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Effect of *SLC5A8* Missense Variants on Its Tumor-Suppressive Function

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ABSTRACT

Background: Solute carrier family-5 member-8 (SLC5A8) serves as a plasma membrane transporter for monocarboxylates, such as lactate, butyrate, pyruvate, acetate, propionate, nicotinate, and β-hydroxybutyrate. SLC5A8 can suppress colorectal cancer (CRC), and its tumor-suppressive function is mainly associated with butyrate, propionate, and pyruvate, which inhibit histone deacetylase. Although SLC5A8 is an important tumor suppressor, the impact of *SLC5A8* variants on its tumor-suppressive function have not been reported. In this study, we investigated the effects of *SLC5A8* missense variants on the expression and tumor-suppressive function of the transporter using various in vitro assays.
Methods: Common *SLC5A8* missense variations were identified using data from the Database of Single-Nucleotide Polymorphisms of the National Center for Biotechnology Information. We generated HCT116 and DLD-1 cell lines stably overexpressing wild-type *SLC5A8* or *SLC5A8* variants. The effect of each variant on SLC5A8 expression was examined via immunoblotting. Finally, using colony-formation, wound-healing, and invasion assays, we investigated whether the decrease in SLC5A8 expression resulting from its variants could affect the tumor-suppressive function of the transporter.
Results: We identified two common missense single-nucleotide polymorphisms of *SLC5A8*, Val193Ile (rs1709189) and Met490Ile (rs164365), and assembled two major haplotypes of *SLC5A8*. Among these haplotypes, haplotype 1 contained wild-type *SLC5A8* mRNA sequences and H2 contained two variants: Val193Ile and Met490Ile. Val193Ile and H2 significantly decreased SLC5A8 expression. These variants significantly increased the proliferation, migration, and invasion abilities of CRC cells.
Conclusion: Decreased SLC5A8 expression caused by missense *SLC5A8* variants appeared to significant impair the tumor-suppressive function of the transporter.

Keywords: SLC5A8; Missense Variant; Colorectal Cancer; Tumor-Suppression

INTRODUCTION

Colorectal cancer (CRC) is the third leading cancer by incidence and the second leading cause of cancer-related mortality.[1] Although the overall mortality due to CRC continues to decrease because of regular screening, surveillance, and advanced treatments, regional spread to nearby organs or lymph nodes dramatically reduces the 5-year survival rate of patients with

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Disclosure

The authors have no potential conflicts of interest to disclose.

Author Contributions

Conceptualization: Choi YH, Choi JH; Formal analysis: Ha SY, Choi JH; Methodology: Ha SY, Song HS, Kim JY; Writing - original draft: Ha SY, Choi JH; Writing - review & editing: Ha SY, Song HS, Kim JY, Choi YH, Choi JH.

CRC.[2,3] Clinical tests have enabled the discovery of new theragnostic biomarkers for CRC.[4] However, due to the strong heterogeneity of CRC, the need for additional predictive factors, such as newly classified genetic or molecular biomarkers, has been emphasized.[5]

Solute carrier family-5 member-8 (SLC5A8), otherwise known as sodium-coupled monocarboxylate transporter, is a plasma membrane-based transporter of monocarboxylates, such as lactate, butyrate, pyruvate, acetate, propionate, nicotinate, and β-hydroxybutyrate.[6-9] SLC5A8 transports nicotinate and various monocarboxylate drugs,[6] and its activity is inhibited by many nonsteroidal anti-inflammatory drugs.[10] SLC5A8 is expressed in various organs, such as the colon, lungs, brain, and thyroid gland.[7,11,12] In contrast, SLC5A8 expression is silenced because of aberrant DNA methylation in cancer of the colon, thyroid, breast, and other organs in humans.[11,12-18] SLC5A8 was first identified as a tumor suppressor in the colon by Li et al.[19] The tumor-suppressive function of SLC5A8 is mainly related to butyrate, propionate, and pyruvate, which can inhibit histone deacetylase (HDAC) activity.[7,11,20] Independent of that mechanism, ectopic SLC5A8 expression in cancer cells suppressed tumor progression by reducing tumor cell survival.[14,21] Because the tumor-suppressive function of SLC5A8 has been observed in CRC and non-CRCs, such as cervical and pancreatic cancer, SLC5A8 has attracted attention as a tumor biomarker.[18,22,23]

Although SLC5A8 has been established as an important tumor suppressor, the molecular and cellular effects of *SLC5A8* variants on its tumor-suppressive function are not well understood. Therefore, in this study, we investigated the effects of *SLC5A8* missense variants on the expression and tumor-suppressive function of this transporter through various in vitro assays.

METHODS

Identifying *SLC5A8* missense variations

We used data from the Database of Single Nucleotide Polymorphisms (dbSNP) of the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/snp/) to identify common *SLC5A8* missense variations (minor allele frequency ≥ 5%). The frequencies of each variant were obtained from the 1,000 genomes project (phase 3) across four ethnic groups: 661 Africans, 347 Americans, 504 East Asians, and 503 Europeans. Haplotype assembly was performed using Haploview 4.3 software (Broad Institute, Cambridge, MA, USA). Nucleotide location numbers were assigned based on the translational start site of the *SLC5A8* mRNA sequence (GenBank accession number: NM_145913.5).

Constructing plasmids expressing wild-type *SLC5A8* or *SLC5A8* variants

Wild-type *SLC5A8* complementary DNA (Origene Technologies, Inc., Rockville, MD, USA) was subcloned into the p3XFLAG-CMV-10 vector. Plasmids encoding *SLC5A8* variants were generated using the QuikChange® II Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA). The relevant primer sequences used are listed in **Supplementary Table 1**. All DNA sequences were confirmed by direct sequencing.

Establishing stable cell lines expressing wild-type *SLC5A8* or *SLC5A8* variants

To produce stable lines overexpressing wild-type *SLC5A8* or *SLC5A8* variants, we grew HCT116 (human colon carcinoma) and DLD-1 (human colorectal adenocarcinoma) cells in RPMI1640 medium (WELGENE, Inc., Gyeongsan, Korea) and transfected them with the corresponding expression plasmids using the Lipofectamine LTX and Plus reagents (Life Technologies,

Carlsbad, CA, USA). After 24 hours, the transfected cells were selected in cell culture medium containing 1 mg/mL G418 (InvivoGen, San Diego, CA, USA) for 7–10 days. Individual colonies were transferred to new cell culture plates and incubated with decreasing concentrations of G418. Stable cells were incubated in modified RPMI1640 medium containing 1 mM sodium pyruvate and 100 μg/mL G418.

