Methylation of *SLFN11* is a marker of poor prognosis and cisplatin resistance in colorectal cancer

Aim: The expression of human *SLFN11* was reported to sensitize cancer cells to DNA damaging agents. This study is to explore the epigenetic change and the function of *SLFN11* in human colorectal cancer (CRC). **Materials & methods:** Six CRC cell lines and 128 primary CRC samples were used. **Results:** *SLFN11* was methylated in 55.47% (71/128) of primary CRC. The expression of *SLFN11* was regulated by promoter region methylation. Methylation of *SLFN11* was significantly associated with age, poor 5-year overall survival and 5-year relapse-free survival (all $p < 0.05$). *SLFN11* suppressed CRC cell growth both *in vitro* and *in vivo* and sensitized CRC cells to cisplatin. **Conclusion:** *SLFN11* is frequently methylated in human CRC, and the expression of *SLFN11* is regulated by promoter region methylation. Methylation of *SLFN11* reduced the sensitivity of CRC cells to cisplatin.

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Colorectal cancer (CRC) is the third most common cancer and the fourth most common cause of cancer-related death worldwide [1]. Incidence is low at ages younger than 50 years but increases significantly with age. Median age at diagnosis is approximately 70 years in developed countries. However, rapid increases of CRC incidence have been noted with the changes in dietary patterns in China [2]. Most sporadic cases have chromosomal instability, an allelic imbalance at several chromosomal loci, chromosomal amplification and translocation [3]. Defects in DNA damage repair and checkpoint control can result in gene mutations and chromosomal instability to promote tumorigenesis [4,5]. Familial adenomatous polyposis and Lynch syndrome, which are caused by gene mutations in the DNA mismatch repair system, account for less than 5% of all CRC [6]. Aberrant methylation changes in DNA damage repair genes were reported to be an important mechanism of

CRC development [7].

The *Schlafen* (*SLFN*) gene family was first identified by Schwarz and co-workers in 1998 [8]. To date, five human *SLFN* genes have been identified [9,10]. *SLFN* gene products have been demonstrated to be involved in cell biological processes including proliferation, differentiation and immune function [11]. From the Cancer Genome Atlas database, *SLFN11* has a wide expression range [12]. The expression of human *SLFN11* was reported to sensitize cancer cells to DNA-damaging agents [13,14]. High *SLFN11* expression independently predicts overall survival (OS) in a group of ovarian cancer patients treated with cisplatin-containing regimens [13]. The role and epigenetic regulation of *SLFN11* in human CRC remain to be elucidated.

Materials & methods

Human tissue samples & cell lines

Six CRC cell lines (RKO, DLD1, SW620, LOVO, Ls180 and DKO) were examined

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Table 1. Primer sequences and PCR conditions.

Gene	Primer	Sense primer	Antisense primer	Amplicon size (bp)	Annealing temp (°C)	PCR cycles
<i>CANA1G</i>	U	5'-TTT TTT TGT TTT GTG TTT AGG TTT T-3'	5'-CCC TCT CAA AAC AAC TTC ACC A-3'	71	64	35
<i>CANA1G</i>	M	5'-TCG TTT CGC GTT TAG GTT TC-3'	5'-CTC GAA ACG ACT TCG CCG-3'	62	64	35
<i>IGF2</i>	U	5'-GGA GTG GTT TTG GTG TTG TTA TT-3'	5'-CCC AAC TCA ATT TAA ACC AAC A-3'	90	66	35
<i>IGF2</i>	M	5'-GCG GTT TCG GTG TCG TTA TC-3'	5'-CCA ACT CGA TTT AAA CCG ACG-3'	86	68	30
<i>RUNX3</i>	U	5'-GTT GGT GGA TTA TGT AGG TGA GTT T-3'	5'-ACT CAC CTT AAA AAC AAC AAA CAA CA-3'	115	66	35
<i>RUNX3</i>	M	5'-GCG GAT TAC GTA GGC GAG TTC-3'	5'-ACC TTA AAA ACG ACG AAC AAC G-3'	107	66	35
<i>MLH1</i>	U	5'-TTT TGA TGT AGA TGT TTT ATT AGG GTT GT-3'	5'-ACC ACC TCA TCA TAA CTA CCC ACA-3'	124	60	35
<i>MLH1</i>	M	5'-ACG TAG ACG TTT TAT TAG GGT CGC-3'	5'-CCT CAT CGT AAC TAC CCG CG-3'	115	60	35
<i>KRAS</i> exon 2		5'-AAG GTG AGT TTG TAT TAA AAG GTA CTG G-3'	5'-TGG TCC TGC ACC AGT AAT ATG C-3'	264	58	35
<i>BRAF-600E</i>		5'-TCA TAA TGC TTG CTC TGA TAG GA-3'	5'-CTT TCT AGT AAC TCA GCA GC-3'	251	60	35

in this study and maintained in 90% Roswell Park Memorial Institute (RPMI) 1640 media supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin. 293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. All cell lines were cultured in an atmosphere of 5% carbon dioxide at 37°C.

