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The *set* gene is a potential oncogene in human colorectal adenocarcinoma and oral squamous cell carcinoma

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Abstract. The purpose of this study was to determine whether *set* gene plays a role in the tumorigenesis of human colorectal adenocarcinoma and oral squamous cell carcinoma. We used the human colon carcinoma cell line Ls174 and oral squamous cell carcinoma cell line HSC3 to evaluate the effect of set suppression on cancer cell proliferation and apoptosis. Using real-time PCR, we examined *set* gene expression in human colorectal adenocarcinoma tissues and matched normal colorectal tissues. Thirty pairs of colorectal adenocarcinoma tissues and matched normal colorectal tissues were used for real-time PCR. We transfected human colon carcinoma cell line Ls174 and oral squamous cell carcinoma cell line HSC3 with siRNA against the *set* gene. The effect of *set* gene suppression on cancer cell proliferation and apoptosis were studied by MTT assay and flow cytometry. Real-time PCR indicated that *set* gene expression was up-regulated in 70% of tumor samples (21 out of 30 samples). siRNA2 sequences significantly decreased *set* mRNA levels in Ls174 and HSC3 cells. The inhibitory rate in the two cell lines was 55.91 and 71.57%, respectively. MTT assay revealed a 21.4% inhibition on cell proliferation in HS174 cells and a 20.2% inhibition in HSC3 cells. Flow cytometry data indicated that the cell apoptosis rate was 18.37% in Ls174 cells and 17.97% in HSC3 cells; these rates were significantly higher than those of the control groups. In conclusion, the *set* gene was found to play a role in the tumorigenesis of human colorectal adenocarcinoma. It may promote tumorigenesis by enhancing cancer cell proliferation and inhibiting cancer cell apoptosis.

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Introduction

Colorectal adenocarcinoma is one of the most common malignant tumors, and its prevalence has been on the increase. Colorectal adenocarcinoma has the second highest incidence among all cancers, and is the second most common cause of cancer-related death worldwide (1,2). On the other hand, the incidence of oral cancer has also exhibited an increase in numerous countries, accounting for 5.6% of all malignant tumors in China (3). Tumorigenesis is a multi-step process that involves multi-gene change. The investigation of the molecular mechanism of tumorigenesis may aid in improving cancer prevention, early diagnosis, and effective treatment (4).

Using cDNA subtractive library and cDNA microarray techniques, we previously identified a total of 86 cDNA sequences differentially expressed between human colorectal adenocarcinoma tissues and normal colorectal tissues (5). In the present study, we focused on one differentially expressed sequence (Genbank accession number: ES274071), identified its full-length cDNA using BLASTn of NCBI, and identified the *set* gene (Genbank accession number: NM003011.3). This gene contains 2936 base pairs, with an 834-bp open reading frame. The *set* gene is located on human chromosome 9p34. It is predicted to encode a 277 amino acid protein with a molecular weight of 39 kDa. The *set* gene has been found to play a role in leukemia and ovarian cancer, but its role in the development of colorectal adenocarcinoma and oral squamous cell carcinoma remains to be elucidated (10-15).

To determine whether the *set* gene is involved in colorectal adenocarcinoma and oral squamous cell carcinoma, we investigated the *set* gene expression profile in human colorectal adenocarcinoma and normal colorectal tissues using real-time PCR. RNAi was used to suppress the *set* gene expression in the human colorectal cancer cell line Ls174 and the human oral squamous cell carcinoma cell line HCS3, and to observe the effect of *set* gene on cancer cell proliferation and apoptosis.

Materials and methods

Collection of tissue samples. This study was approved by the medical research ethics committee of Sichuan University. Written informed consent was obtained from the patients. Colorectal adenocarcinoma tissues and adjacent normal colorectal tissues were obtained from 30 colorectal adeno-

Table I. PCR primers.

Gene	Primer sequences	Product location	Product length (bp)
set	Forward: 5'-GCTCAACTCCAACCACGAC-3'	389-407	120
	Reverse: 5'-TCCTCACTGGCTTGTTCATTA-3'	488-508	
GAPDH	Forward: 5'-GGAAGGTGAAGGTCGGAGT-3'	107-11	117
	Reverse: 5'-TGAGGTCAATGAAGGGGTC-3'	205-223	

Table II. RT-PCR reaction.