Immunoblotting

For immunoblotting, HCT116 and DLD-1 cells were lysed in NP-40 solution (Invitrogen, Waltham, MA, USA) containing protease inhibitors. Protein extracts were separated on a 4–12% sodium dodecyl sulfate gel (Bio-Rad Laboratories, Hercules, CA, USA), and the separated proteins were transferred using a trans-blot Turbo RTA Transfer Kit and a trans-blot turbo transfer system (Bio-Rad Laboratories). An anti-FLAG M2 antibody (Merck, Rahway, NJ, USA) and an anti-β-actin antibody (Santa Cruz Biotechnology, Dallas, TX, USA) were used as primary antibodies. The blots were imaged using an Amersham ImageQuant 800 biomolecular imager (Cytiva, Seoul, Korea), and the intensity of each band was measured using ImageJ software (National Institutes of Health, Bethesda, MD, USA). β-actin expression was detected as an internal control for immunoblotting. HCT116 or DLD-1 cells were treated with 10 μM MG132 (Sigma-Aldrich, St. Louis, MO, USA) or 10 nM bafilomycin A_1 (MedChemExpress, Monmouth Junction, MJ, USA) for 24 hours and immunoblotted to examine the mechanisms underlying the altered membrane expression of *SLC5A8* variants. In addition, HCT116 cells expressing wild-type *SLC5A8* or the Val193Ile variant were treated with 10 μM MG132 for 3, 6, 9, or 24 hours and subjected to immunoblotting.

Immunofluorescence assay

HCT116 and DLD-1 cells were seeded in 4-well chamber slides (Thermo Fisher Scientific, Waltham, MA, USA). After 24 hours, the cells were fixed and permeabilized with 100% methanol (prechilled at −20°C) at room temperature for 5 minutes and blocked with 1% bovine serum albumin for 1 hour. The cells were incubated overnight with primary antibodies against FLAG M2, BiP (Abcam, Cambridge, UK), or giantin (Abcam). Subsequently, the cells were washed thrice with phosphate-buffered saline (PBS) and incubated with secondary Alexa Fluor® 488-conjugated rabbit anti-mouse IgG or Alexa Fluor® 594-conjugated goat anti-rabbit IgG antibodies (Thermo Fisher Scientific). Nucleic acids were stained with 4′,6-diamidino-2-phenylindole (Vector Laboratories, Newark, CA, USA). Images were obtained using a confocal laser-scanning microscope and analyzed using an LSM image examiner (Carl Zeiss, Oberkochen, Germany).

Colony-formation assay

HCT116 and DLD-1 cells (500 cells/well) were seeded in 6-well plates. After 8 days, the cells were washed with ice-cold PBS and fixed for 10 minutes with a methanol and acetic acid solution. The cells were then stained with crystal violet solution (Sigma-Aldrich).

Wound-healing assay

HCT116 and DLD-1 cells (1.8×10^5 cells/well) were seeded in 24-well plates. After scratches were created using a 24-well plate cell scratcher (SPL Life Sciences Co., Pocheon, Korea), images were captured at 0 and 48 hours under an IN300T-FL microscope (AmScope, Irvine, CA, USA).

Invasion assay

HCT116 and DLD-1 cells (0.5×10^5 cells/mL) were seeded into the inserts, and invasion assays were performed using a Cell Invasion Kit (Sigma-Aldrich) per the manufacturer's instructions. After 48 hours, the cells were stained with crystal violet solution, and images

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were captured under a microscope. The stained cells were resuspended in 10% acetic acid to quantify the number of cells, and the absorbance was measured at 560 nm using VERSAmax™ tunable microplate reader (Molecular Devices, San Jose, CA, USA).

Cell-viability assay

HCT116 and DLD-1 cells (0.5×10^4 cells/mL) were seeded in 96-well plates and incubated with or without 1 mM pyruvate. After 48 hours, cell viabilities were measured using the Cell Counting Kit-8 per the manufacturer's instructions (Dojindo Laboratories, Rockville, MD, USA). The absorbance at 450 nm was measured using a VERSAmax™ tunable microplate reader.

Flow cytometry

HCT116 and DLD-1 cells were incubated with or without 1 mM pyruvate for 48 hours and stained with annexin V and propidium iodide using the FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen™; Becton, Dickinson and Company, Franklin Lakes, NJ, USA). The stained cells were analyzed via flow cytometry (NovoCyte 3000; ACEA Biosciences, Inc., San Diego, CA, USA) and the data were analyzed using Novoexpress software (ACEA Biosciences, Inc.).

Statistical analyses

Statistical analyses were performed using GraphPad Prism software, version 8.0 (GraphPad Software Inc., San Diego, CA, USA). Student's *t*-test was used to compare the results before and after MG132 or bafilomycin A_1 treatment, as well as the flow cytometry data, and calculate the corresponding *P* values. Student's *t*-test was also used to calculate *P* values comparing the wild-type versus the empty vector. Other *P* values were calculated using one-way analysis of variance followed by Dunnett's two-tailed test. $P < 0.05$ was considered to indicate a statistically significant difference.

RESULTS

Missense variations of *SLC5A8*

Two common missense SNPs of *SLC5A8*, namely Val193Ile (rs1709189) and Met490Ile (rs164365), were identified using the NCBI dbSNP. The frequencies of each *SLC5A8* variant in the four ethnic groups studied are shown in **Table 1**. Using the SNP data, two major *SLC5A8* haplotypes were assembled (**Table 2**). Haplotype 1 (H1) contained wild-type *SLC5A8* mRNA (NM_145913.5), and H2 contained the Val193Ile and Met490Ile variants.

Table 1. Frequencies of *SLC5A8* missense variations

rs number	Variant	Amino acid substitution	Minor allele	Minor allele frequency			
				African	American	East Asian	European
rs1709189	c.577G > A	Val193Ile	A	0.222	0.127	0.544	0.248
rs164365	c.1470G > T	Met490Ile	T	0.149	0.127	0.544	0.249

The data represented were obtained from the 1,000 genomes project (phase 3).
SLC5A8 = solute carrier family-5 member-8.

Table 2. Frequencies of *SLC5A8* haplotypes

Haplotype ID	c.577G > A	c.1470G > T	Frequency			
			African	American	East Asian	European
H1	G	G	0.754	0.872	0.456	0.753
H2	**A**	**T**	0.126	0.125	0.544	0.247
H3	**A**	G	0.097	-	-	-
H4	G	**T**	0.023	-	-	-

The single nucleotide polymorphisms are indicated with bold text.
SLC5A8 = solute carrier family-5 member-8.

