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## Original Research Article

# Septin 9 hypermethylation contributes to migration and resistance to drug treatments in colon cancer

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### Abstract

**Purpose:** To examine septin 9 gene-promoter methylation content in colorectal cancer and establish its significance in cancer progression and chemoresistance.

**Methods:** Patient samples and colorectal cancer cell lines (CRC) were evaluated for septin 9 expression and promoter hypermethylation content. Septin 9 promoter methylation and expression in cells were perturbed by 5-AZA (5-aza-2'-deoxycytidine) treatments or overexpression and probed for changes in Rho A signaling, cell proliferation, and migration. Finally, the significance of septin 9 methylation in chemoresistance was probed using apoptotic assays in CRC cells and in a xenograft tumor model.

**Results:** Expression analysis showed a reduction in septin 9 levels in tumor tissues ( $p < 0.001$ ) and cell lines ( $p < 0.01$ ), while an increase in septin 9 promoter methylation was seen, respectively ( $> 2$ -fold;  $p < 0.01$ ). Increasing septin 9 levels in CRC cells by 5-AZA treatments or overexpression showed decreased Rho A signaling and cell migration ( $p < 0.01$ ), whereas cell proliferation remained unaffected. Furthermore, increasing septin 9 levels also exhibited increased cisplatin-induced apoptosis in CRC cells and reduced chemoresistance in the mouse (~2-fold;  $p < 0.01$ ).

**Conclusion:** Septin 9 promoter hypermethylation reduces septin 9 expression and promotes migration and chemoresistance.

**Keywords:** Septin 9, Hypermethylation, Colorectal cancer, Drug resistance, Rho A signaling

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## INTRODUCTION

Colorectal cancer (CRC) is one of the leading cancer types causing mortality worldwide [1]. Treatment options such as surgical resection, radiotherapy and chemotherapy are widely used but its outcomes are largely based on the stage of cancer [2,3]. The patients with stage I CRC exhibit 94.1 % five-year survival rate while it is significantly reduced to 24 % in stage IV disease [2,3]. Thus early detection of the cancer has a significant impact on the treatment outcome.

Changes in DNA methylation profile of critical genes in cells is one of the early molecular changes in cancer progression [4,5]. These epigenetic events combined with disease-causing mutations provide a selective advantage for cancer initiation and progression. Multiple studies have shown that septin 9 gene spanning a region of 219 kb in the genome and encoding different isoforms is epigenetically modified in the promoter region, and can be used as a diagnostic test for CRC [6-8].

Recent studies have shown that septin 9 promotes angiogenesis and facilitate tumor

growth in the hetero-transplant model [9]. In contrast, breast cancer-related studies have indicated distinct cellular functions and suggested a tumor-suppressor role [10]. Despite that, it remains unclear how septin 9 hypermethylation affects gene expression in normal and neoplastic colon tissues and cells, and the underlying molecular mechanisms triggered due to aberrant septin 9 methylation in tumor development and chemoresistance, particularly colorectal cancer (CRC).

## EXPERIMENTAL

### Cells and tissue samples

SW480, CCD-18Co, HT-29, HS6751, HCT-116 and LoVo cell lines were purchased from American Type Culture Collection (ATCC) and maintained as per the instructions provided. For patient samples, colon neoplastic and adjacent non-transformed tissue samples were obtained from institute's pathology archive following institutional review board approval from The People's Hospital of Laiwu City, Laiwu, Shangdong (Protocol no. 2705S). Tissue samples were then processed accordingly for different procedures.

### Quantitative realtime polymerase chain reaction (qRT-PCR)

Total RNA was extracted from cells and tissue samples using RNAeasy kit (Qiagen) and cDNA synthesis was performed as described previously [11]. Gene-specific PCR was performed CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, USA) using specific TaqMan probes and universal PCR Master Mix (Life Technologies, USA). The TaqMan probes used for analyzing the gene expressions were: Septin 9-Hs00246396\_m1; GAPDH -Hs02758991\_g1 (Applied Biosystems, USA)

### Pyrosequencing assays targeting *Septin 9* promoter regions

DNA was extracted from cells and patient samples and evaluated for the status of methylation in CpG dinucleotides located in septin 9 promoter region. We utilized the Qiagen PyroMark assay kit (Qiagen, USA) and investigated the methylation status as described previously [12] using the primer sets: Fwd: GATCTAGCCTAGGGTTCCAG, Rev: ACCGTTTCGTGCAGACGAG (5'-biotin labeled) and the sequencing probe: TTGACGCGGTGCTAG. Briefly, the PCR products were suspended using the PyroMark Q24 (Qiagen) following the manufacturer's

protocol, and the methylation status was quantified with an estimated score between 0-100 with 0 being no methylation and 100 represents complete methylation for all of the CpG dinucleotide in the region. The threshold in the assay was > 5 %.

