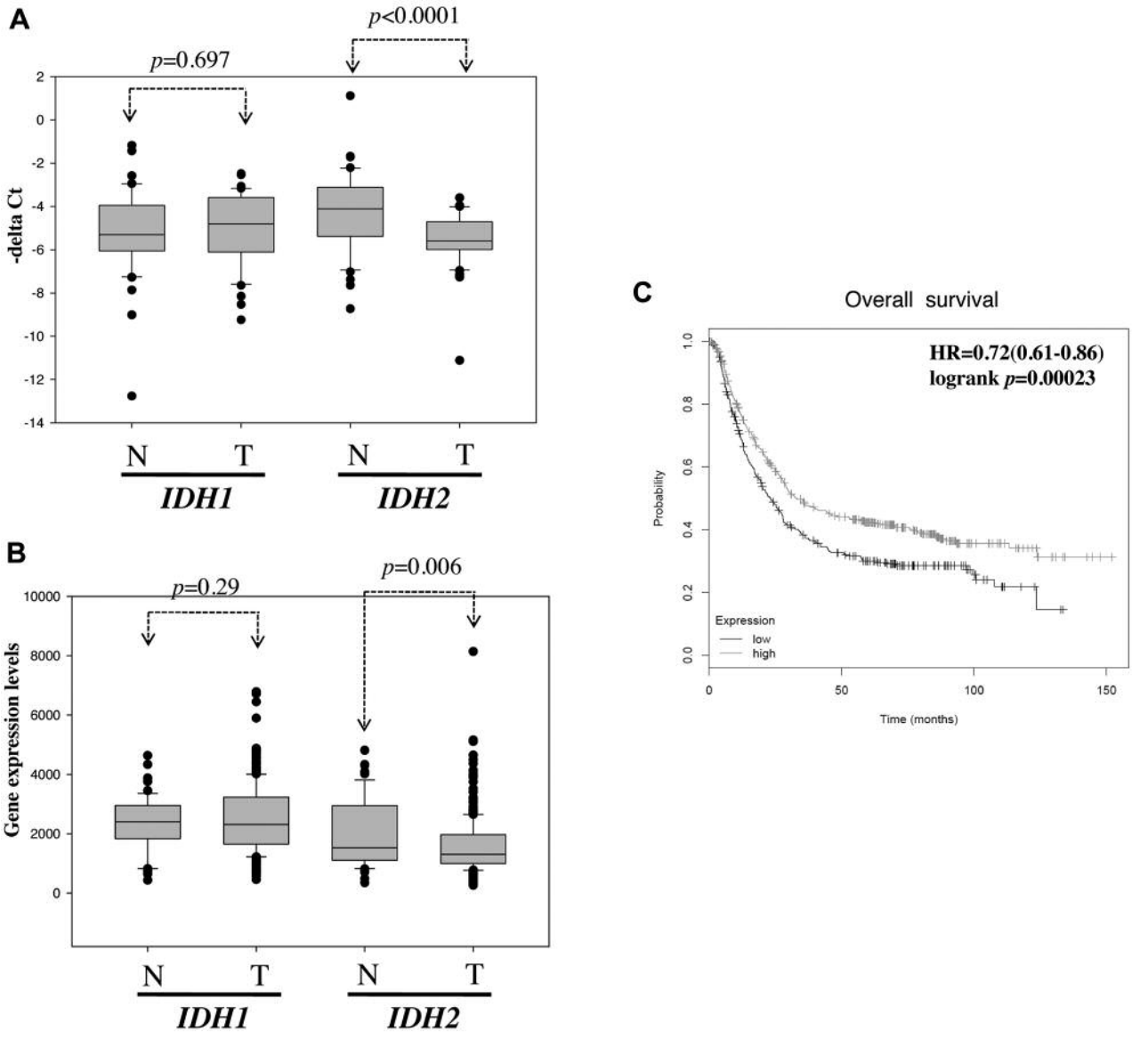

Figure 2. *Expression levels of TET1 and IDH2 in gastric cancer cells. (A) The expression levels of IDH1 and IDH2 were examined in gastric cancer and corresponding adjacent normal tissues from 41 patients through real-time PCR. The expression level was compared between tumor and normal cells by using paired t-tests. (B) The expression levels of IDH1 and IDH2 were analyzed in 311 gastric cancer and 57 adjacent normal tissues from the GENT database. The expression level was compared between tumor and normal cells by using the Student's t tests. (C) IDH2 expression levels were associated with overall survival. These survival data were obtained using a Kaplan-Meier survival plot).*

tissues. Compared to normal tissues, only the 5hmC level tended to decrease in the gastric cancer tissues (48 out of 58, 82%). By contrast, 5mC, 5fC and 5caC levels tended to increase in the gastric cancer tissues (Figure 1B). In addition, we observed a weak correlation between these three DNA modifications and 5hmC levels (Figure 1C). These results indicate that, although 5mC is required as a substrate for oxidation to generate 5hmC, 5hmC depletion is not caused by low 5mC levels in gastric cancer cells.

