

## OIP5 is a highly expressed potential therapeutic target for colorectal and gastric cancers

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Previously, we reported that overexpression of Opa (*Neisseria gonorrhoeae* opacity-associated)-interacting protein 5 (OIP5) caused multi-septa formation and growth defects, both of which are considered cancer-related phenotypes. To evaluate OIP5 as a possible cancer therapeutic target, we examined its expression level in 66 colorectal cancer patients. OIP5 was upregulated about 3.7-fold in tumors and over 2-fold in 58 out of 66 colorectal cancer patients. Knockdown of OIP5 expression by small interfering RNA specific to OIP5 (siOIP5) resulted in growth inhibition of colorectal and gastric cancer cell lines. Growth inhibition of SNU638 by siOIP5 caused an increase in sub-G1 DNA content, as measured by flow cytometry, as well as an apoptotic gene expression profile. These results indicate that knockdown of OIP5 may induce apoptosis in cancer cells. Therefore, we suggest that OIP5 might be a potential cancer therapeutic target, although the mechanisms of OIP5-induced carcinogenesis should be elucidated. [BMB reports 2010; 43 (5): 349-354]

### INTRODUCTION

The human genome project has greatly increased our knowledge of the molecular mechanisms of human cancer. The identification of drug targets with high anti-tumor activity and low toxicity is crucial for the development of anti-tumor agents. In this manner, a variety of platform technologies have been developed for the identification and verification of anti-cancer therapeutic targets. In the era of personalized medicine, the development of patient-oriented cancer-specific gene therapies may become invaluable for the treatment of cancer.

OIP5 (GenBank Acc. No. NM\_007280) was originally iden-

tified as an Opa (*Neisseria gonorrhoeae* opacity-associated)-interacting protein (1). OIP5 is homologous to OIP4, which is expressed in tumors and some normal tissues (2). Recently, OIP5 was reported as LINT-25, since it interacts with lamina-associated polypeptide 2 $\alpha$  (LAP2 $\alpha$ ) (3). The level of LINT-25 protein was upregulated during G1 phase in proliferating cells and upon cell cycle exit in quiescent, senescent and differentiating cells. OIP5 was also reported as Mis18beta, which is essential for the structure and function of centromeres/kinetochores and is accumulated specifically at the telophase-G1 centromere (4). Overall, OIP5 may have unique, LAP2 $\alpha$ -linked functions in chromatin organization and cell cycle control (2, 3).

OIP5 has gained interest in the identification of molecular targets in gastric/liver carcinogenesis due to its biological function (5). In a previous report, we demonstrated that overexpression of OIP5 in the fission yeast *Schizosaccharomyces pombe* caused multi-septa formation and growth defects, both of which are considered cancer-related phenotypes (5). OIP5 was expressed at a high level in liver and gastric cancer cell lines. The transient expression of OIP5 in NIH3T3 cells resulted in a 2-fold increase in proliferation rate, highlighting its oncogenic properties (5). Further, Nakamura *et al.* reported that OIP5 was upregulated in tumor tissue compared to normal tissue in gastric cancer patients, suggesting that OIP5 may be a novel immunotherapy target for patients with gastric cancer (6).

In this study, OIP5 was evaluated as a possible cancer therapeutic target by examining its expression levels in 66 colorectal cancer patients and its knockdown effects in colon and gastric cancer cell lines. Here, we report that OIP5 was expressed at high levels in 87.9% of colorectal cancer tissues and that knockdown of OIP5 expression by siRNA induced apoptosis, thus suggesting that OIP5 is a potential therapeutic target for the treatment of colorectal and gastric cancers.

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## RESULTS AND DISCUSSION

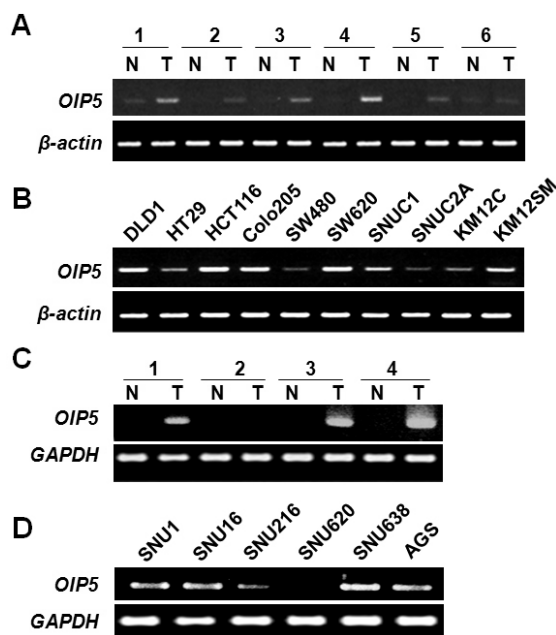
### Upregulation of OIP5 in colorectal cancer tissues was identified by microarray