Name	PCR Cycle
set	95˚C 30 sec; 95˚C 5 sec, 62˚C 30 sec, 40 cycles; 95˚C 15 sec, 60˚C 30 sec, 95˚C 15 sec
GAPDH	95˚C 30 sec; 95˚C 5 sec, 60˚C 30 sec, 40 cycles; 95˚C 15 sec, 60˚C 30 sec, 95˚C 15 sec

Table III. Target sites and siRNA sequences.

Name	Target site	siRNA sequences
siRNA1	GGAGGAAGGATTAGAAGAT	5'-GGAGGAAGGAUUAGAAGAUdTdT-3'
		3'-dTdTCCUCCUUCCUAAUCUUCUA-5'
siRNA2	CTGGAAAGGATTTGACGAA	5'-GGAGGAAGGAUUAGAAGAUdTdT-3'
		3'-dTdTGACCUUUCCUAAACUGCUU-5'
siRNA3	GATTGAACACATTGATGAA	5'-GAUUGAACACAUUGAUGAAdTdT-3'
		3'-dTdTCUAACUUGUGUAACUACUU-5'

carcinoma patients at the West China Hospital of Sichuan University, China. Normal colorectal tissues were collected from the area at least 5 cm from the edge of the tumor. Pathological examination further confirmed no cancer cells were evident in the collected normal tissues. The tissues were snap-frozen and stored in liquid nitrogen for real-time PCR analysis.

Real-time PCR. Total RNA from tumor and matched normal tissues was isolated using TRIzol RNA isolation reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Total RNA (1 μg) was reverse transcribed using the M-Mulv reverse transcriptase kit (Fermentas, Ontario, Canada).

Real-time PCR was performed using SYBR Premix Ex Taq (Takara, Japan) according to the manufacturer's instruction and related international standards (6-8). cDNA (2 μl) from 1 μg RNA template was used for each PCR reaction. The amplification was performed on the Bio-Rad C1000 Real-Time Thermal Cycler (Bio-Rad, Philadelphia, PA, USA). *GAPDH* gene was used as an endogenous control. PCR primers of the *set* gene and *GAPDH* control are shown in Table I. The conditions of PCR reactions are shown in Table II.

siRNA selection. Based on the mRNA sequence of *set* gene, three pairs of siRNA (small interfering RNA) sequences were selected by siRNA design software on line (http://www.ambion.com). The target sites and corresponding siRNA sequences are shown in Table III. Using the BLASTn program, we confirmed that the selected siRNA sequences lacked homology to any other human coding sequences. Negative control siRNA did not cause any gene silencing, and was only used to test the specificity of the siRNA.

Human colon carcinoma cell line Ls174 (ATCC, Manassas, VA, USA) and oral squamous cell carcinoma cell line HSC3 (Tissue and Cell Culture Center, Japan), were transfected with *set* siRNA. Ls174 and HSC3 cells were cultured in DMEM (10% FBS, 100 U/ml penicillin and 100 U/ml streptomycin) at 37˚C with 5% CO_2. Each type of cell was divided into five groups: the non-treatment control group, negative control group (control siRNA), and the siRNA1, siRNA2 and siRNA3 groups. Following transfection with siRNA sequences or controls for 48 h, total RNA was isolated from each group. After reverse transcription, the *set* gene expression level was examined by real-time PCR. The *GAPDH* gene was used as the endogenous control. The siRNA sequences, which exhibited the highest inhibition effect on *set* gene expression, were selected for subsequent experiments.

MTT assay. MTT assay was performed to examine the cell proliferation. Cells in the logarithmic phase were cultured in 96-well culture plates. Three groups were set up as follows: the non-treatment control, negative control (control siRNA),

Table IV. *set* gene expression in colorectal adenocarcinoma and normal colorectal tissue.

Name	Min	Max	Mean	Standard error
Adenocarcinoma	0.025011	0.47049	0.0996757	0.09177069
Normal	0.02764	0.26622	0.0697897	0.04925080

Table V. *set* gene expression change and patient characteristics such as gender, age, Dukes' stage and differentiated degree.