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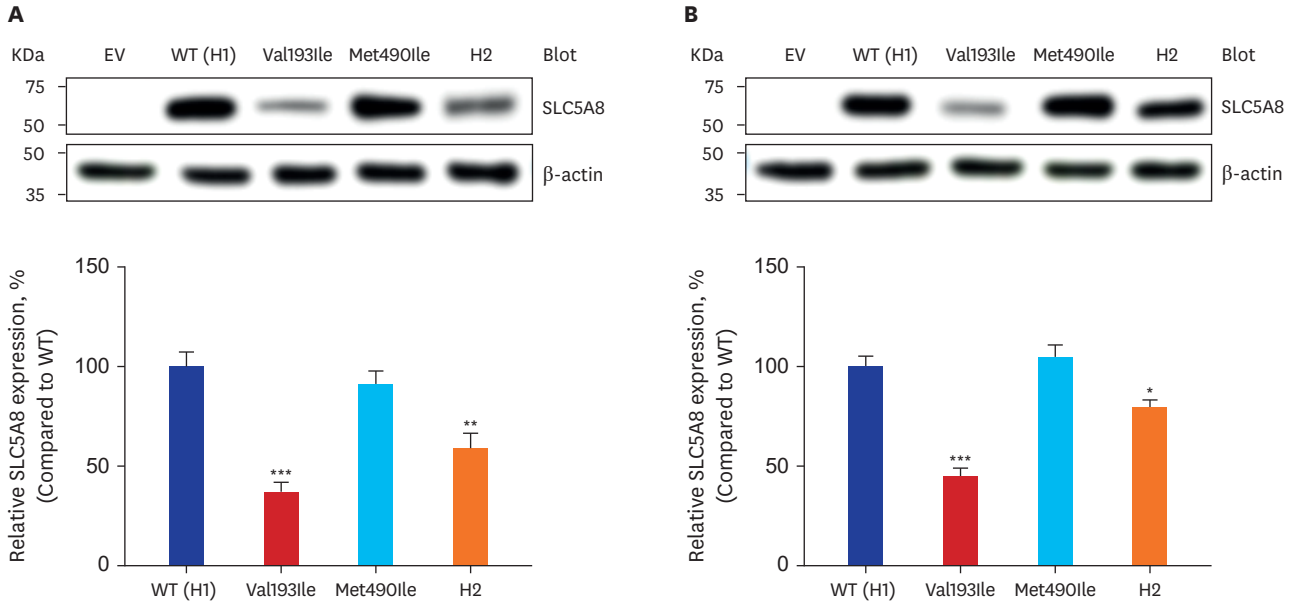


Fig. 1. Effects of *SLC5A8* variants on SLC5A8 expression. SLC5A8 expression was measured by immunoblotting with whole-cell lysates from HCT116 (**A**) or DLD-1 (**B**) cells stably expressing WT *SLC5A8* or its variants. The data shown represent the mean ± standard deviation from three independent experiments, analyzed by one-way analysis of variance followed by Dunnett's two-tailed test.
EV = empty vector, WT = wild-type, SLC5A8 = solute carrier family-5 member-8.
$^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. WT.

Effects of *SLC5A8* variations on SLC5A8 expression

Immunoblotting and immunofluorescence assays were performed to investigate the effects of *SLC5A8* variants on SLC5A8 expression. Val193Ile and H2 showed significantly lower SLC5A8 expression in HCT116 cells (62.8% and 40.9%, respectively) than the wild-type ($P < 0.001$ and $P = 0.006$, respectively; **Fig. 1A**). Likewise, Val193Ile and H2 showed significantly lower SLC5A8 expression in DLD-1 cells (55.2% and 20.7%, respectively) than the wild-type ($P < 0.001$ and $P = 0.037$, respectively; **Fig. 1B**). In contrast, SLC5A8 expression of the Met490Ile variant was slightly higher in HCT116 and DLD-1 cells than the wild-type; however, this difference was not statistically significant (**Fig. 1**).

After treatment with MG132, a proteasomal proteolysis inhibitor, SLC5A8 expression of Val193Ile and H2 in HCT116 cells recovered significantly more than naïve SLC5A8 expression ($P = 0.001$ and $P = 0.004$, respectively, **Fig. 2A**). Specifically, Val193Ile and H2 showed relative expression-level recoveries of 131.72% and 90.43%, respectively, when compared with the expression of wild-type SLC5A8. Similarly, after treating DLD-1 cells with MG132, SLC5A8 expression of Val193Ile and H2 recovered significantly more than naïve SLC5A8 expression ($P < 0.001$ and $P = 0.012$, respectively; **Fig. 2C**). The variants recovered to 81.40% and 98.31% of the level of wild-type SLC5A8 expression, respectively.

After treating HCT116 cells with bafilomycin A_1, a lysosomal degradation inhibitor, SLC5A8 expression of Val193Ile and H2 recovered significantly, when compared with naïve SLC5A8 expression ($P < 0.001$ and $P = 0.002$, respectively; **Fig. 2B**). The SLC5A8 expression of those variants recovered to 83.81% and 97.52% of wild-type SLC5A8 expression, respectively. Further, after treatment with bafilomycin A_1, SLC5A8 expression of Val193Ile and H2 in DLD-1 cells was significantly restored ($P < 0.001$; **Fig. 2D**). That is, expression of Val193Ile and H2 recovered to 113.46% and 117.99% of wild-type SLC5A8 expression, respectively.