Currently, colorectal cancer is the second most common cancer both in terms of incidence and mortality in Europe and the US. To evaluate whether or not OIP5 can be used as a potential therapeutic target for colorectal cancer, the expression profile of OIP5 in colorectal cancer patients was examined. Microarray analysis of tissues from 66 tumors and 9 normal tissue samples was carried out using a 48K Illumina oligonucleotide chip as described in the Materials and Methods section. Expression of OIP5 in colorectal cancer tissues was significantly upregulated 3.7-fold on average ( $P < 2.52E-06$ ; unpaired t-test) compared to that in normal tissues (Fig. 1). Further, OIP5 was upregulated over 2-fold in 58 of 66 (87.9%) tumors. However, there were no significant differences in OIP5 expression between recurrent and non-recurrent ( $P = 0.4597$ ) or between stage II and III ( $P = 0.5612$ ) colon tumors (Fig. 1C, D).

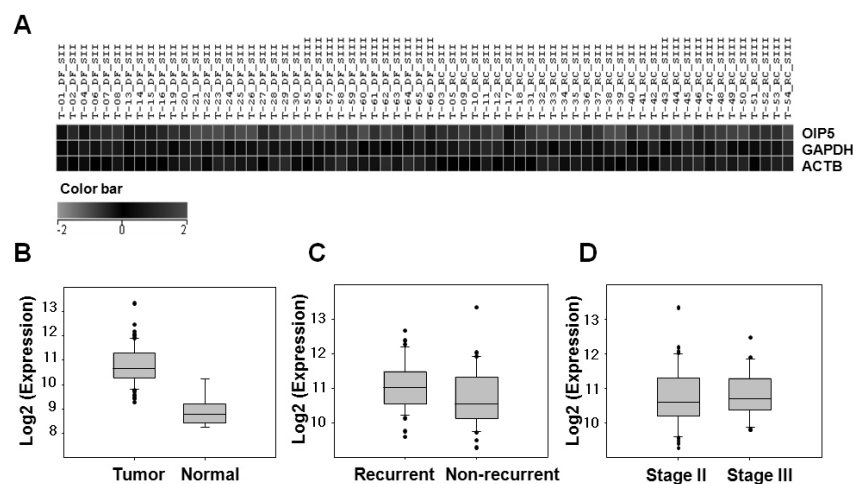
### Overexpression of OIP5 mRNA by RT-PCR in colorectal and gastric cancer tissues

The levels of OIP5 mRNA in 6 colorectal cancer tissues paired with adjacent nontumorous region tissues were examined by reverse transcription polymerase chain reaction (RT-PCR) (Fig. 2A). OIP5 expression was higher in colorectal cancer tissues compared to normal tissue, which is consistent with the results of the microarray analysis. When the expression of OIP5 in colon cancer cell lines was examined, it was observed that all cells differentially expressed the OIP5 gene (Fig. 2B), suggesting that OIP5 is regulated in a cell type-dependent manner. In addition, we examined whether OIP5 was overexpressed in gastric cancer tissues and cell lines (Fig. 2C, D). As expected, the gastric cancer cell lines SNU1, SNU16, SNU216, SNU638

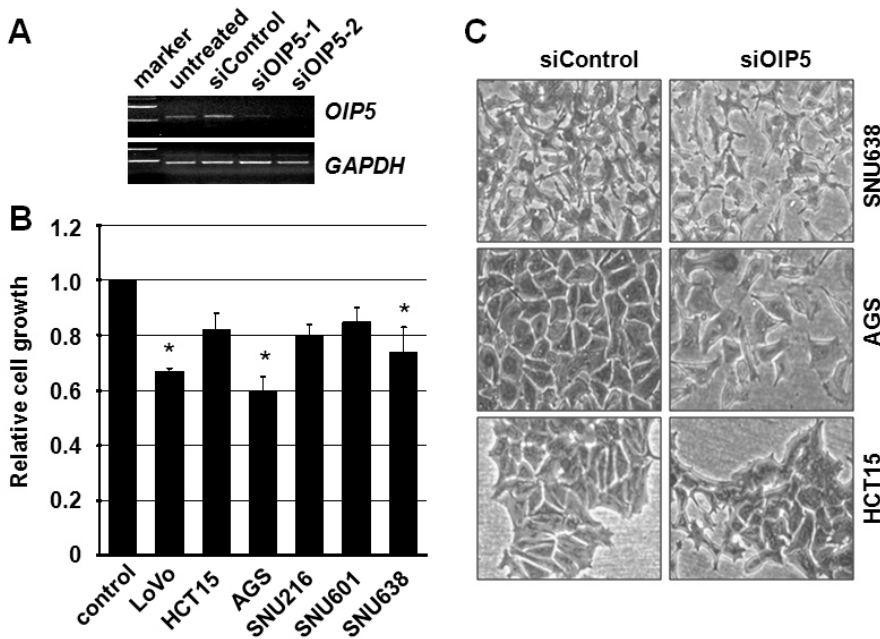
and AGS strongly expressed OIP5 mRNA. These results imply that OIP5 may be used as a biomarker for the diagnosis of colorectal and gastric cancer, or as a candidate therapeutic target for cancer treatment.