	Gender		Age		Dukes stage			Differentiated degree		
Expression	Male	Female	≥50	<50	B	C	D	High	Middle	Low
Up-regulation	10	10	17	3	3	12	5	1	15	4
Down-regulation	7	3	9	1	3	5	2	0	8	2

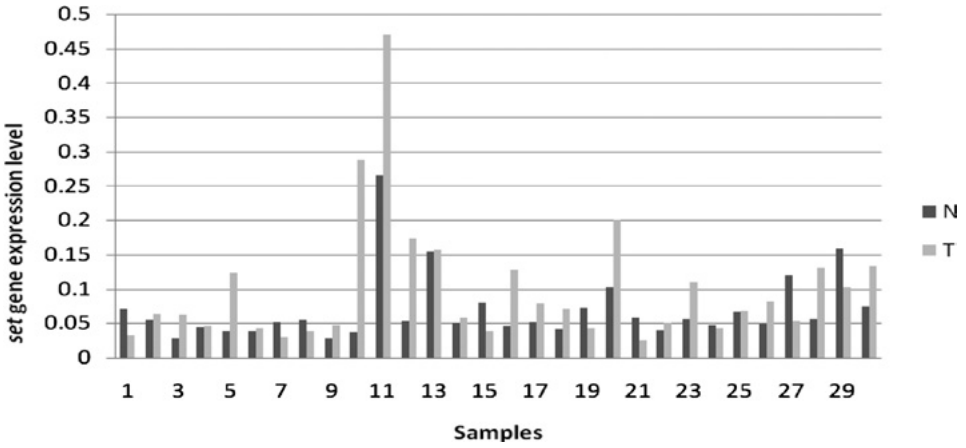

Figure 1. Expression level of the *set* gene (T=135, P=0.045 by Wilcoxon signed-rank test). T, colorectal adenocarcinoma sample; N, matched normal sample).

and experimental groups. Each group had six replicates. MTT reagent (20 μl) (Sigma, St. Louis, MO, USA) were added at each time point (6, 12, 36 and 48 h). After the cells were cultured for a further 4 h, the medium was removed and 150 μl DMSO was added into each well, and incubated for 15 min. The plate was read at an absorbance wavelength of 490 nm by a microplate reader (Bio-Rad, Model 680, USA). The experiment was repeated three times.

Flow cytometry. *set* siRNA effect on cell apoptosis was studied by flow cytometry (BD FACSAria II Cell Sorter, BD Co., USA). Cells in the logarithmic phase were collected and cultured in six-well culture plates. Three groups were set up as follows: the non-treatment control, negative control (control siRNA) and experimental groups. Each group had triplicates. In the cell apoptosis experiment, cells were collected 24 h post-transfection, using PBS to wash the cells twice (2000 rmp, 5 min, 4˚C). The cells were floated in 400 μl PBS, 5 μl Annexin V-FITC was added and agitated gently, and the mixture was placed for 10 min in room temperature. The mixture was placed into 10 μl PI (20 μg/ml), and was incubated for 30 min in 4˚C, and then cell apoptosis was tested by flow cytometry. The experiment was repeated three times.

Statistical analysis. Values are given as the means ± standard error of the mean (SEM). Statistical analysis was performed using the Student's t-test, Wilcoxon signed-rank test, Fisher's exact probabilities test, or ANOVA with SPSS17.0. P<0.05 was considered to be statistically significant.

Results

set gene expression level. To determine the *set* gene expression profile in human colorectal adenocarcinoma tissues, we collected 30 pairs of tumor samples and matched normal tissue samples. Real-time PCR was used to detect the mRNA level of the *set* gene. *set* gene expression was up-regulated in 70% of tumor samples (21 out of 30 samples). Of these, 6 samples had a fold change >2-fold, ranging from 2.24- to 7.9-fold, compared to the adjacent normal colorectal tissues (Table IV, Fig. 1). No significant correlation was found between *set* gene expression and gender, age, Dukes' stage, and differentiated degree by Fisher's exact probabilities test (Table V).

siRNA selection. To examine the effect of *set* siRNA on cancer cell lines, the human colon carcinoma cell line Ls174 and oral squamous cell carcinoma cell line HSC3, were transfected with