### Immunoblotting

Total cell lysates were prepared using RIPA buffer and proteins were separated on a 4 - 15 % SDS-PAGE gel under denaturing conditions. Proteins were then transferred to PVDF membrane, blocked and probed for specific proteins using antibodies: Septin 9 (1: 1000, Novus Biologicals, USA); Actin (1:2000, Cell Signaling Technology, USA); Rho A (1: 500, Cell Signaling Technology, USA). The membranes were then incubated with secondary antibodies conjugated with horseradish peroxidase followed by chemiluminescence substrate based development (Super Signal West Dura, Pierce Biotechnology, USA).

### Cell proliferation assays

Cell proliferation assays were performed using Alamar Blue assay (Invitrogen, USA) [11]. Briefly, control or overexpressing cells ( $1 \times 10^4$  per well) were seeded in a 96-well plate and after 16 h, 50  $\mu$ L of Alamar reagent (1 mg/mL) was added and incubated for 2 h. Change in Alamar blue oxidation is noted as a measure of cell density. These values were noted at different time points as indicated. Optical density values were noted at 570 nm using a Microplate reader and proliferation trend over time was plotted.

### Cell migration assays

Cell migrations assays were performed using a chemotaxis chamber (NeuroProbe, Gaithersburg, MD) [13]. Briefly, cells were seeded in the upper chemotaxis chamber and allowed to migrate for 24 h. After scraping the non-migrated cells from the top side, the cells that are migrated to the bottom side were stained using Diff-Quik Stain Set (Dade Behring, Eschborn, Germany) and quantified. At least five independent fields were randomly picked per sample and quantified.

### Immunohistochemistry

Tissue sections were deparaffinized with xylene and gradient ethanol washes, rehydrated, and antigen retrieved in sodium citrate buffer (pH 6.0). Tissue sections were then blocked with 5 % BSA and 0.2 % goat serum. Primary antibodies specific to septin 9 (Novus Biologicals, USA) were incubated (1:50 v/v), followed by secondary

antibody (1:100) and counterstained for the nucleus.

### Overexpression experiments

For overexpression, the coding DNA sequence of septin 9\_v 1 isoform was cloned into pcDNA3.1 and transfected into cells. Stable cells were selected with geneticin selection (G418-2 µg/mL; Invitrogen).

### Drug treatments

For cell-based experiments, cells were treated with cisplatin (5 µM) 24 h and evaluated for apoptosis by TUNEL assay. For mouse experiments, BALB/c (nu/nu) mice were inoculated with SW480 or LoVo cells (2.5 X 10<sup>6</sup> per mouse) subcutaneously and administered with cisplatin (5 µM/mouse) intraperitoneally (i.p.) on days 3, 6, 10, 14, 20. As the mice used in the study ranged in similar weight we used a same dose (5 µM/mouse) of injection. Tumor size was shown as long diameter time short diameter (mm<sup>2</sup>). All mouse experiments were performed with prior approval from the institute's animal care committee at The People's Hospital of Laiwu City, Laiwu, Shangdong (protocol no: 251216). Housing and all procedures using animals were performed in accordance with the Animals (Scientific Procedures) Act, 1986 (UK) (amended 2013) and reported as per the ARRIVE Guidelines for reporting animal research [14]. All mice were housed in individually ventilated cages (5 per cage) under specific pathogen-free (SPF) condition, temperature-controlled, and in a 12 h/12 h light/dark cycle. Animal weights were routinely monitored every other day.

### Detection of apoptosis

Apoptosis was detected using a TUNEL staining kit (Promega, WI) [15]. TUNEL staining was performed directly in cells and an absolute number of TUNEL positive cells in randomly picked microscopy fields were noted.