**Fig. 2.** RT-PCR analysis of OIP5 in colorectal and gastric cancer tissues and cell lines. Total RNAs were extracted from paired tumorous (T)-nontumorous (N) tissues and from cancer cell lines, and the mRNA expression levels of OIP5 were examined by RT-PCR. GAPDH and  $\beta$ -actin were used as internal reaction controls. (A) Colorectal cancer tissues. (B) Colon cancer cell lines. (C) Gastric cancer tissues. (D) Gastric cancer cell lines.



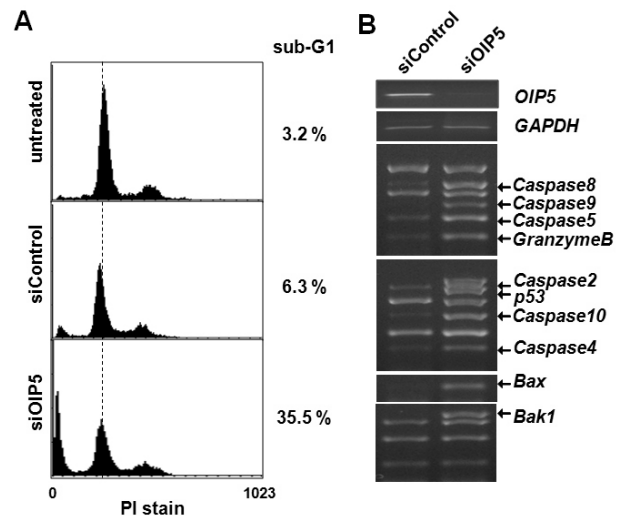
**Fig. 1.** Overexpression of OIP5 in colorectal cancer tissues. (A) Pattern of OIP5 expression in 66 colon tumors. Array data were processed and analyzed by Illumina BeadStudio version 3.0 software. Red and green colors represent higher and lower relative expression of OIP5, respectively, compared to mean OIP5 expression in 9 normal colon tissue samples. The expression levels of GAPDH and ACTB are shown for comparison. DF and RC indicate non-recurrent and recurrent cancers, respectively. SII and SIII indicate stage II and stage III cancers, respectively. (B) Comparison of OIP5 expression in tumor and normal tissues. (C, D) Comparison of OIP5 expression in colon cancer subgroups: (C) Recurrent ( $n = 32$ ) vs. Non-recurrent ( $n = 34$ ); (D) Tumor stage II ( $n = 42$ ) vs. stage III ( $n = 24$ ).



**Fig. 3.** Cell growth inhibition in OIP5-suppressed cells. (A) After SNU638 cells were treated with siOIP5-1 and siOIP5-2, the mRNA level of OIP5 was determined using RT-PCR. siRNA specific to GFP was used as a negative control. (B, C) After colorectal and gastric cancer cell lines were treated with siOIP5-2 or siControl, relative cell growth was determined (B) and SRB-stained cells were photographed (C) as described in the Materials and Methods section. \*P < 0.05.