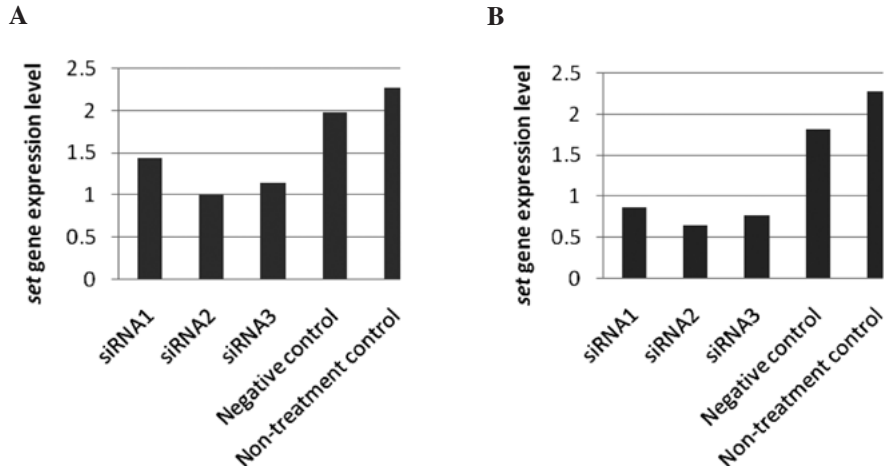

Figure 2. (A) *set* expression level in Ls174 cells. (siRNA inhibition of the siRNA1, siRNA2 and siRNA3 groups, and the negative control group was 36.82, 55.91, 49.71 and 12.77%, respectively). (B) *set* expression level in HSC3 cells. (siRNA inhibition of the siRNA1, siRNA2 and siRNA3 groups, and the negative control group was 62.38, 71.57, 61.16 and 20.06%, respectively).

Table VI. *set* gene expression in each group.

Groups	siRNA1	siRNA2	siRNA3	Negative control	Non-treatment control
Ls174	1.433±0.0453	1±0.0374	1.141±0.166	1.978±0.041	2.268±0.129
HSC3	0.856±0.037	0.647±0.155	0.77±0.038	1.818±0.35	2.275±0.139

Table VII. LS174 cell proliferation.

Time points (h)	*set* siRNA	Negative control	Non-treatment control	F	P
6	0.414±0.011	0.417±0.015	0.452±0.026	5.483	0.028
12	0.455±0.021	0.493±0.033	0.524±0.031	5.5	0.028
24	0.505±0.03	0.582±0.043	0.633±0.032	13.06	0.002
36	0.674±0.035	0.746±0.031	0.787±0.043	9.625	0.006
48	0.82±0.018	0.89±0.035	0.909±0.04	8.12	0.01

set siRNA. Compared to the non-treatment control group, the siRNA2 group indicated a 55.91% inhibition in Ls174 cells and 71.57% in HSC3 cells. This inhibition was greater than that of the siRNA1 and siRNA3 groups (Table VI, Fig. 2). Therefore, siRNA2 sequences were used for subsequent experiments.

Cell proliferation. Using the selected siRNA sequences against the *set* gene, we examined its role in cell proliferation in the Ls174 and HSC3 cells. MTT assay showed that after interference for 24 h, cell proliferation of both Ls174 and HSC3 cells was significantly inhibited in the *set* siRNA group compared to the non-treatment control and negative control groups. The inhibition rate was 21.4% in the Ls174 cell lines (Table VII), and 20.2% in the HSC3 cell lines (Table VIII). This result suggested that siRNA inhibition of the *set* gene suppressed cancer cell proliferation.

Cell apoptosis. To examine the role of the *set* gene in cell apoptosis, we performed flow cytometry in Ls174 and HSC3 cells transfected with the selected siRNA sequences against the *set* gene. After transfection for 24 h, the cell apoptosis rate of the *set* siRNA, negative siRNA control and non-treatment control groups in Ls174 cells was 18.37±2.97, 12.23±0.87 and 11.32±1.15% (Fig. 3A). The cell apoptosis rate of the *set* siRNA group was significantly higher than that of the negative siRNA control (t=-4.854, p=0.003) and non-treatment control groups (t=-5.421, p=0.001). No statistical significance was noted between the negative siRNA control and non-treatment control groups (t=-1.552, p=0.154). The apoptosis rate of the *set* siRNA, negative siRNA control and non-treatment control groups in HSC3 cells was 17.97±2.77, 8.53±0.91 and 7.85±0.62%, respectively (Fig. 3B). The cell apoptosis rate of the *set* siRNA group was significantly higher than that of

Table VIII. HCS3 cell proliferation.