### Pulldown assay

RhoA activity in cell lysates was measured using Rho-binding pull-down assay as per the manufacturer's instructions (EMD Millipore, Germany). The ratio of guanosine triphosphate (GTP-bound) to total Rho was measured and used as an index for its activity.

### Statistical analysis

Data were analyzed using one-way ANOVA with statistical significance defined as  $p < 0.05$ . All analyses were performed using GraphPad Prism (version 6) and all values are listed as mean ± standard deviation (SD).

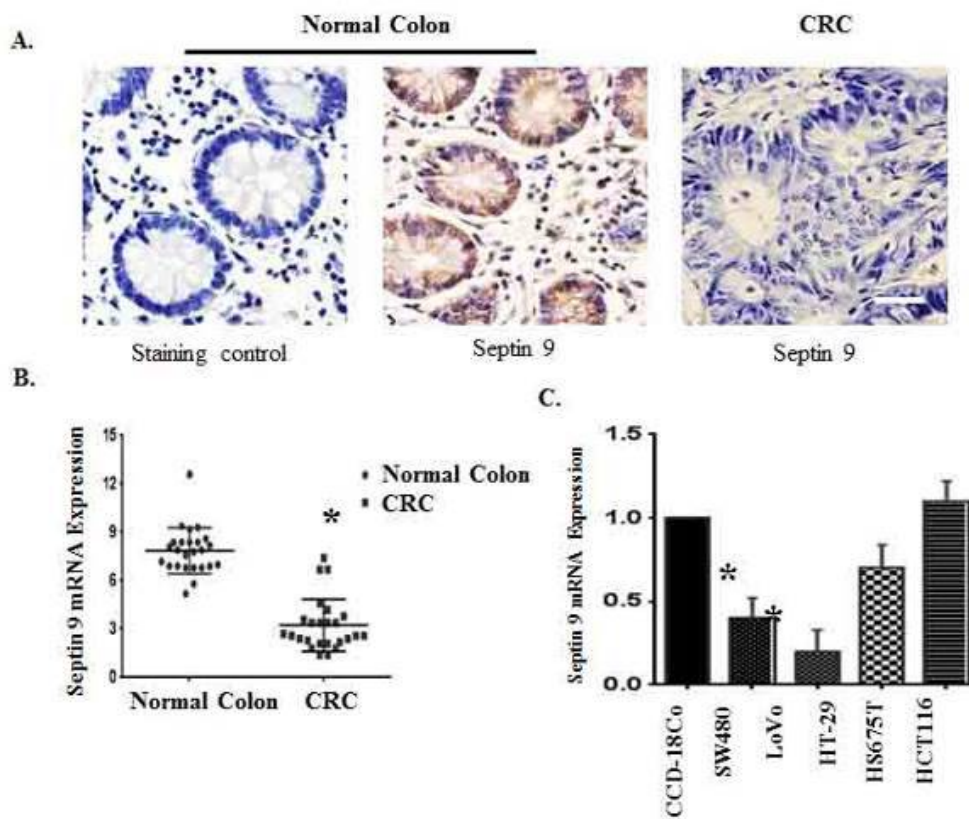
## RESULTS

### Septin 9 expression in colon cancer tissues and cell lines

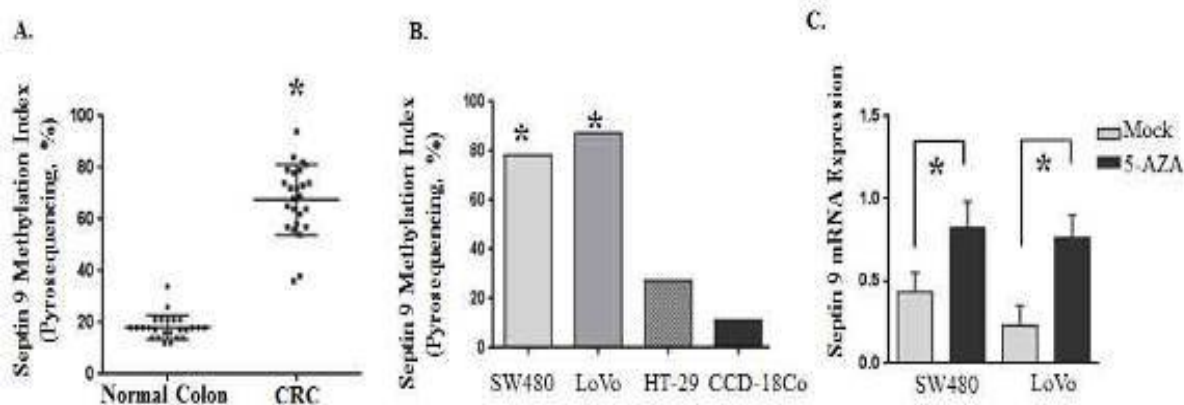
To begin with, we evaluated the expression of septin 9 in primary colon cancer tissues with their corresponding adjacent normal tissue regions as respective controls. The results as evaluated through immunostaining showed an absence or decreased expression of septin 9 in tumor tissues as compared to normal regions (Figure 1A). In addition, septin 9 mRNA analysis using PCR showed a similar decrease in septin 9 expressions in tumor tissues (~58 %;  $p < 0.001$ ) (Figure 1B). While, these results confirmed the septin 9 decrease in tumor samples, CRC cell lines showed a varied expression profile for Septin 9 in different cell lines. Our results in CRC lines as evaluated through septin 9 specific PCR showed that maximum decrease was seen in the most invasive cell line such as SW480 and LoVo cells (~50 %;  $p < 0.01$ ) while in other cell lines tested septin 9 mRNA were reduced but to varying levels (Figure 1C).