#### OIP5-suppressed cells showed a decreased cell growth

RNA interference (RNAi), which is a normal cellular mechanism leading to the highly specific degradation of corresponding mRNA, can be used to study the function of genes of interest and for reverse genetics in invertebrate model systems (7-9). RNAi using small interfering RNA (siRNA) (19-21 bp) has been recognized as an efficient method for identifying gene therapy targets in various diseases (8, 10). Since OIP5 was highly expressed in colorectal cancer tissues, which suggests it may be involved in carcinogenesis, we investigated whether or not OIP5 knockdown induces growth inhibition of cancer cells. Two siRNAs specific to OIP5, siOIP5-1 and siOIP5-2, were introduced into various cancer cell lines, and the degree of cell growth inhibition was determined by SRB staining assay (Fig. 3). Cells treated with siRNA specific to GFP were used as a control. After SNU638 cells were transfected with siOIP5-1 and siOIP5-2, cells were analyzed by RT-PCR to verify OIP5 suppression (Fig. 3A). When colorectal and gastric cancer cell lines were treated with siOIP5-2, growth inhibition assay (Fig. 3B) and microscopic observation (Fig. 3C) were carried out. Treatment with siOIP5 caused significant growth inhibition of the gastric cancer cell lines AGS, SNU216, SNU601 and SNU638, as well as of the colorectal cancer cell lines LoVo and HCT15. The growth of colo205 cancer cells was only weakly inhibited, indicating a cancer cell-specific RNAi effect for siOIP5. The siOIP5-induced growth inhibition of cells was observed in SNU638, AGS and HCT15 cells under a microscope (Fig. 3C), and these observations were consistent with the SRB assay. These data suggest that OIP5 contributes to cell



**Fig. 4.** Apoptosis induction in OIP5-suppressed cells. SNU638 cells were treated with siOIP5 or siControl, and cells were analyzed. (A) Cells were harvested, stained with PI and analyzed using a flow cytometer. Cell population (%) in sub-G1 phase was indicated. (B) Cells were lysed and their RNAs were extracted for RT-PCR analysis. Induced pro-apoptotic genes (arrows) are indicated at the right.

growth and may be involved in colorectal and gastric tumorigenesis. Therefore, OIP5 may be a potential therapeutic cancer target.

### Induction of apoptosis in OIP5-suppressed cells

Apoptosis, an essential physiological process and type of cell death, is associated with a variety of biological events. We next examined whether or not the knockdown of OIP5 caused growth inhibition of colorectal and gastric cancer cells as shown in Fig. 3 was due to the induction of apoptosis. Growth inhibition of SNU638 by siOIP5 was examined by flow cytometric analysis (Fig. 4A). When siOIP5 was introduced into SNU638 cells and incubated for 72 h, the sub-G1 portion of DNA was increased 6-fold higher than that of control cells treated with siRNA specific to GFP, indicating that OIP5-suppressed cells may easily advance through the apoptotic process. To further investigate the knockdown effect of OIP5 in SNU638 cells, RT-PCR of apoptosis-related genes was performed in OIP5-suppressed cells (Fig. 4B). We detected increased mRNA levels of the pro-apoptotic Bax and Bak factors (11, 12). These proteins regulate mitochondria-mediated apoptosis by directly modulating mitochondrial membrane permeability (13) to initiate a parallel pathway of caspase activation and apoptosis (14). Further, the expression level of Granzyme B was increased in OIP5-suppressed cells. Granzyme B, a serine protease of cytotoxic T-lymphocytes (CTL) and NK cells, triggers apoptotic cell death (15) and is able to directly activate caspases 3, 7, 8 and 10. In addition, knockdown of OIP5 resulted in the upregulation of pro-apoptotic proteins, including caspases 8, 9, 2, 5, 10 and 4. Expression of anti-apoptotic Mcl-1, which inhibits the function of Bax downstream (16), was decreased in OIP5-suppressed cells (data not shown). These results suggest that OIP5 suppression might be a therapeutic target in colorectal and gastric cancers, although further studies to determine OIP5-directed function in tumorigenesis are needed.

In this study, we evaluated OIP5 as a potent target for cancer therapeutics using RNAi in colorectal and gastric cancer cells. We performed the knockdown of OIP5 via siOIP5 and induced growth inhibition in both cell lines. Growth inhibition in OIP5-suppressed cells caused an increase in sub-G1 DNA content and resulted in the expression of pro-apoptotic genes such as caspases and Bax. Recently, several studies using siRNA-based therapeutics have been carried out in various tumor models for the development of therapeutic siRNA agents (17-20). Extensive efforts are being made to overcome critical constraints, including off-target and non-specific effects, delivery system, as well as the relatively poor stability of siRNA (10, 17, 21-23). Many trials evaluating siRNA for therapeutic purposes in various diseases have been performed, and some siRNAs are currently being tested in human clinical trials or will be tested in the near future (10, 22). We suggest that OIP5 might be a promising siRNA cancer target for patient-oriented cancer therapeutics. Further, OIP5 could be a potential target for the identification of compounds that mitigate growth inhibition by targeting OIP5 genes.