A total of 128 cases of primary CRC and five cases of noncancerous colorectal mucosa were collected from the Chinese PLA General Hospital in Beijing between May 2009 and November 2013. All cancer samples were classified according to WHO Classification of Digestive Tumors: the Fourth Edition [15]. This study was approved by the Chinese PLA General Hospital's institutional review board.

5-aza-2'-deoxycytidine treatment

CRC cell lines (RKO, DLD1, SW620, LOVO and Ls180) were split to a low density (30% confluence) 12 h before treatment with 2 μ M 5-aza-2'-deoxycytidine (5-AZA, Sigma, MO, USA). Growth medium conditioned with 5-AZA at 2 μ M was exchanged every 24 h for a total of 96 h. At the end of the treatment course, RNA was extracted from the cells as described below.

RNA isolation & semiquantitative reverse transcription PCR

Total RNA was extracted using Trizol reagent; cDNA

was synthesized according to the manufacturer's instructions (Invitrogen, CA, USA) and glyceraldehyde-3-phosphatedehydrogenase (*GAPDH*) was used as control. The *SLFN11* PCR primer sequences were as follows: 5'-AACGCCCGATAACCTTCACA-3' (forward) and 5'-CTAAGGGGAGGCCCACTAGA-3' (reverse). Thermal cycling parameters were as follows: 95°C 5 min; (95°C 30 s, 60°C 30 s and 72°C 30 s) 35 cycles; 72°C 5 min. The *GAPDH* PCR primer sequences were as follows: 5'-GACCACAGTCCATGCCATCAC-3' (forward) and 5'-GTC-CACCACCCTGTTGCTGTA-3' (reverse). Thermal cycling parameters were as follows: 95°C 5 min; (95°C 30 s, 63°C 30 s and 72°C 40 s) 25 cycles; 72°C 5 min.

KRAS & BRAF mutation detection, bisulfite modification, methylation-specific PCR & bisulfite sequencing

Genomic DNA from colorectal cell lines and CRC tissue samples were prepared by the proteinase-K method. *KRAS* codons 12 and 13 and *BRAF* codon 600 were amplified by PCR and sequenced according to previously reports [16,17]. The primer sequences are listed in Table 1. *SLFN11* methylation-specific PCR (MSP) and bisulfite sequencing (BSSQ) were performed as described previously [18]. DNA samples were then purified with the Wizard DNA Clean-Up System (Promega, CA, USA). MSP primers were designed as follows: 5'-ATTATTAG-

TAGCGTGACGGTTATC-3' (M-forward), 5'-CGA-CAAATATACAAATTAACCGCG-3' (M-reverse), 5'-TATATTATTAGTAGTGTGATGGTTATT-3' (U-forward) and 5'-ATACAACAAATATACAAATTAACACACA-3' (U-reverse). Thermal cycling parameters were as follows: 95°C 5 min; (95°C 30 s, 58°C 30 s and 72°C 40 s) 35 cycles; 72°C 5 min. BSSQ primers were designed including the MSP region in a 225-bp region upstream of the *SLFN11* transcription start site (-319 to -544 bp). BSSQ primers were designed as follows: 5'-TAGAAAAGTAGAAYGTTGGTAG-3' (forward) and 5'-CAAAAAATAAATCTTAAAAAC-3' (reverse). Thermal cycling parameters were as follows: 95° 5 min; (95° 30 s, 55° 30 s and 72° 40 s) 35 cycles; 72° 5 min. Methylation status was detected by MSP in four genes (*RUNX3*, *CACNA1G*, *IGF2* and *MLH1*) to represent CpG island methylator phenotype (CIMP) as described previously [19]. The primer sequences are listed in Table 1.