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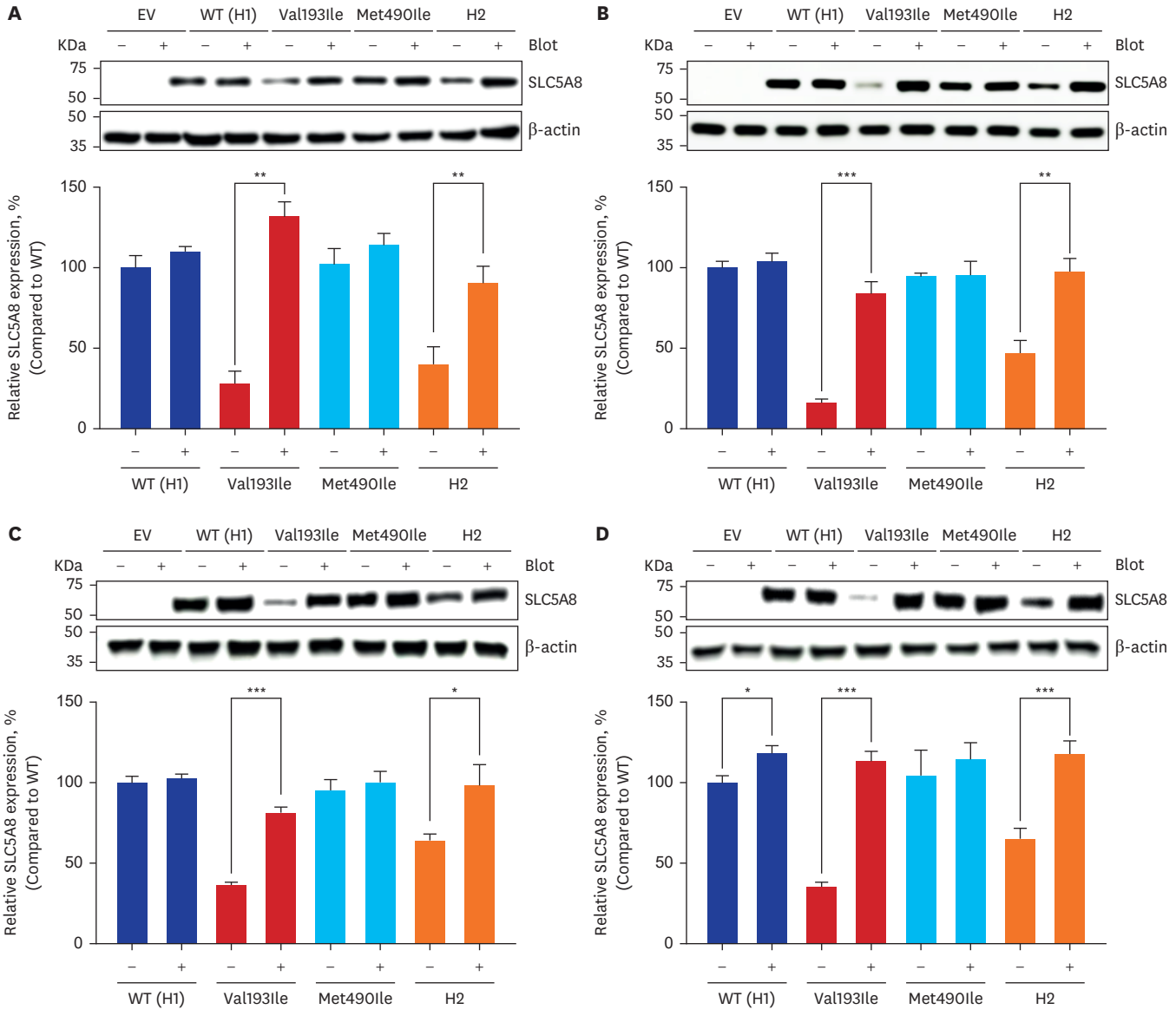


Fig. 2. Effects of MG132 or bafilomycin A_1 on SLC5A8 expression. HCT116 or DLD-1 cells stably expressing WT *SLC5A8* or its variants were treated with MG132 (**A**, **C**) or bafilomycin A_1 (**B**, **D**). Then, cell lysates were prepared and immunoblotted. The data shown represent the mean ± standard deviation from three independent experiments, analyzed by Student's *t*-test.
EV = empty vector, WT = wild-type, SLC5A8 = solute carrier family-5 member-8.
$^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. native SLC5A8 expression without MG132 or bafilomycin A_1 treatment.

To confirm the proteasomal degradation of SLC5A8, we conducted a more detailed time-course analysis, treating HCT116 cells expressing wild-type *SLC5A8* or the Val193Ile variant with MG132 at successive intervals and then measuring SLC5A8 expression via immunoblotting. We observed that SLC5A8 expression of the Val193Ile increased significantly after 3, 6, 9, and 24 hours, when compared with the naïve SLC5A8 expression ($P = 0.013$, $P = 0.003$, $P < 0.001$, and $P < 0.001$, respectively). The Val193Ile variant showed expression-recovery levels of 87.49%, 99.98%, 114.63%, and 138.58%, respectively, when compared with wild-type SLC5A8 expression (**Supplementary Fig. 1**). Collectively, our data indicate that Val193Ile and H2 downregulation may reflect proteasomal or lysosomal degradation. Finally, our immunofluorescence assays revealed that plasma membrane SLC5A8 expression decreased in HCT116 and DLD-1 cells harboring Val193Ile and H2 (**Fig. 3**).

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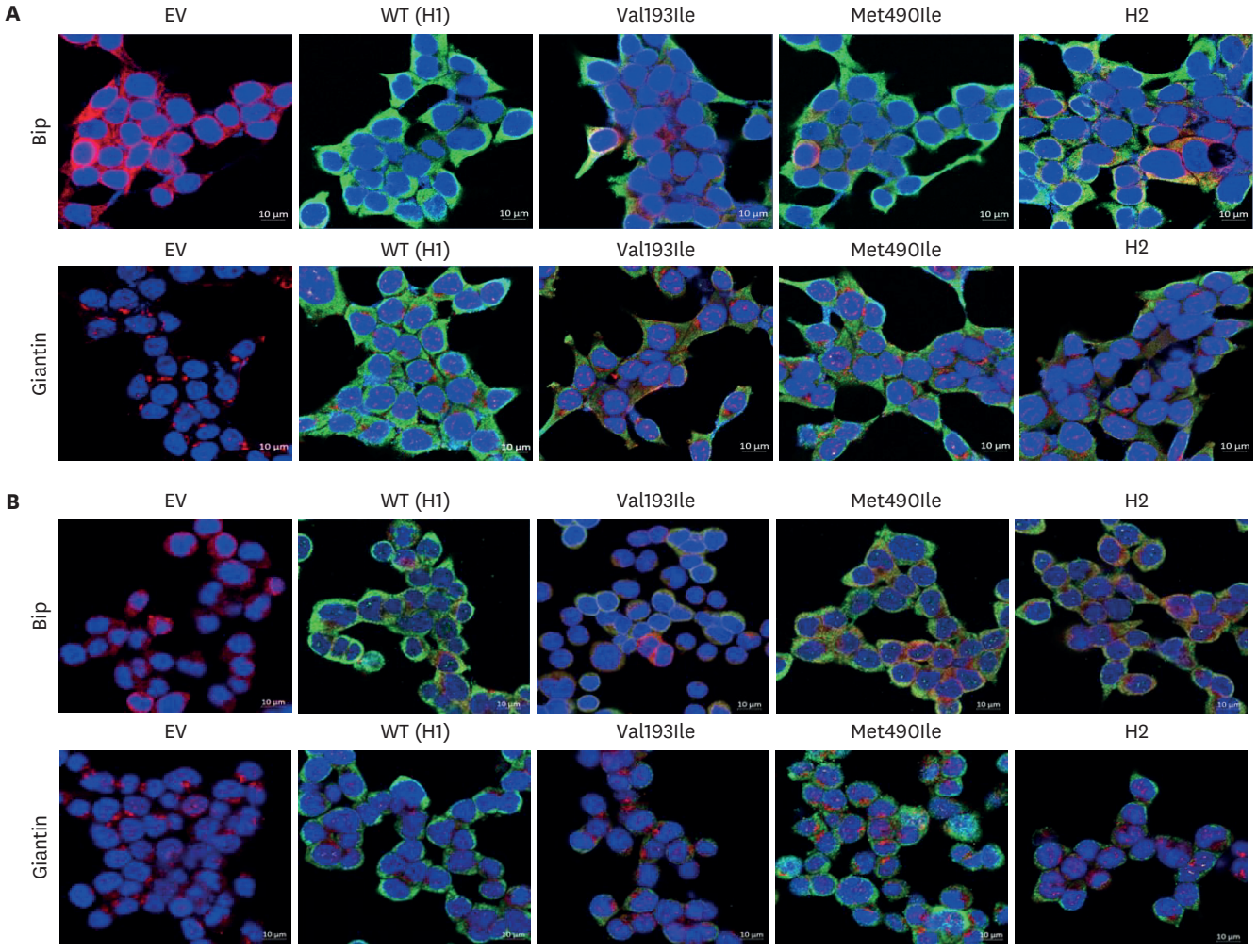