Expression of IDH1 and IDH2 genes in gastric cancer cells. The results of the dot-blot assay analysis revealed that compared to adjacent normal tissues, the correlation between 5mC and 5hmC staining levels was not strong in gastric cancer tissues. Therefore, we speculated that the potential mechanism underlying global 5hmC depletion may be the aberrant expression of metabolic enzymes involved in the conversion of 5mC to 5hmC. Studies have reported that TET1 and TET2 are highly associated with 5hmC depletion

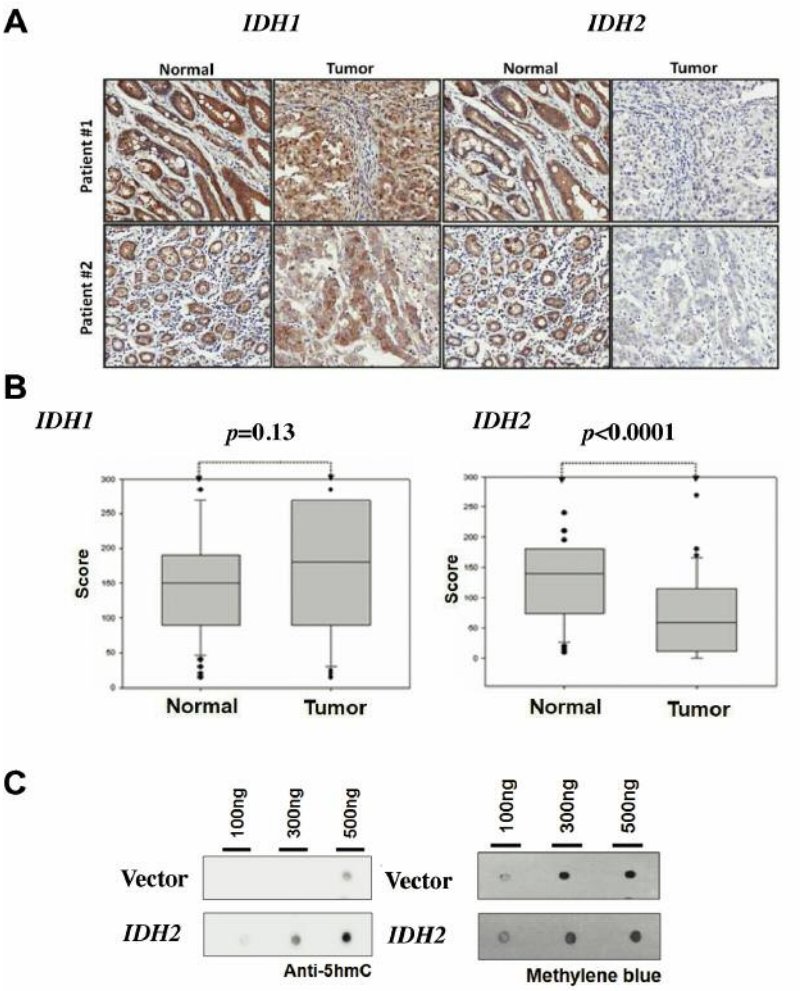

Figure 3. *Expression levels of IDH1 and IDH2 analyzed using immunohistochemistry (IHC) staining in gastric cancer tissue arrays. Two representative cases are shown. (A and B) The expression levels of IDH1 and IDH2 proteins were examined through IHC analysis in gastric cancer tissues and corresponding adjacent normal tissues obtained form 59 patients with gastric cancer. (C) IDH2 overexpression increased the global 5hmC status in the genomic DNA of AGS cells. The loading control was visualized using methylene blue staining.*

and are frequently down-regulated in human cancer cells (6). We examined the expression levels of *IDH1* and *IDH2* transcripts in gastric cancer cells through real-time PCR and by analyzing the GENT database. As presented in Figure 2A, the transcriptional levels of *IDH2* were substantially lower in gastric cancer tissues than in the corresponding adjacent normal tissues obtained from 41 patients; however, a similar result was not observed for *IDH1*. In addition, we analyzed the expression levels of *IDH1* and *IDH2* in gastric cancer cells from the GENT database. Similar data demonstrated that only *IDH2* was significantly lower in the gastric cancer tissues than in the adjacent normal tissues (Figure 2B). Furthermore, we evaluated the association of *IDH2* expression with survival in patients with gastric cancer by using a Kaplan-Meier survival plot (22). As presented in Figure 2C, low *IDH2* expression levels significantly correlated with poor overall survival (OS) in patients with gastric cancer (hazard ratio (HR)=0.72; 95% confidence interval (CI)=0.61-0.86; p=0.00023).