Construction of human full length *SLFN11* cDNA lentiviral expression vector & transient full length *SLFN11* cDNA pcDNA3.1(+) expression vectors

The human full length *SLFN11* cDNA

(NM_001104587) was cloned into the PCDH (pCDH-CMV-MCS-EF1-Puro) lentiviral plasmid (Genetimes ExCell Technology, Shanghai, China). Transfection was performed at a ratio of 1:3 (DNA mass:lipo mass) following the manufacturer's instructions for Lipofectamine 3000 Reagent (Invitrogen, CA, USA). Packaged recombinant lentiviruses were harvested from the supernatant of cell cultures at 48 h post-transfection. RKO and DLD1 cells were then infected with viral supernatant. Cells stably expressing *SLFN11* were selected with puromycin at a concentration of 2 µg/ml for 2 weeks.

For transient transfection, the human full length *SLFN11* cDNA (NM_001104587) was cloned into the pcDNA3.1(+) plasmid (Era Biotech, Shanghai, China). Transfection was performed according to the manufacturer's instructions by using Lipofectamine 2000 in SW620 cell (Invitrogen, CA, USA).

siRNA knockdown technique

Selected siRNAs targeting *SLFN11* and RNAi negative control duplex (Gene Pharma, Shanghai, China) were used in this study. The sequences were as follows: siRNA-1726 duplex (sense: 5'-CCAGGAUUAU-

Figure 1. Expression of *SLFN11* is silenced by DNA methylation in colorectal cancer cell lines. (A) RT-PCR shows the levels of *SLFN11* expression – (-) untreated; (+) 5-AZA treated; H₂O: double distilled water. GAPDH was used as internal control. (B) MSP results of *SLFN11* in colorectal cancer cell lines. (C) Bisulfite sequencing results: the double-headed arrow indicates the region of the MSP product. 5-AZA: 5-Aza-2'-deoxycytidine; Filled circles: Methylated CpG sites; IVD: *In vitro* methylated DNA; M: Methylated alleles; NL: Normal lymphocyte DNA; Open circles: Unmethylated CpG sites; RT: Reverse transcription; TSS: transcription start site; U: Unmethylated alleles.

Figure 2. *SLFN11* is frequently methylated in human primary colorectal cancer tissues and the role of *SLFN11* in overall survival and relapse-free survival in colorectal cancer patients. (A) Representative MSP results of *SLFN11* in normal colorectal tissues (N1, N2, N3, N4 and N5) and primary CRC tissues. (B) *SLFN11* methylation status is associated with RFS and OS of CRC patients.

CRC: Colorectal cancer; OS: Overall survival; RFS: Relapse-free survival.

UUGCGAUUAUATT-3'; antisense: 5'-UAUAUCG-CAAAUAUCCUGGTT-3'); RNAi negative control duplex (sense: 5'-UUC UCC GAA CGU GUC ACG UTT-3'; and antisense: 5'-ACG UGA CAC GUU CGG AGA ATT-3'). RNAi oligonucleotide or RNAi negative control duplex was transfected into *SLFN11* highly expressed DKO cells using Lipofectamine RNAiMAX (Invitrogen, CA, USA).

Cell viability assay

Cells were plated into 96-well plates at a density of 2×10^3 cells per well, and cell viability was measured by the methyl thiazolyl tetrazolium assay (KeyGEN Biotech, Nanjing, China) at 0, 24, 48 and 72 h. Absorbance was measured on a microplate reader of Thermo Multiskan MK3 (Thermo Fisher Scientific, MA, USA) at a wavelength of 490 nm.

The IC_{50} was also detected by the MTT assay. Cells were plated at a density of 3000 cells per well into 96-well plates. After overnight incubation, cells were treated with cisplatin at concentrations of 0, 1, 2.5, 5, 10, 25 and 50 μ M for 48 h.

Colony formation assay

SLFN11 stably expressed and unexpressed RKO and

DLD1 cells were plated onto 6-well plates in a total of 200 cells per well. After 2 weeks, cells were fixed with 75% ethanol for 30 min. Colonies were then stained with 0.5% crystal violet solution and counted. The experiment was performed in triplicate. Another method was employed in this study to further validate the results. The detailed protocol was described as previously [20]. In brief, *SLFN11* stably expressed and unexpressed RKO and DLD1 cells were plated at a confluency of 6×10^4 cells per 6-cm dish. Media were replenished every 2 days. After 7–10 days, cells were stained with crystal violet in formalin for 30 min. After excess stain was washed away, colonies were imaged and the percent plate coverage is indicated as determined from five independent areas per plate using ImageJ software.

After 48 h of transfection, the *SLFN11* expressing DKO cells and DKO cells with knockdown of *SLFN11* were treated as mentioned above.