### Septin 9 hypermethylation causes reduced expression in tumor tissues and cell lines

To determine whether methylation status of the septin 9 promoter is involved in the decreased expression of septin 9 in tumor tissues and cell lines, we first evaluated the promoter methylation using a pyrosequencing assay targeting the promoter region of septin 9. The results showed an increased methylation in the septin 9 promoter region in tumor tissues compared to the adjacent normal tissues (> 3fold;  $p < 0.01$ ) (Figure 2A). Similarly, SW480 and LoVo cells exhibited hypermethylation in the promoter regions, compared to other cell lines (> 2.5 fold;  $p < 0.01$ ). In addition, 5-AZA (5-aza-2'-deoxycytidine), a demethylation agent, treatments of SW480 and LoVo cells increased septin 9 expression (Figure 2C). These results together indicate that hypermethylation of septin 9 promoter regions negatively regulates septin 9 expression.



**Figure 1:** Septin 9 expression in colon cancer patient samples and cell line  
**(A)** Septin 9 expression levels were evaluated by immunostaining in primary tissue samples. Representative cases are shown (40X). Normal colon cells exhibited expression of septin 9, while it is minimal or absent in carcinoma samples. Scale bar 5 μm. **(B)** Septin 9 mRNA expression profiles in patient samples. Expression levels from all the 25 CRC region specimens and matched normal colon region specimens were quantified by qRT-PCR. Septin 9 expression was significantly reduced in cancerous regions as compared to normal regions ( $8.1 \pm 0.95$  fold in normal regions versus  $3.4 \pm 1.72$  in cancer, \*  $p < 0.001$ ). **(C)** Septin 9 mRNA expression profiles in CRC cell lines. SW480 and LoVo cells showed a significant reduction compared to normal cells (CCD-18Co) (\*  $p < 0.01$ )