Fig. 3. Effects of *SLC5A8* variants on SLC5A8 subcellular localization. HCT116 (**A**) or DLD-1 (**B**) cells stably expressing WT *SLC5A8* or its variants were fixed, permeabilized, and immunostained using primary antibodies against FLAG M2, BiP, or giantin. Scale bars, 10 μm.
EV = empty vector, WT = wild-type, SLC5A8 = solute carrier family-5 member-8.

Effects of *SLC5A8* variations on the tumor-suppressive function of the transporter

Colony-formation, wound-healing, and invasion assays were performed to investigate the effects of *SLC5A8* variants on the tumor-suppressive function of the transporter. The colony-formation ability of cells expressing wild-type *SLC5A8* was significantly lower than cells transfected with the empty vector, with both HCT116 ($P < 0.001$, **Fig. 4A**) and DLD-1 ($P < 0.001$, **Fig. 4B**) cells. These results are consistent with those of a previous study showing that SLC5A8 can suppress CRC progression.[21] Val193Ile and H2 showed significantly higher colony-formation abilities with both HCT116 and DLD-1 cells than wild-type ($P < 0.001$ and $P = 0.002$ in HCT116 cells and $P < 0.001$ and $P = 0.013$ in DLD-1 cells, respectively; **Fig. 4**). However, the Met490Ile variant showed significantly lower colony-formation abilities with both HCT116 and DLD-1 cells than wild-type ($P = 0.014$ and $P = 0.004$, respectively, **Fig. 4**). Previous findings identified pyruvate-dependent apoptosis or cell death due to survivin depletion as tumor-suppressing mechanisms mediated by SLC5A8 in in breast cancer cells.[9,16]

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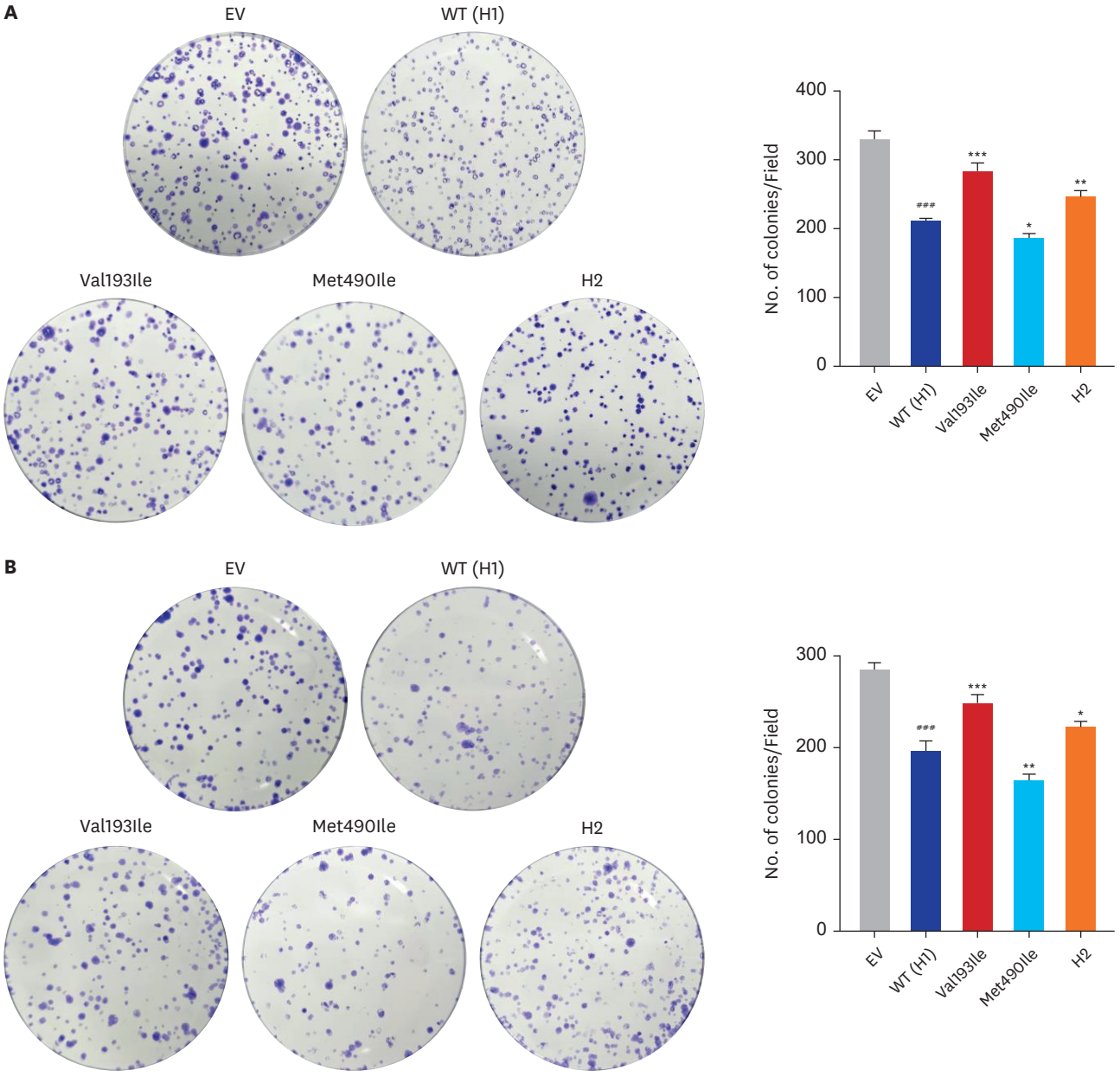