Colony formation assay treated with cisplatin

This was performed as previously reported [21]. For the colony-forming assay, *SLFN11* stably expressed and unexpressed RKO and DLD1 cell lines were plated onto 6-well plates in a total of 1000 cells per well.

After overnight incubation, cells were treated with cisplatin (2 μ M) for 24 h and maintained in a drug-free medium for an additional 2 weeks until visible colonies were evident. After staining with the 0.5% crystal violet, the number of colonies of >0.1 mm in diameter was counted. After 48 h post transfection, DKO cells and DKO cells with knockdown of *SLFN11* were treated as mentioned above.

Flow cytometry for cell cycle & apoptosis

SLFN11 stably expressed and unexpressed RKO

and DLD1 cell lines were plated into 6-well plates at 1×10^6 cells per well. The cells were harvested at 48 h, washed with phosphate-buffered saline twice and stained according to manufacturer's instructions of Cycletest™ Plus DNA Reagent Kit (Becton, Dickinson and company, CA, USA). Samples were analyzed with an FACS Caliber flow cytometer (Becton, Dickinson and company).

Apoptosis was detected and quantified by Annexin V-FITC and propidium iodide staining. *SLFN11* stably expressed and unexpressed RKO and DLD1

Table 2. Clinic-pathological features and *SLFN11* methylation status in colorectal cancer patients.

Clinical parameter	Number (n = 128)	Methylation status		p-value
		Methylated n = 71 (55.47%)	Unmethylated n = 57 (44.53%)	
Age				0.02
	<50	30	19	
	≥50	98	38	
Gender				0.26
	Male	84	34	
	Female	44	23	
Tumor differentiation				1.00
	Poor	35	16	
	Moderate and well	93	41	
Lymphatic metastasis				0.86
	No	69	30	
	Yes	59	27	
Size				0.22
	<5 cm	66	33	
	≥5 cm	62	24	
Anatomical position				0.58
	Proximal	83	35	
	Distal	45	22	
T stage				1.00
	Stages I and II	64	29	
	Stages III and IV	64	28	
KRAS mutation				0.136
	Wide-type	99	48	
	Mutation	29	9	
CIMP				0.173
	CIMP(-)	90	44	
	CIMP(+)	38	13	
BRAF 600E mutation				1.00
	Wide-type	125	56	
	Mutation	3	1	

CIMP: CpG island methylator phenotype.

Table 3. Univariate and multivariate analysis of *SLFN11* methylation status with relapse-free survival or overall survival in colorectal cancer patients.

Clinical parameter	RFS				OS			
	Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
	HR (95% CI)	p-value	HR (95% CI)	p-value	HR (95% CI)	p-value	HR (95% CI)	p-value
Age (<50 vs ≥50 years)	1.391 (0.613–3.16)	0.430			1.395 (0.614–3.168)	0.427		
Gender (male vs female)	1.15 (0.60–2.22)	0.681			1.105 (0.571–2.137)	0.767		
Tumor location (proximal colon vs distal colon or rectum)	0.631 (0.307–1.3)	0.212			0.634 (0.308–1.306)	0.216		
Tumor size (<5 vs ≥5 cm)	1.25 (0.661–2.363)	0.492			1.228 (0.65–2.322)	0.527		
Differentiation (high or middle vs low differentiation)	0.465 (0.244–0.886)	0.020	0.225 (0.335–1.293)	0.225	0.465 (0.244–0.885)	0.020	0.672 (0.341–1.325)	0.251
Pathologic T stage (T3–4 vs T1–2)	1.797 (0.638–5.065)	0.268			1.864 (0.661–5.255)	0.239		
Pathologic N stage (N1–2 vs N0)	3.369 (1.668–6.805)	0.001	3.061 (1.465–6.394)	0.003	3.367 (1.668–6.795)	0.001	3.053 (1.456–6.401)	0.003
<i>SLFN11</i> absence of methylation	0.461 (0.229–0.93)	0.031	0.442 (0.219–0.891)	0.023	0.457 (0.227–0.923)	0.029	0.439 (0.217–0.887)	0.022
<i>KRAS</i> mutation (wild-type and mutation)	1.804 (0.909–3.580)	0.091			1.799 (0.906–3.572)	0.93		
CIMP(-) and CIMP(+)	1.277 (0.643–2.535)	0.485			1.299 (0.654–2.579)	0.455		

CIMP: CpG island methylator phenotype; HR: Hazard ratio; OS: Overall survival; RFS: Relapse-free survival.

cell lines were seeded in 6-well plates at a density of 1.5×10^6 cells/ml. Cells were treated with a concentration of 10 μ M cisplatin for 36 h. The cells were then harvested using 0.2% trypsin (no ethylenediaminetetraacetic acid), stained according to manufacturer's instructions of FITC Annexin V apoptosis Detection kit I (Becton, Dickinson and company), and analyzed by flow cytometry. After 48 h of transfection, DKO cells with and without knockdown of *SLFN11* were treated with a concentration of 10 μ M cisplatin for 36 h; apoptosis was detected.