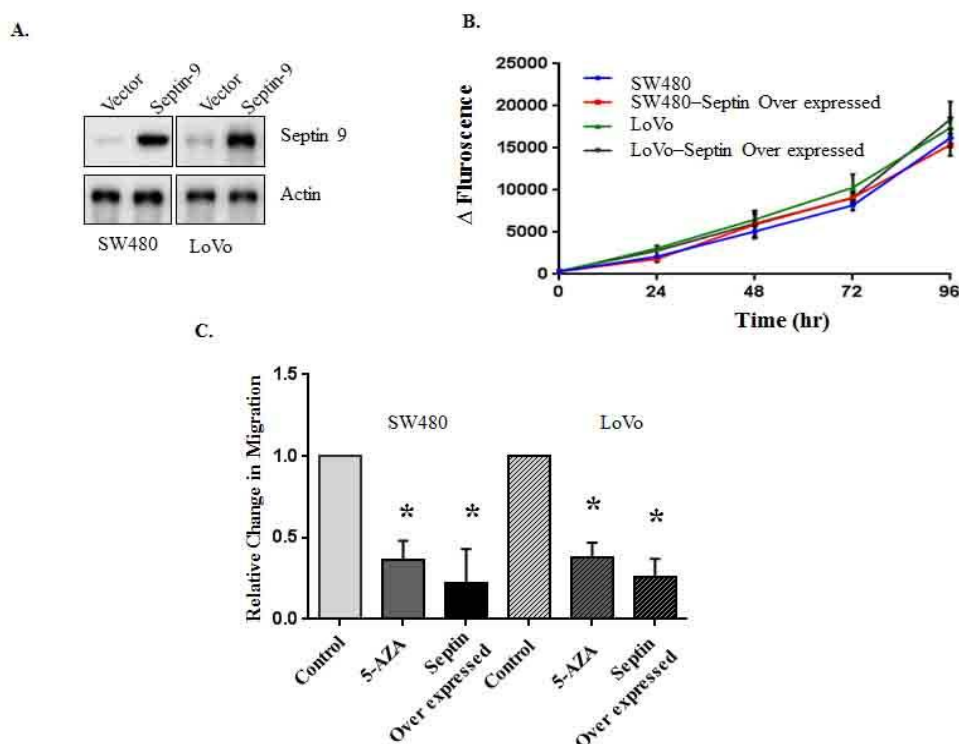


**Figure 2:** DNA methylation status inversely correlates with loss of septin 9 expression in CRCs.  
**(A)** The septin 9 promoter regions are highly methylated in CRC patients as opposed to normal colon regions (\*  $p < 0.001$ ). **(B)** SW480 and LoVo cell lines exhibited the high methylation in promoter regions of septin 9, whereas the HT-29 and CCD-18Co (normal) cells showed minimal promoter methylation. (\*  $p < 0.01$ ) **(C)** 5-AZA treatments in SW480 and LoVo cell lines led to significantly increased septin 9 levels. Values were normalized against baseline expression in CCD-18Co cells (\*  $p < 0.01$ )

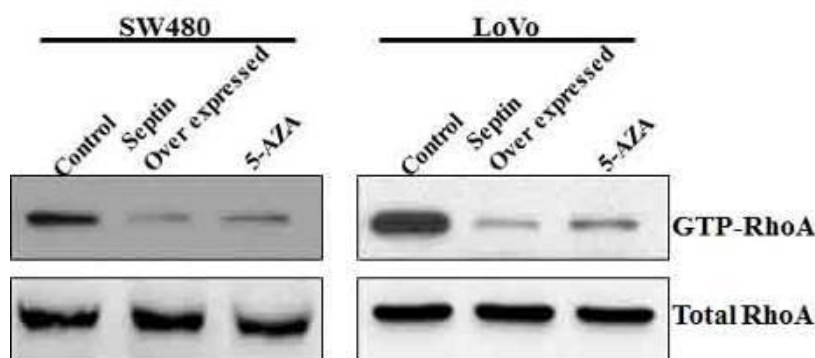
### Septin 9 suppresses colorectal cancer migration, but not proliferation *in vitro*

To understand the consequence of hypermethylation induced decreased septin 9 expressions in cancer progression, we constitutively overexpressed the septin 9 in SW480 cells and LoVo cells (~ 50 % reduced;  $p < 0.01$ ) (Figure 3A) and evaluated their proliferation and migration. The result showed that septin 9 overexpression significantly decreased migration for both the cell types with septin 9 overexpression, while no significant