We examined the expression levels of IDH1 and IDH2 through IHC analysis in a gastric cancer tissue array (n=59 cases). The protein levels of IDH2 were substantially lower in the gastric cancer tissues than in the adjacent normal tissues. The expression levels of IDH1 did not significantly differ between the gastric cancer and normal tissues (Figure 3A and B). The transfection of AGS cells with *IDH2* for 24 h resulted in an increase in global 5hmC levels (Figure 3C). In summary, these results indicated that the decrease in 5hmC levels in gastric cancer cells may be attributable to low *IDH2* expression levels.

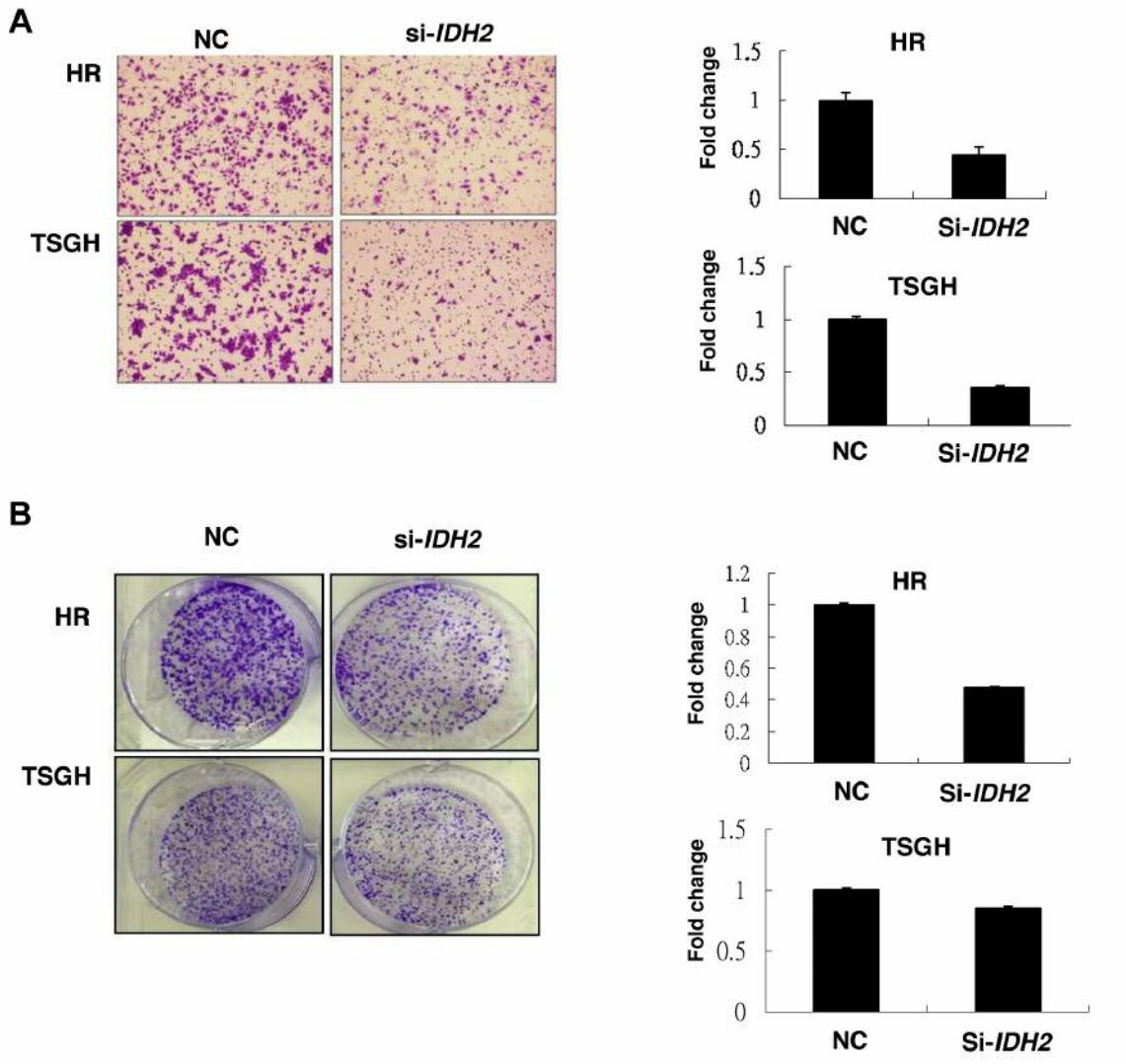

Figure 4. *IDH2 expression knockdown affected gastric cancer cell growth and invasion. (A) The colony formation assay was performed after transfection of HR and TSGH cells with si-IDH2 or a control for 2 weeks. The cell images from a representative experiment are shown and a graph shows quantified values. (B) Data are reported as the number of colonies relative to the control (means±SD). ***p<0.001 vs. control. (C) The invasion abilities of HR and TSGH cells transfected with si-IDH2 or the control for 24 h were assessed using the transwell assay. Subsequently, cells were stained with a crystal violet solution and the number of cells that migrated was quantified by counting cells in three fields under a phase-contrast microscope. The cell images of a representative experiment are shown and the graph shows values quantified using the Ascent software. Data are reported as the number of colonies relative to the control (means±SD). ***p<0.001 vs. control.*

IDH2 influences the growth and motility of gastric cancer cells. Studies have reported that *IDH1* and *IDH2* mutations have a crucial role in the progression of several human cancers. However, the role of *IDH1* and *IDH2* dysfunction in gastric cancer remains largely unknown. In this study, we observed that the expression levels of *IDH2* were significantly lower in the gastric cancer tissues than in the adjacent normal tissues. To examine the biological function, we knocked-down *IDH2* expression in gastric cancer cells by transfecting the cells with si*IDH2* and control scrambled oligonucleotides, respectively. As presented in Figure 4A, knockdown of *IDH2* expression reduced the colony formation ability of gastric cancer cells. Furthermore, *IDH2* knockdown significantly suppressed the invasion ability of HR and TSGH cells (Figure 4B). Overall, *IDH2* knockdown in gastric cancer cells revealed that *IDH2* might have an oncogenic role in gastric cancer cells. This finding contradicts the finding of lower *IDH2* expression in gastric cancer tissues than in the adjacent normal tissues. The discrepancy in these results must be examined in future studies.

Discussion