Fig. 4. Effects of *SLC5A8* variants on colorectal cancer cell proliferation. Colony-formation assays were performed using HCT116 (**A**) or DLD-1 (**B**) cell stably expressing WT *SLC5A8* and its variants. After 8 days, the cells were stained with crystal violet solution, and the number of colonies was counted. The data shown represent the mean ± standard deviation from three independent experiments, analyzed by Student's *t*-test and one-way analysis of variance followed by Dunnett's two-tailed test.
EV = empty vector, WT = wild-type, *SLC5A8* = solute carrier family-5 member-8.
$^{###}P < 0.001$ vs. EV; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. WT.

To investigate the tumor-suppressive mechanism of SLC5A8 in CRC cells, we performed cell-viability and apoptosis assays. The viability of HCT116 or DLD-1 cells expressing wild-type *SLC5A8* was significantly lower than cells transfected with the empty vector ($P = 0.002$ and $P < 0.001$, respectively). In contrast, the reduced expression of SLC5A8 associated with the Val193Ile variant significantly increased the viability with both HCT116 ($P < 0.001$) and DLD-1 cells ($P < 0.001$). However, in the absence of pyruvate, no significant difference was observed (**Supplementary Fig. 2**). We also observed that SLC5A8 induced apoptosis in CRC cells in a

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pyruvate-dependent manner. After incubation with pyruvate, HCT116 or DLD-1 cells expressing wild-type *SLC5A8* showed significantly more early apoptotic cells than those transfected with the empty vector (16.92-fold [$P = 0.002$] and 7.57-fold [$P < 0.001$], respectively) (**Supplementary Fig. 3**).

In wound-healing assays, expressing wild-type *SLC5A8* significantly decreased the migration of HCT116 (**Fig. 5A**) and DLD-1 (**Fig. 5B**) cells by 29.1% and 35.0%, respectively, when compared with those of control cells transfected with the empty vector ($P < 0.001$). Val193Ile and H2 significantly increased the migration ability of HCT116 cells by 19.5% and 13.8%, respectively ($P < 0.001$, **Fig. 5A**), whereas Met490Ile significantly reduced the migration ability by 9.3%, compared with wild-type ($P = 0.006$, **Fig. 5A**). Val193Ile and H2 significantly increased the migration ability of DLD-1 cells by 21.4% and 11.8%, respectively, compared with the wild-type ($P < 0.001$, **Fig. 5B**). Further, Met490Ile decreased the migration ability of DLD-1 cells by 4.5%; however, this difference was not statistically significant (**Fig. 5B**).

The results of the invasion assay revealed that expressing wild-type *SLC5A8* significantly decreased the invasion ability of HCT116 or DLD-1 cells, compared with the empty vector ($P < 0.001$ and $P = 0.001$, respectively, **Fig. 6**). Val193Ile and H2 significantly increased the invasion ability of both HCT116 and DLD-1 cells, compared with wild-type ($P < 0.001$ and $P = 0.003$ in HCT116 and $P < 0.001$ and $P = 0.005$ in DLD-1 cells, respectively; **Fig. 6**). In addition, Met490Ile significantly reduced the invasion ability of both HCT116 or DLD-1 cells, compared with wild-type ($P = 0.040$ and $P = 0.029$, respectively, **Fig. 6**).