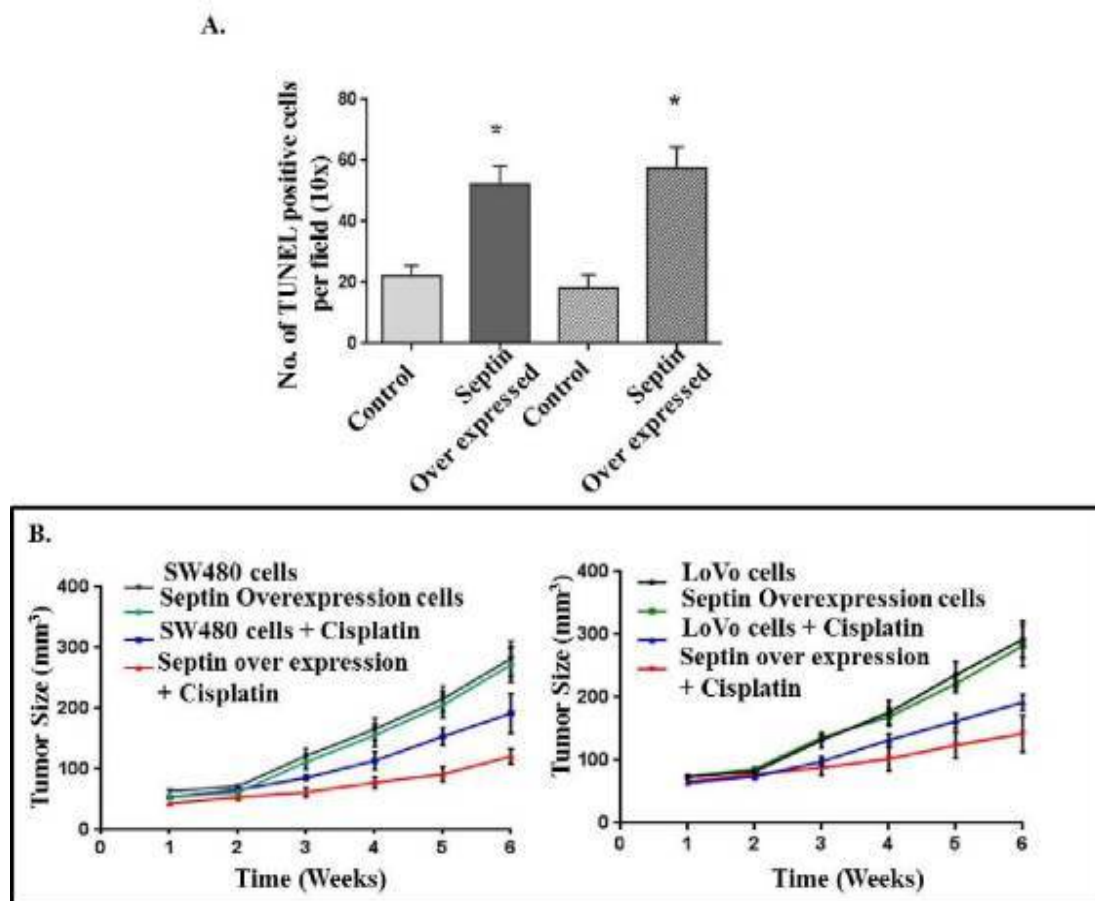
change in their cell proliferation rate *in vitro* (Figure 3B and Figure 3C) were noted. In addition, as septin 9 is modulating migration in both the cell types, which is usually regulated by GTPases, we chose to evaluate for changes in Rho A. The results showed that septin 9 overexpression significantly decreased the Rho A activation pathway as determined by reduced RhoA-GTP levels as compared to total Rho A levels (~ 80 % reduced;  $p < 0.01$ ). These results suggest that septin 9 suppresses cell migration by negatively modulating the Rho A pathway (Figure 4).



**Figure 3:** Septin 9 overexpression correlates with decreased migration but not proliferation (A) Stable cells of both SW480 and LoVo cells overexpressing septin 9 were prepared. Immunoblot of septin 9 and actin was shown. (B) Alamar blue assay showing no change in proliferation with overexpressing septin 9. (C) Decrease in migration were noted in both SW480 and LoVo cells upon overexpressing septin 9 or with 5-AZA treatment (\* $p < 0.01$ )



**Figure 4:** Septin 9 expression levels are associated with altered RhoA signaling. Increased Rho-GTP was present in SW480 cells and LoVo cells, and is reduced with overexpression of septin 9 or 5-AZA treatments (~ 80 % reduced;  $p < 0.01$ ). Representative immunoblots are shown