Protein preparation & western blotting

Cells were lysed in icecold Tris buffer (20 mmol/l Tris; pH 7.5) containing 137 mmol/l NaCl, 2 mmol/l ethylenediaminetetraacetic acid, 1% Triton X, 10% glycerol, 50 mmol/l NaF, 1 mmol/l DTT and a protease inhibitor cocktail (Roche Applied Science, IN, USA). Protein concentrations were quantified using the BCA protein assay kit (CWBIO, Beijing, China). The protein lysates were then separated by SDS-PAGE and electroblotted onto polyvinylidene fluoride (PVDF)

membranes (Hybond-P). After blocking with 5% nonfat milk and 0.1% Tween-20 in tris buffered saline (TBS), the membranes were incubated with antibodies.

The antibodies for immunoblot analysis were as follows: *SLFN11* (1:200, Santa Cruz Biotechnology, CA, USA), cyclin D1 (1:500, Proteintech, IL, USA), cyclin E1 (1:500, Proteintech, IL, USA) and β -actin (1:1000 Beyotime Biotech, Jiangsu, China). β -actin antibody was used as a loading control. The blots were visualized using enhanced chemiluminescence (Pierce Bioscience, IL, USA).

SLFN11 unexpressed & re-expressed DLD1 cell xenograft mouse model

Animal experiments were reviewed and approved by the Chinese PLA General Hospital Experimental Animal Committee. *SLFN11* unexpressed and *SLFN11* stably expressed DLD1 cells were injected subcutaneously into the dorsal left side of 4-week-old male Balb/c nude mice ($n = 10$) weighing 12–18 g. Tumor volumes were measured every 4 days for 20 days starting 4 days after implantation. Tumor volumes were calculated accord-

ing to the formula: $V = L \times W^2/2$, where V represents the volume (mm^3), L represents largest length (mm) and W represents width (mm), respectively. Mice were sacrificed after 20 days.

Statistical analysis

Statistical analysis was performed using SPSS 17.0 software (SPSS, IL, USA). Either χ^2 or Fisher's exact tests were used to evaluate the relationship between methylation status and clinicopathological characteristics. The two-tailed independent samples' t-test was applied to determine the statistical significance of the differences between the two experimental groups. Survival rates were calculated by the Kaplan–Meier method, and differences in survival curve were evaluated using the log-rank test. Cox proportional hazards models were fit to determine independent associations

of *SLFN11* methylation with OS and relapse-free survival (RFS) outcome. Two-sided tests were used to determine significance, and $p < 0.05$ was considered statistically significant.

Results

The expression of *SLFN11* is regulated by promoter region methylation in CRC

The expression levels of *SLFN11* were examined by semiquantitative reverse transcription PCR (RT-PCR) to explore the regulation of *SLFN11* in CRC. *SLFN11* was expressed in Ls180 and DKO cells and unexpressed in RKO, DLD1, SW620 and LOVO cells (Figure 1A). Complete methylation was observed in RKO, DLD1, SW620 and LOVO cells; partial methylation was detected in Ls180; and unmethylation was found in DKO cells (Figure 1B). MSP results were

Figure 3. *SLFN11* suppresses colorectal cancer cell proliferation. (A) The effect of *SLFN11* on cell proliferation was measured by the MTT assay for 72 h – *SLFN11*: *SLFN11* re-expressed CRC cells. Control: empty vector. * $p < 0.05$, ** $p < 0.01$. (B) The effects of *SLFN11* on colony formation in colorectal cells; ** $p < 0.01$.