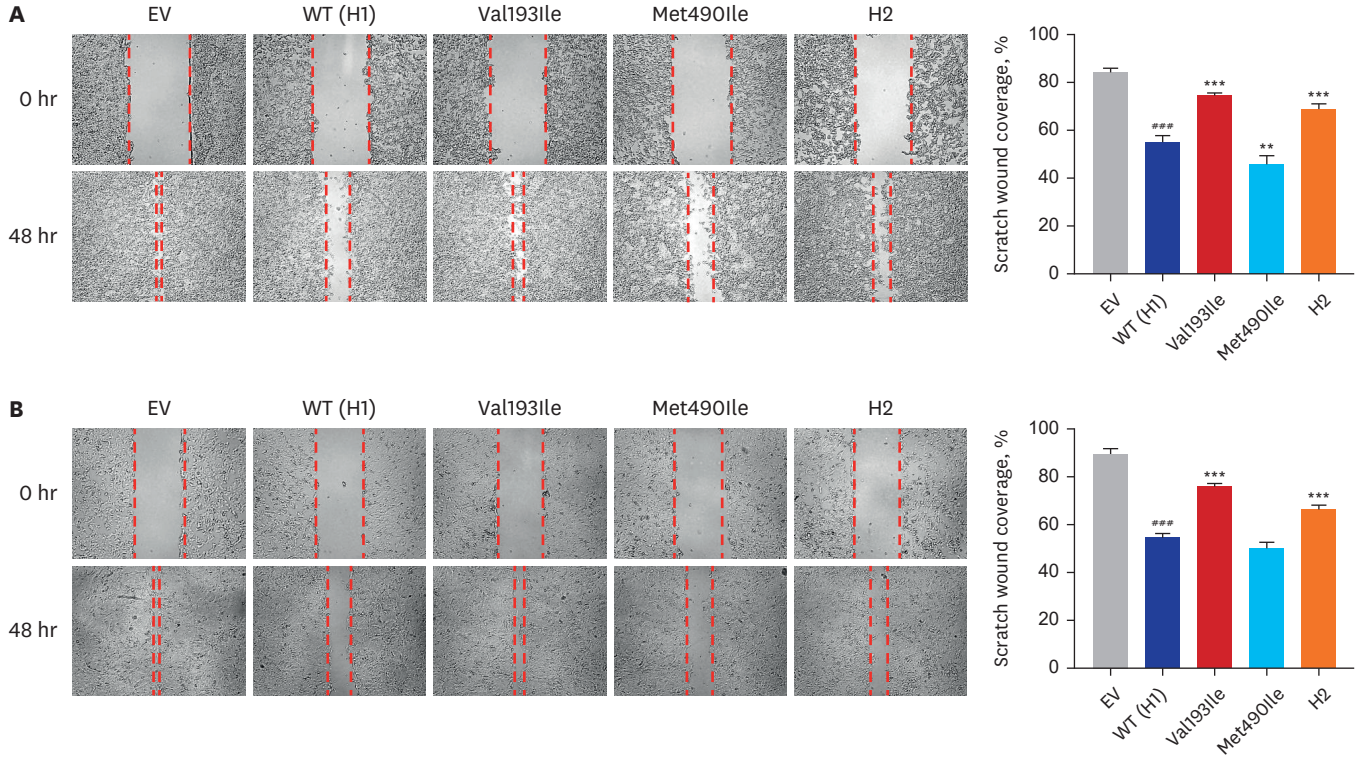


Fig. 5. Effects of *SLC5A8* variants on colorectal cancer cell migration. HCT116 (**A**) or DLD-1 (**B**) cells stably expressing WT *SLC5A8* and its variants were scratched and photographed after 0 and 48 hours. The graphs on the right show the results after 48 hours. The data shown represent the mean ± standard deviation from three independent experiments, analyzed by one-way analysis of variance followed by Student's *t*-test and Dunnett's two-tailed test.
EV = empty vector, WT = wild-type, *SLC5A8* = solute carrier family-5 member-8.
$^{###}P < 0.001$ vs. EV, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. WT.

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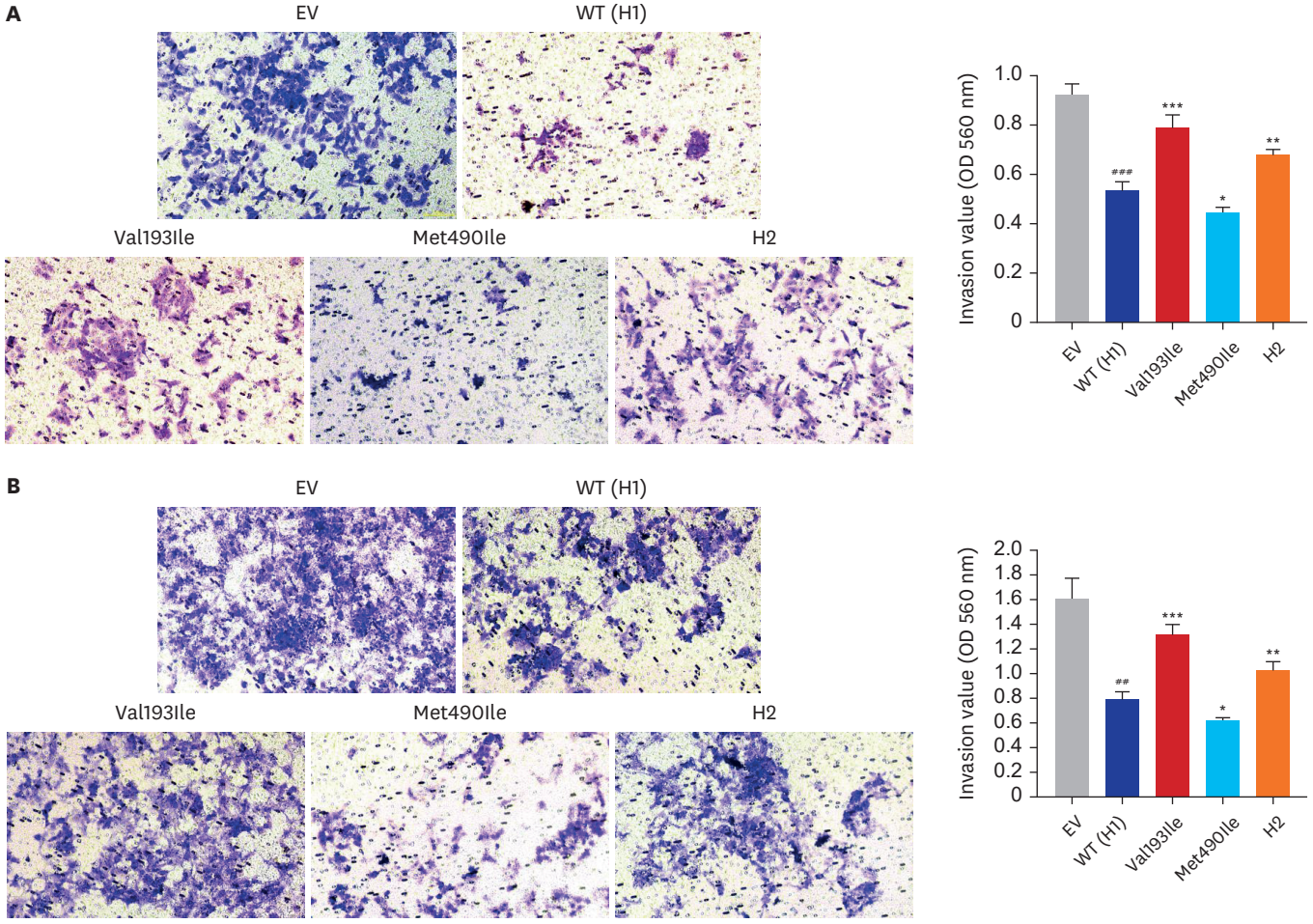


Fig. 6. Effects of *SLC5A8* variants on colorectal cancer cell invasion. HCT116 (**A**) or DLD-1 (**B**) cells stably expressing WT *SLC5A8* and its variants were used in cell-invasion assays and photographed after a 48 hours-incubation. The data shown represent the mean ± standard deviation from three independent experiments, analyzed by one-way analysis of variance followed by Student's *t*-test and Dunnett's two-tailed test.
EV = empty vector, WT = wild-type, *SLC5A8* = solute carrier family-5 member-8.
$^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. EV, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. WT.