Time points (h)	*set* siRNA	Negative control	Non-treatment control	F	P
6	0.394±0.017	0.395±0.018	0.461±0.023	15.913	0.001
12	0.437±0.029	0.477±0.033	0.536±0.031	10.350	0.005
24	0.493±0.039	0.574±0.035	0.627±0.034	13.820	0.002
36	0.7±0.046	0.803±0.054	0.816±0.038	7.429	0.012
48	0.83±0.045	0.941±0.041	0.953±0.033	13.827	0.002

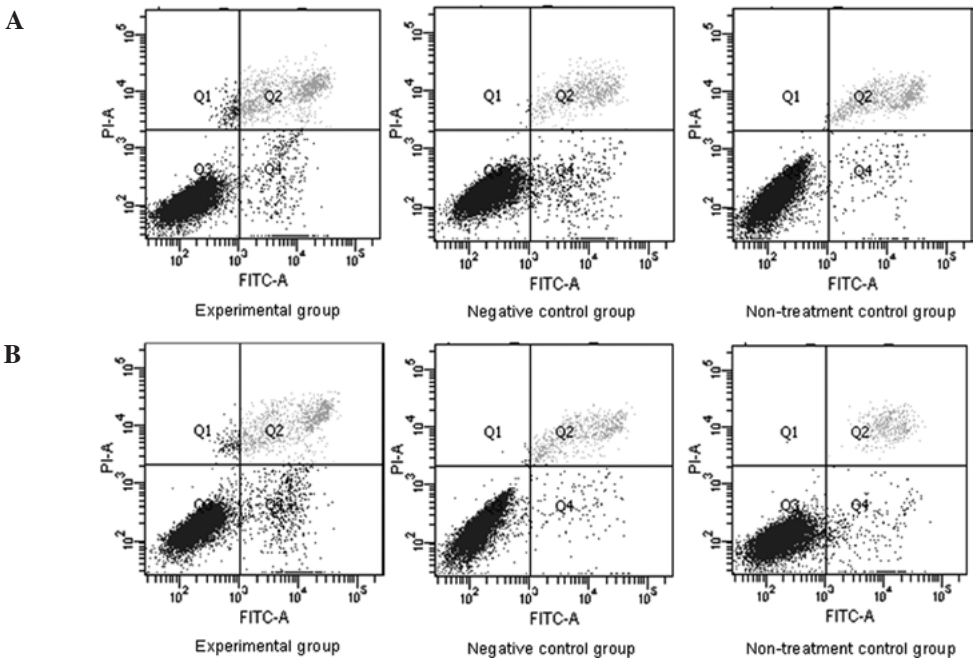

Figure 3. (A) Cell apoptosis of Ls174 and (B) HSC3 cells.

the negative siRNA control (t=7.916, p=0) and non-treatment control groups (t=8.721, p=0). No statistical significance was found between the negative siRNA control and non-treatment control groups (t=1.524, p=0.163).