Figure 3. *SLFN11* suppresses colorectal cancer cell proliferation (cont. from previous page). (C) Cell phase distribution in *SLFN11* re-expressed cells (RKO and DLD1) and the effects of knockdown of *SLFN11* (DKO) on cell cycle. * $p < 0.05$, ** $p < 0.01$. (D) *SLFN11*, cyclin D1 and cyclin E1 were detected by western blot in *SLFN11* re-expressed (RKO and DLD1) cells and control cells. (E) *SLFN11*, cyclin D1 and cyclin E1 were detected by western blot in *SLFN11* knockdown DKO cells and control cells.

validated by BSSQ in RKO, DLD1 and Ls180 cells (Figure 1C). Loss of/reduced expression of *SLFN11* was correlated with promoter region methylation. Upon treatment with 5-AZA, a demethylating agent, restoration of *SLFN11* expression was observed in RKO, DLD1, SW620 and LOVO cells, and increased expression was detected in Ls180 (Figure 1A). No expression changes were detected in DKO cells. These results suggest that the expression of *SLFN11* is regulated by promoter region methylation.

SLFN11 is frequently methylated in human CRC

The methylation status of *SLFN11* was detected by MSP (Figure 2A). *SLFN11* was methylated in 55.47% (71/128) of CRC patient samples. No methylation was found in five cases of noncancerous colorectal mucosa. Methylation of *SLFN11* was significantly associated with age, poor 5-year OS and 5-year RFS (all $p < 0.05$; Figure 2B & Table 2), while no association was found between *SLFN11* methylation and gender, tumor differentiation, lymphatic metastasis, location, stage, size (all $p > 0.05$, Table 2). Among 128 cases of CRC, *KRAS* mutation was found in 29 cases (22.66%), and *BRAF* mutation in 3 cases (2.34%). No association was found between *SLFN11* methylation and *KRAS* or *BRAF* mutation (all $p > 0.05$, Table 2). Among 128 cases of CRC, CIMP (+) was detected in 38 cases (29.69%) and CIMP (-) in 90 cases (70.31%). No association was found between *SLFN11* methylation and CIMP (+) in CRC ($p > 0.05$, Table 2).

Cox proportional hazards model analysis indicated that *SLFN11* methylation was an independent prognostic factor for OS and RFS after adjusting for tumor differentiation and N stage (both $p < 0.05$, Table 3).

SLFN11 suppresses CRC cell proliferation

SLFN11 stably expressing cells were established by transfecting PCDH-*SLFN11* expression vector into *SLFN11* unexpressed RKO and DLD1 cells. MTT and colony formation assays were employed to evaluate the effects of *SLFN11* on cell proliferation. The optical density (OD) values were 0.533 ± 0.037 versus 0.349 ± 0.019 ($p < 0.05$) in DLD1 cells and 0.968 ± 0.037 versus 0.782 ± 0.065 ($p < 0.01$) in RKO cells before and after restoration of *SLFN11* expression (Figure 3A). Re-expression of *SLFN11* significantly suppressed colony formation in RKO and DLD1 cells (Figure 3B & Supplementary Figure 1). The effects of *SLFN11* on cell cycle were analyzed by flow cytometry. The cell phase distributions before and after re-expression of *SLFN11* in DLD1 cells were as follows: G0/G1 phase: $45.93 \pm 2.05\%$ versus $50.23 \pm 0.95\%$, S phase: $17.51 \pm 0.24\%$ versus $14.72 \pm 0.59\%$ and G2/M phase: $36.55 \pm 1.84\%$ versus $35.05 \pm 0.57\%$. In RKO cells, the

cell phase distributions before and after re-expression of *SLFN11* were as follows: G0/G1 phase: $35.57 \pm 0.82\%$ versus $38.66 \pm 0.85\%$, S phase: $14.77 \pm 0.98\%$ versus $13.62 \pm 1.12\%$; and G2/M phase: $49.66 \pm 0.75\%$ versus $47.72 \pm 0.29\%$. The number of cells in G1/G0 phase increased significantly after re-expression of *SLFN11* in DLD1 and RKO cells (both $p < 0.05$, Figure 3C). To further validate these results, siRNA knockdown technique was employed. The OD values were 0.591 ± 0.015 versus 0.677 ± 0.022 before and after knockdown of *SLFN11* in DKO cells ($p < 0.05$, Figure 3A). Knockdown of *SLFN11* significantly promotes colony formation in DKO cells (Figure 3B & Supplementary Figure 1). The cell phase distributions before and after knockdown of *SLFN11* were as follows: G0/G1 phase: $37.36 \pm 0.98\%$ versus $34.36 \pm 0.43\%$, S phase: $33.9 \pm 0.61\%$ versus $31.41 \pm 0.05\%$, and G2/M phase: $28.74 \pm 0.91\%$ versus $34.23 \pm 0.44\%$. The number of cells in G0/G1 phase was significantly reduced after knockdown of *SLFN11* in DKO cells ($p < 0.05$, Figure 3C).