DISCUSSION

SLC transporters are vital for the human digestive system, facilitating the absorption of various substances into cells and their movement between cells.[24] SLC transporters have been implicated in the development, progression, and metastasis of various gastrointestinal cancers. Depending on the cancer type, they can function as either oncogenes or tumor suppressors.[21,25] Numerous reports have highlighted the crucial roles of SLC transporters in gastrointestinal cancers. However, the specific impact of genetic variants of these transporters on gastrointestinal cancer remains underexplored. Recently, we examined the effect of missense *SLC22A18* variants on CRC progression. SLC22A18 is a suppressor of CRC.[26] Our findings indicate that certain variations in this transporter significantly decreased SLC22A18 expression. The reduced expression was associated with significant increases in the proliferation, migration, and invasion of CRC cells.[27]

SLC5A8 can suppress cancer in the colon.[19] This transporter is expressed in the epithelial cells of the colon and transports short-chain fatty acids, such as butyrate, pyruvate, and

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propionate, into cells. Butyrate and pyruvate play important roles in suppressing cancer by inhibiting HDAC.[21] Butyrate plays an important role in the colon, as it is produced by the bacterial breakdown of dietary fiber. The blood concentration of butyrate is less than 10 μM, whereas pyruvate levels are more than ten times higher. During the early stages of carcinogenesis, when epithelial cells lose polarity, pyruvate also becomes important in the colon, as SLC5A8 facilitates its transport from the blood into the cells.[9,21] Specifically, inhibiting HDAC plays a key role in suppressing cancer by reactivating tumor-suppressor genes, such as p53; HDAC inhibitors promote histone acetylation, which can loosen the chromatin structure and reactivate silenced tumor-suppressor genes.[28] HDAC inhibitors can modulate the expression of genes involved in cell cycle regulation, leading to cell cycle arrest and apoptosis, thereby effectively preventing the proliferation of cancer cells.[29]

Tumor-suppressor genes are often silenced in cancer cells. In CRC, *SLC5A8* expression is suppressed through DNA methylation in the promoter region.[30] Re-expressing transporters whose expression is suppressed in cancer could be a useful strategy for cancer treatment. For example, previous data showed that DNA-methylation inhibitors, such as 5′-azacytidine and 5′-aza-2′-deoxycytidine, can induce SLC5A8 in cancer cells.[19,31] Among them, 5′-azacytidine is currently used to treat acute myelogenous leukemia and myelodysplastic syndrome.[32,33]

In this study, we investigated the effects of missense *SLC5A8* variants on SLC5A8 expression and functionally characterized each variant in CRC cells. The Val193Ile variation significantly downregulated the transporter. H2, comprising both the Val193Ile and Met490Ile variants, showed significantly lower expression than wild-type. The 193rd amino acid is located within the 6th transmembrane domain of the SLC5A8 transporter, which is composed of an α-helical secondary structure. Both valine and isoleucine are hydrophobic amino acids. Therefore, a substitution between these residues would be expected minimally impact the protein structure.[34] However, even chemically similar amino acid substitutions can affect protein structures and functions through several mechanisms. Substitutions may alter the local folding pathway by disrupting short-range interactions, even if the overall change is minor.[35] In addition, even small changes in the residue size or polarity can alter the binding affinity of a protein to ligands or other molecules, potentially reducing interaction strengths or increasing misfolding risks.[36] Finally, the replacement of residues with similar but not identical properties may affect how tightly side chains pack together, influencing the structural integrity of a protein.[37] In this study, we could not elucidate the specific mechanisms whereby the Val193Ile variation affects the protein stability. Additional experiments will be necessary to investigate this topic in detail.

We investigated whether the decrease in transporter expression caused by such variations affected the tumor-suppressive function of the SLC5A8 transporter in vitro with CRC cells (HCT116 and DLD-1) stably expressing the variants. With all three methods used (colony-formation, wound-healing, and invasion assays), overexpressing wild-type SLC5A8 resulted in significantly lower cancer cell proliferation and progression, when compared with the empty vector. In our colony-formation assays, Val193Ile and H2, which showed significantly lower expression, also showed higher cell proliferation than wild-type. In contrast, the Met490Ile variant significantly decreased cell proliferation. In wound-healing and invasion assays, Val193Ile and H2 caused significantly greater CRC cell migration and invasion than wild-type. In conclusion, our finding suggests that reduced *SLC5A8* expression, resulting from missense variations, can notably impair the tumor-suppressive function of the transporter. In addition, we observed one mechanism whereby SLC5A8 suppressed CRC, namely pyruvate-dependent apoptosis.

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To our knowledge, this study was the first to investigate the effects of missense *SLC5A8* variants on its expression and function in CRC cells. In a previous study, the frequencies of *SLC5A8* genetic variations were compared between patients with prostate cancer and healthy individuals.[38] Three study groups were analyzed: one group consisting of African Americans and two groups of Caucasians. The Val193Ile variant showed no significant difference in frequency between patients and healthy individuals across all analyses. In contrast, the Met490Ile variant exhibited statistically significant differences in frequency between patients and healthy individuals in one Caucasian group but no significant differences in the other two groups. In a subgroup analysis based on family history, the body–mass index, and height, both variants were significantly associated with prostate cancer susceptibility among individuals taller than 70 inches. Notably, the Met490Ile variant significantly impacted cancer progression in a group consisting of African Americans. However, no follow-up studies have validated these findings. Thus, further research is needed to determine whether *SLC5A8* variants influence CRC susceptibility.

SUPPLEMENTARY MATERIALS

Supplementary Table 1

Primers used in the construction of the plasmids expressing wild-type *SLC5A8* or its variants

Supplementary Fig. 1

Effects of MG132 on SLC5A8 expression. HCT116 cells expressing WT *SLC5A8* or Val193Ile were treated with MG132 for 3, 6, 9, and 24 hours and subjected to immunoblotting. The data shown represent the mean ± standard deviation from three independent experiments analyzed by Student's *t*-test.

Supplementary Fig. 2

Effects of *SLC5A8* variants on cell viability. HCT116 (**A**) or DLD-1 (**B**) cells expressing WT *SLC5A8* or its variants were incubated with or without 1 mM pyruvate. After 48 hours, cell-viability assays were performed. The data shown represent the mean ± standard deviation from three independent experiments analyzed by Student's *t*-test and one-way analysis of variance followed by Dunnett's two-tailed test.

Supplementary Fig. 3

Pyruvate-dependent apoptosis in colorectal cancer cells expressing SLC5A8. HCT116 (**A**) or DLD-1 (**B**) cells expressing EV or WT *SLC5A8* were incubated with or without 1 mM pyruvate. After 48 hours, the cells were stained with annexin V and PI and analyzed via flow cytometry. Each bar shows the percentages of annexin V-positive and PI-negative cells. The data shown represent the mean ± standard deviation from three independent experiments analyzed by Student's *t*-test.

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