## MATERIALS AND METHODS

### Cancer tissues and cell lines

Tumor and adjacent normal mucosa tissues of 66 colorectal cancer patients with stages II and III cancer were obtained from Samsung Medical Center (Seoul, Korea). Colorectal cancer cell lines such as LoVo, HCT15, DLD1, HT29, HCT116, Colo205, SW480, SW620, SNUC1, SNUC2A, KM12C and KM12SM, as well as gastric cancer cell lines such as SNU1, SNU16, SNU216, SNU601, SNU620, SNU638 and AGS were used in this study. Cells were cultured in RPMI 1640 medium (Gibco-BRL, Grand Island, NY, USA) or Dulbecco's Modified Eagle's Medium (DMEM; Gibco-BRL), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco-BRL) and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) and maintained in a 37°C incubator containing a humidified atmosphere of 5% CO<sub>2</sub>.

### Microarray analysis of colorectal cancer tissue

Total RNA was extracted from colorectal cancer tissues of 66 patients with stages II and III cancer using a Qiagen kit according to the manufacturer's manual. Quantified RNA was then used for microarray analysis on 48K Illumina chips (Illumina Inc., San Diego, CA, USA). Total RNA samples were labeled using the Illumina TotalPrep RNA Amplification Kit (Ambion, Applied Biosystem, CA, USA) for cDNA synthesis and *in vitro* transcription. Single-stranded RNA (cRNA) was generated and labeled by incorporating biotin-NTP (Ambion). A total of 1.5 µg of biotin-labeled cRNA were hybridized at 58°C for 16 h to Illumina's Sentrix Human-6 v2 Expression BeadChip (Illumina). The hybridized biotinylated cRNA was detected with streptavidin-Cy3 and quantified using a Illumina's BeadArray Reader Scanner (Illumina) according to the manufacturer's instructions. A total of 66 tumors and adjacent normal mucosa samples were analyzed. Array data were processed and analyzed by Illumina BeadStudio version 3.0 software (Illumina). Scanned data were normalized by the quantile-quantile normalization method and log-transformed by base two. The T-test was used to judge the significance of gene expression differences between the two groups. Java Treeview (<http://jtreeview.sourceforge.net/>) was used to visualize the pattern of gene expression in tumors compared to normal tissues.

### Measurement of mRNA levels by RT-PCR

The mRNA levels of OIP5 in the colorectal cancer tissue samples were measured by RT-PCR as previously described (5). The OIP primers used were 5'-GGCTGGGGGCTGAGGAGCC A-3' for the N-terminus and 5'-CACTCACTCAGAATCTTCA-3' for the C-terminus. To determine the mRNA levels of genes involved in apoptosis, the ExprssGene kit (SeeGene Inc., Seoul, Korea) was used as described in the manufacturer's manual.

### Synthesis and transfection of siRNA

The two different siRNAs for OIP5 were used in this study (siOIP5-1, 5'-CAUUGAAGGUUCACUCAAAATT-3'; siOIP5-2, 5'-CCUAGUUGGCAUUGAAGGUTT-3'). Control GFP-siRNA was 5'-CACACCACATCTTCCGTAC-3'. RNA oligonucleotides were custom synthesized by Samchunlly Pharm. Co., LTD (Seoul, Korea) with an overhang of 2 thymidine residues (dTdT) at the 3' end. Cells were seeded in 6-well plates in RPMI-minimal essential medium (OptiMEM; Invitrogen, Grand Island, NY, USA) supplemented with 10% Fetal Bovine Serum (FBS) to give 40% to 50% confluency at the time of transfection. The siRNAs were introduced into cells at a concentration of 50 nM using Hiperfect (Gibco BRL) according to the manufacturer's recommendations. Control cells were transfected with siRNA specific to GFP. After incubating cells for 72 h, knock-down of OIP5 was examined by RT-PCR, and the growth inhibition of cells was observed under a microscope by SRB staining.

### SRB assay

The sulforhodamine B (SRB) assay was used for cytotoxicity of siRNA-treated cells. After siRNA was transfected for 72 h, cells were stained with SRB, washed with 1% acetic acid, and SRB-mediated color development was measured at 510 nm with SRB-stained cells being photographed.

### Flow cytometry analysis

Analysis of cellular DNA content by flow cytometry was performed. Cells were fixed with 5 ml of ice-cold 75% ethanol for at least 24 h at 4°C and then stained with propidium iodide solution (2 mg/ml in distilled water) in the dark for at least 30 min. The stained cells were analyzed using a flow cytometer (FACSCalibur; Becton Dickinson, San Jose, CA, USA). Histograms were analyzed using Modifit software (Verity Software House, Tosham, ME, USA).

### Statistics

The data are expressed as mean  $\pm$  SD and statistical significance was assessed by 2-tailed unpaired Student's t test.  $P < 0.05$  was considered statistically significant.

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