To further validate the effects of *SLFN11* on cell cycle, the levels of cyclin D1 and cyclin E1 were examined by western blot in DLD1 and RKO cells before and after re-expression of *SLFN11*. The expression levels of cyclin D1 and cyclin E1 were reduced after re-expression of *SLFN11* in RKO and DLD1 (Figure 3D), while the expression levels of cyclin D1 and cyclin E1 were increased after knockdown of *SLFN11* in DKO (Figure 3E). These results further suggest that *SLFN11* inhibits cell proliferation in CRC.

SLFN11 sensitized CRC cells to cisplatin

The expression of *SLFN11* has been reported to sensitize ovarian cancer to cisplatin-containing regimens [13]. To explore the sensitizing effects of *SLFN11* on CRC cells, the MTT assay was employed. The IC_{50} values of cisplatin were 21.77 ± 0.46 versus $9.99 \pm 1.12 \mu M$, 6.23 ± 0.77 versus $2.59 \pm 0.28 \mu M$ and 17.56 ± 0.30 versus $13.19 \pm 1.48 \mu M$ before and after re-expression of *SLFN11* in RKO, DLD1 and SW620 cells, respectively. The IC_{50} values were significantly reduced after re-expression of *SLFN11* (all $p < 0.05$, Figure 4A). The IC_{50} values were 14.19 ± 0.21 versus $24.20 \pm 2.51 \mu M$ in DKO cell before and after knockdown of *SLFN11*. The IC_{50} value was significantly increased after knockdown of *SLFN11* (all $p < 0.05$, Figure 4A). These results suggest that *SLFN11* sensitizes CRC cells to cisplatin.

Under treatment with $2 \mu M$ of cisplatin, the number of colonies was 111 ± 20 versus 26.33 ± 8.14 , 137.67 ± 8.02 versus 9.33 ± 3.51 and 47.50 ± 10.61 versus 10.00 ± 4.00 in RKO, DLD1 and SW620 cells, respectively, before and after re-expression of *SLFN11*. The colony numbers were reduced significantly after re-expression of *SLFN11* in RKO, DLD1 and SW620

Figure 4. SLFN11 promotes chemosensitivity of colorectal cancer cells to cisplatin *in vitro*. (A) Cell viability determined by MTT assays following exposure to cisplatin. (B) Colony formation under treatment with 2 μM of cisplatin in RKO, DLD1 and SW620 cells before and after re-expression of SLFN11, and in DKO cells before and after knockdown of SLFN11.

cells (all $p < 0.01$, Figure 4B). Upon treatment with 2 μM of cisplatin, the number of colonies was 71.33 ± 15.95 versus 204.66 ± 28.15 in DKO cells before and after knockdown of SLFN11. The colony numbers were increased significantly after knockdown of SLFN11 in DKO cells ($p < 0.01$, Figure 4B). These results further demonstrate that SLFN11 sensitizes CRC cells to cisplatin.

To further validate these results, the effects of SLFN11 on apoptosis were analyzed upon cisplatin treatment. Before and after re-expression of SLFN11 in RKO and DLD1 cells upon treatment with 10 μM of cisplatin, the percentages of early apoptosis in lower right quadrant (LR) cells were $7.26 \pm 2.2\%$ versus $16.82 \pm 1.81\%$ ($p < 0.01$) and $6.85 \pm 0.87\%$ versus $9.68 \pm 0.64\%$; the percentages of necrosis/late apoptosis in upper left quad-

rant (UL) and upper right quadrant (UR) cells were $3.97 \pm 0.96\%$ versus $10.15 \pm 1.61\%$ and $3.89 \pm 0.05\%$ versus $5.17 \pm 0.63\%$, respectively ($p < 0.05$) (Figure 4C). In DKO cells before and knockdown of *SLFN11*, the percentages of early apoptotic cells (LR) were $15.22 \pm 2.36\%$ versus $9.37 \pm 0.49\%$ ($p < 0.05$), and necrosis/late apoptosis (UL + UR) cells were $18.76 \pm 1.25\%$ versus $15.09 \pm 0.69\%$ ($p > 0.05$), respectively (Figure 4C).

These results demonstrate that the amount of apoptosis induced by cisplatin treatment significantly increased after re-expression of *SLFN11* and significantly decreased after knockdown of *SLFN11*. These

results further suggest that *SLFN11* sensitizes CRC cells to cisplatin.