**Figure 5:** Septin 9 might contribute to drug-induced apoptosis *in vitro* and *in vivo*

(A) SW480 cells and LoVo cells with or without overexpression of septin 9 were treated with cisplatin (5  $\mu$ M) and evaluated for apoptosis. TUNEL positive staining was quantified. Overexpression of septin 9 enhances apoptosis (\* $P < 0.05$ ) (B) Xenograft models of SW480 and LoVo cells with or without septin 9 overexpression were established and treated with cisplatin. Changes in tumor size were measured over time. Septin overexpression enhanced the effect of cisplatin-induced reduction in tumor size in both cell types from third week onwards (\* $P < 0.01$ )

### Septin 9 expressions contribute to drug-induced apoptosis *in vitro* and *in vivo*

We evaluated the efficacy of cisplatin in SW480 and LoVo CRC cells with the over-expression of septin 9. The results showed an increased drug-induced apoptosis as measured by TUNEL assay in overexpressing cells than control cells (> 2 fold enhanced;  $p < 0.05$ ) (Figure 5A). In addition, overexpression of septin 9 significantly enhanced the drug efficiency in a xenograft tumor model (~ 2 fold enhanced;  $p < 0.01$ ). These increased efficiencies were correlating with increased TUNEL positive cells in septin 9 overexpressing cells (Figure 5B).

## DISCUSSION

It is well established now that DNA methylation in the genome could modulate gene expression [16]. In particular, multiple CpG-rich promoter regions were extensively studied over the last

decade and associated the methylation content with effect on transcription [17,18]. Similar hypermethylation content was also previously identified in promoter regions of septin 9 and implicated for modulating expression in breast cancer cells [19]. In the current study, we observed that septin 9 expressions in tissues correlate with methylation content in the promoter regions in CRC patient samples. This notion is further supported by the decreased septin 9 expressions in colon cancer cells that are hypermethylated in promoter regions. In addition, we have shown that demethylation treatment restored at least partially the expression levels of septin 9, thereby suggesting that expression regulation in tumor conditions is achieved by aberrant mode of epigenetic modifications.

Since septin 9 is involved in modulating cytoskeleton [20], it is conceivable that suppression of septin 9 could modulate multiple

related processes. In accordance with this notion, other studies have also shown a function of septin 9 in multiple processes such as migration and cilia formation. Our data have shown that suppressed septin 9 expression facilitate migration and altered Rho A signaling, while proliferation remains unaffected. This suggests that septin 9 functions are context-dependent, and identification of other binding proteins or other means of context could aid us to understand septin 9 mediated effects in these processes further.

Multiple studies have highlighted increased resistance to chemotherapy in advanced CRC patients [21,22]. Given that septin 9 suppression is one of the early biomarker and prerequisite for CRC progression, we reasoned, if this would facilitate the development of chemoresistance in later stages. Our data have shown that septin 9 overexpression in cells increases efficiency in drug-induced apoptosis. While the specific molecular mechanism through which septin 9 modulates drug efficiency needs further investigation, similar proteins such as septin 4 effects in hepatocellular carcinoma suggests the involvement of increased expression of p21 and decrease in pro-caspases such as pro-caspase 7 and 3 [23].

Together, our data indicate that septin 9 plays a role in tumor suppression and the particular mode of its regulation such as hypermethylation affects its expression and related functions. However, it also worth mentioning that some of the earlier evidence have highlighted oncogenic function for septin 9 in breast cancer cells and attributed it to the different isoforms of septin 9 expression and localization. An intriguing question raised by our study in the context of these data is, how septin 9 hypermethylation contributes to variations in isoform expression in colon tissues, and how the different isoforms would affect CRC progression. While we have limited our analysis to septin 9\_v1 isoform, future studies aimed at understanding these different isoform expression patterns in colon tissues and its relation to hypermethylation content and chemoresistance would provide more insights into these complex processes.

## CONCLUSION

The findings of this study provide the first set of evidence for hypermethylation-induced reduction of septin 9 expression, which facilitates the migration and invasion of CRC cells and development of chemoresistance. Thus, chemotherapy options with concurrent

treatments to increase septin 9 expression may improve outcomes in colorectal cancers.

## DECLARATIONS

### Acknowledgement

None declared.

### Conflict of Interest

No conflict of interest associated with this work.

### Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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