Our study results revealed that 5hmC levels were substantially lower during gastric cancer progression and that 5mC, 5fC and 5caC levels tended to increase or did not change in gastric cancer cells (Figure 1). Therefore, 5hmC depletion is not because of global genomic hypomethylation and 5hmC levels might be reduced through the 5hmU pathway in gastric cancer.

The IDH family of enzymes comprises the key functional metabolic enzymes in the Krebs cycle that catalyze the conversion of isocitrate to α-KG, which acts a cofactor in the conversion of 5mC to 5hmC. Studies have indicated that *IDH1* and *IDH2* mutations have a crucial role during the progression of several human cancers. Somatic heterozygous mutations in *IDH1* and *IDH2* have been identified in various human cancers, including glioma and leukemia (13, 14, 16). In addition, studies have reported a low frequency of *IDH1* and *IDH2* mutations in solid tumors, including gastric cancer (14, 23). Fassan *et al*. reported that only one *IDH2* mutation was identified in 15 invasive early gastric cancers (24). However, the role of IDH1 and IDH2 protein expression levels in the prognosis of human gastric cancer and their biological function remain largely unknown. In this study, we observed that IDH2 expression was significantly lower in gastric cancer tissues and that low IDH2 expression levels were significantly associated with poor survival in patients with gastric cancer.

IDH2 overexpression can increase 5hMC levels in gastric cancer cells and low *IDH2* expression might contribute to 5hmC depletion in gastric cancer cells. However, we observed a conflicting finding that *IDH2* knockdown suppressed the growth and motility of gastric cancer cells, suggesting that *IDH2* has an oncogenic role in gastric cancer cells. In addition, to maintaining hydroxymethylation, IDH2 is involved in energy metabolism and the redox homeostasis pathway in human cancers. To support rapid proliferation, cancer cells consume substantial energy through the activation of the glucose metabolism pathway (25). Geiger *et al*. reported that high *IDH2* levels are poor prognostic biomarkers and indicate low survival in patients with breast cancer. They suggested that increased *IDH2* expression causes over-production of NAPDH and regulates the oxidative state in transformed cells (26). A recent study reported that IDH-dependent reductive carboxylation and redox homeostasis are involved in modulating anchorage-independent growth (27). Ward *et al*. reported that knockdown of *IDH1* and *IDH2* can significantly reduce cell proliferation (18). Therefore, *IDH2* may play an oncogenic role through modulation of glucose metabolism and redox homeostasis in cancer cells. In conclusion, knockdown of *IDH2* expression suppressed gastric cancer cell growth and motility through a 5hmC-independent pathway in gastric cancer progression.

Conflicts of Interests

The Authors declare that they have no competing interests.

Acknowledgements

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