SLFN11 suppresses human CRC cell xenograft growth in mice

To further investigate the effects of *SLFN11* on CRC, *SLFN11* unexpressed and re-expressed DLD1 cell xenograft mouse models were employed (Figure 5A). The tumor volumes were 372.83 ± 79.59 versus $133.11 \pm 46.41 \text{ mm}^3$ in *SLFN11* unexpressed and re-expressed DLD1 cell xenografts ($p < 0.01$; Figure 5B). The tumor weights were 0.15 ± 0.04 versus $0.07 \pm 0.02 \text{ g}$

Figure 4. *SLFN11* promotes chemosensitivity of colorectal cancer cells to cisplatin *in vitro* (cont. from facing page). (C) Apoptosis was detected and quantified by annexin V-FITC and propidium iodide staining.

Figure 5. Inhibits tumor growth in colorectal cancer cell xenograft mice. (A) Results of SLFN11 re-expressed and unexpressed DLD1 cell xenografts in mice – top: SLFN11 re-expressed colorectal cells group; Bottom: control group. (B) Tumor growth curves and average weights of SLFN11 re-expressed and unexpressed DLD1 cell xenografts.

** $p < 0.01$.

in SLFN11 unexpressed and re-expressed DLD1 cell xenografts ($p < 0.01$; Figure 5B).

Discussion

In humans, there are only five SLFN members, SLFN12 is located in cytoplasm and the remaining members (SLFN5, SLFN11, SLFN13 and SLFN14) are localized to the nucleus [10]. The function of human *SLFN* has not been extensively studied. Previous proteomic studies pointed to SLFN11 as a putative interactor of several key proteins of the DNA damage cell response system, such as replication proteins RPA1, RPA2 and RPA3 or BRCA1-associated Ring Domain protein [14]. Nogales *et al.* found that *SLFN11* methylation is a predictive biomarker of platinum resistance in ovarian and non-small-cell lung cancer by analyzing the DNA methylome database [14]. In another study, the authors found that SLFN11 sensitizes CRC cells to irinotecan [22]. Our study demonstrated that *SLFN11* is methylated in 55.47% of human CRC samples, and the expression of *SLFN11* is regulated by promoter region methylation. The results suggest that methylation of SLFN11 is involved in human CRC

development and is a potential CRC detection marker. *SLFN11* suppressed CRC growth both *in vitro* and *in vivo*. Methylation of *SLFN11* may serve as a potential therapeutic target in CRC. Methylation of *SLFN11* was significantly associated with age, poor 5-year OS and 5-year RFS, suggesting that *SLFN11* methylation is a poor prognostic marker in CRC.

Defects of the DNA damage repair system can contribute to tumorigenesis and progression [23]. On the other hand, defects of DNA damage repair pathways may sensitize cancer cells to chemotherapy [24,25]. Human *SLFN11* interacts with RPA and negatively regulates the DNA damage response by inhibiting checkpoint maintenance and homologous recombination repair [26]. Our study demonstrated that SLFN11 suppresses CRC cell proliferation by inducing G1/S arrest. In addition, *SLFN11* sensitizes CRC cells to cisplatin. Thus, *SLFN11* methylation may serve as a cisplatin insensitive marker. However, the chemotherapeutic regimens and other therapies were different in this population, it limited further analysis the association between *SLFN11* methylation and each chemical agent. Because there is no reliable

antibody available for *SLFN11*, we were not able to perform immunohistochemistry staining in primary CRC.

In conclusion, *SLFN11* is frequently methylated in human CRC, and the expression of *SLFN11* is regulated by promoter region methylation. Methylation of *SLFN11* is associated with age, poor 5-year OS and 5-year RFS. *SLFN11* suppresses CRC growth both *in vitro* and *in vivo* and *SLFN11* sensitizes CRC cells to cisplatin.

Future perspective

SLFN11 is a candidate tumor suppressor in CRC. Methylation of *SLFN11* may be a frequent event in CRC and is a potential detection and prognostic marker in CRC. Methylation of *SLFN11* may serve as a cisplatin-resistant marker in CRC.

Financial & competing interests disclosure

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Summary points

- *SLFN11* is frequently methylated in human colorectal cancer (CRC).
- The expression of *SLFN11* is regulated by promoter region methylation.
- Methylation of *SLFN11* is associated with age, poor 5-year overall survival and 5-year relapse-free survival.
- *SLFN11* suppresses CRC growth both *in vitro* and *in vivo*.
- Methylation of *SLFN11* reduced the sensitivity of CRC cells to cisplatin.

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