Discussion

In 1992, von Lindern *et al* (9) first identified the *set* gene in a patient with acute undifferentiated leukemia. Translocation of chromosome 9 resulted in the generation of the SET-CAN fusion protein. To study the role of the *set* gene in acute undifferentiated leukemia, Adachi *et al* (10,11) identified the SET protein by immunoprecipitation using rabbit antiserum, and observed that this nuclear phosphoprotein was expressed ubiquitously in various human cell lines. These authors proposed that SET played a key role in the mechanism of leukemo-genesis in acute undifferentiated leukemia, possibly by activating CAN in nuclei and stimulating the transformation potential of SET-CAN. Saito *et al* (12) developed a transgenic mice strain-expressing SET-CAN protein. This transgenic mouse showed the characteristics of anemia, thrombocytopenia, and splenomegaly, with a great quantity of blood cells in peripheral blood. These symptoms were in line with those of acute undifferentiated leukemia. Furthermore, van Vlierberghe *et al* (13) reported set-can gene fusion in T-cell acute lymphoblastic leukemia. These authors found that the fusion protein appeared to promote an elevated expression of the HOXA cluster genes (homeobox A, HOXA), which are key genes involved in hematopoietic stem cell proliferation and differentiation, that may cause leukemia, such as T-cell acute lymphoblastic leukemia. After screening 141 human leukemia and lymphoma cell lines, Quentmeier *et al* (14) found that only the T-cell acute lymphoblastic leukemia cell line Loucy and the acute myeloid leukemia cell line Megal expressed the set-can fusion gene transcript. In addition to leukemia, the *set* gene was also found to be correlated to human ovarian cancer. In their study, Ouellet *et al* found that the SET complex was highly expressed in invasive grade 3 tumors in human ovarian cancer by immunohistochemistry. Notabley, overexpression of this complex was correlated with tumor differentiation (15). These studies indicate that *set* gene is involved in the tumorigenesis of multiple malignant tumors. However, the role of the *set* gene in colorectal adenocarcinoma and oral squamous cell carcinoma has yet to be determined.

Using real-time PCR, our results showed that *set* gene expression was significantly up-regulated in human colorectal adenocarcinoma tissues, compared to adjacent normal colorectal tissues. Although we did not observe any significant correlation between *set* gene expression and patient characteristics such as gender, age, Dukes' stage, or tumor differentiation a higher rate was noted in Dukes' C (70.59%) and D stages (71.43%) as compared to Dukes' B stage (50%). This tendency was in agreement with Ouellet's finding in human ovarian cancer (16). Our findings suggest that the *set* gene plays a role in cancer cell invasion in human colorectal adenocarcinoma. We hypothesize that *set* is a diagnostic marker for prognosis, particularly neoplasm invasiveness.

Our real-time PCR data indicated that *set* gene may be involved in the development of colorectal adenocarcinoma. To examine the role of *set* in the tumorigenesis of colorectal adenocarcinoma, we performed a *set* siRNA interference experiment to determine the effect of set inhibition on cancer cell proliferation and apoptosis. We selected human colon carcinoma cell line Ls174 and oral squamous cell carcinoma cell line HSC3 to perform these experiments.

In our study, three pairs of siRNA sequences were synthesized as candidates for RNAi. siRNA2 sequences demonstrated the highest inhibition on *set* gene expression in Ls174 and HSC3 cells, and were selected for the present study. MTT assay and flow cytometry results suggest that the interfering *set* gene inhibited cell proliferation while promoting cell apoptosis in colorectal adenocarcinoma and oral squamous cell carcinoma cell lines.

It appears that *set* has a different mechanism of action in tumors. Scientists (16-19) have reported that *set* is capable of inhibiting the acetylation of histone and regulating transcription. On the other hand, investigators (20-23) have also reported that SET regulates the process of cell division, including promoting DNA replication in adenovirus, regulating nucleosome assembly, chromosome resolution and promoting gene transcription as molecular chaperones of histone. Moreover, SET is capable of inhibiting the anti-oncogene PP2A (24,25). It has been found that *set* increased the activity of the (17,20) splitting enzyme in CYP17 and prompted the creation of γ-interferon in natural killer cells by inhibiting PP2A. PP2A plays a crucial role in cell cycle, DNA replication, signal conduction, cell differentiation and malignant tumor transformation. Therefore, *set* may promote tumorigenesis by inhibiting PP2A. We hypothesized that *set* is a potential oncogene in colorectal adenocarcinoma and oral squamous cell carcinoma, and that during tumorigenesis, it may utilize a mechanism of inhibition of histone acetylation, or PP2A.

In conclusion, our study showed that the *set* gene is overexpressed in human colorectal adenocarcinoma, and that the inhibition of its gene expression effectively suppresses cell proliferation and promotes cell apoptosis. We hypothesized that the *set* gene is a diagnostic marker and treatment target for multiple malignant tumors. However, further studies are required to confirm this hypothesis and investigate the mechanism.

Acknowledgements

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