MicroRNAs that Potentially Regulate SOS1 Expression in Colon Cancer

Fung Lin Yong, Chee Wei Law, and Chee Woon Wang

***Abstract*—Colon cancer is one of the leading causes of cancer-associated morbidity and mortality worldwide. The development of colon cancer is closely related to epidermal growth factor receptor (EGFR) pathway. Son of Sevenless Homolog 1 (SOS1) gene is a key component in the EGFR pathway that has been reported to be overexpressed in cancer. The aim of the study was to investigate the microRNAs that potentially regulate SOS1 expression in colon cancer patients. A total of 60 cancerous and adjacent non-cancerous tissues were collected. Western blot, microRNA microarray and quantitative real-time PCR analyses were carried out. Significant overexpression of SOS1 and downregulation of miR-195 were determined ($p < 0.05$). The findings suggested a potential regulation of SOS1 expression by miR-195.**

***Index Terms*—Colon cancer, microRNA, SOS1, TNM.**

I. Introduction

Colon cancer is one of the leading causes of cancer-associated morbidity and mortality worldwide. A population-based study by Bray *et al.* [1] revealed an estimation of 1.2 million new cases per year and more than 600,000 deaths, primarily through liver metastasis. The pathogenesis of colon cancer is heterogeneous, multi-factorial and may take several decades. Several risk factors that might contribute to colon carcinogenesis include environmental exposure to carcinogens, diet, lifestyle and inflammation [2]. Colon cancer could develop sporadically through randomly acquired somatic mutations (92%) or as part of hereditary cancer syndromes such as Familial Adenomatous Polyposis (FAP) (<1%) and Hereditary NonPolyposis Colorectal Cancer (HNPCC) (8%) [3]-[6].

Son of Sevenless Homolog 1 (SOS1) is a gene of 8331 base pairs which is mapped to chromosome 2p21 (RefSeq Accession Number: NM_005633.3). SOS1 encodes a protein that is a guanine nucleotide exchange factor (GEF) that binds guanine nucleotides and prepares the GTP binding site for Ras proteins binding in the epidermal growth factor receptor (EGFR) pathway [7]. EGFR signaling is a common pathway that contributes to the progression of colon cancer [8]. SOS1 also possesses an opposite role in Ras proteins inactivation by facilitating the exchange of GTP for GDP [7]. Generally, a stimulation in the EGFR pathway would initiate the activation of adaptor proteins such as SH-2 containing protein (SHC) and growth-factor-receptor bound protein 2 (GRB2) that recruit SOS1 protein to the cell membrane [9]. The association with SOS1 will activate KRAS and further activate both RAF kinases and PI3Ks of the MAPK and PI3K/AKT pathways, respectively [9]. Gain-of-function mutations and/or overexpression of SOS1 gene has been found to be associated with Noonan syndrome type 4 [10] and gingival fibromatosis type 1 [11]. Although a DNA sequencing study by Swanson *et al.* [12] did not conclude SOS1 as a significant human oncogene, the overexpression of SOS1 is implicated in the pathobiology of various solid tumors. For instance, a study by Chen *et al.* [13] revealed the function of SOS1 in mediating Ras-induced Rac activation and metastatic colonization with the association with adaptor proteins EPS8 and ABI1. The silencing of any factor in the SOS1/EPS8/ABI1 tri-complex was reported to inhibit ovarian cancer cell migration and metastatic progression [13]. Timofeeva *et al.* [14] supported the role of SOS1 in cancer promotion and progression through a study in prostate cancer. Their group has successfully blocked prostate cancer cell migration and invasion through siRNA silencing of SOS1 [14].

MicroRNAs (miRNAs) are short (19-22 nucleotides), non-coding RNA molecules that act as regulators of gene expression [15]. MiRNAs are evolutionary conserved across species and play important roles in cancer pathophysiology such as cell proliferation, differentiation, apoptosis and metastasis [16]-[18]. MiRNAs can confer both oncogenic and tumor suppressive roles, depending upon their downstream targets [19]. Recently, miRNA-based study has become an important area in cancer research. In this study, differential expressions of miRNAs that correlate to the increased expression of SOS1 in colon cancer patients were evaluated.

II. Methods

A. Sample Collection

A total of 60 cancerous and adjacent non-cancerous tissues were collected from patients having surgical resection for primary colon cancer at the University of Malaya Medical Centre (UMMC), Malaysia between January 2011 and June 2012. The histology was confirmed by pathological analysis and staged according to the tumor-node-metastasis (TNM) staging system of the International Union against Cancer. The recruited patients were of sporadic cases and without

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family history of colon cancer. The patients did not receive any preoperative chemotherapy and/or radiotherapy prior to surgical treatment. The research was carried out according to the principles of the Declaration of Helsinki and was performed with the approval from Medical Ethics Committee of UMMC (reference number 805.9). Written informed consent has been obtained from each patient. The patient demographics were shown in Table I.

TABLE I: DEMOGRAPHICS OF COLON CANCER PATIENTS

Characteristics		Tissue specimens (n = 60), n (%)
Average age (years)		63.8 ±10.6
Gender	Male	33 (55.0%)
	Female	27 (45.0%)
Race	Malay	10 (16.7%)
	Chinese	38 (63.3%)
	Indian	12 (20.0%)
TNM stage	I-II	20 (33.3%)
	III	20 (33.3%)
	IV	20 (33.3%)
pT	pT1-T2	6 (10.0%)
	pT3	43 (71.7%)
	pT4	11 (18.3%)
pN	pN0	20 (33.3%)
	pN1	36 (60.0%)
	pN2	4 (6.7%)
M	M0	40 (66.7%)
	M1	20 (33.3%)
Histology	Conventional adenocarcinoma	55 (91.7%)
	Mucinous adenocarcinoma	5 (8.3%)
Tumor differentiation	Well differentiated	13 (21.7%)
	Moderately differentiated	43(71.7%)
	Poor differentiated	4 (6.7%)
Location of metastasis	None	40 (66.7%)
	Liver	14 (23.3%)
	Liver and other	4 (6.7%)
	Non-liver	2 (3.3%)

B. Western Blot

Tissues were homogenized in T-PER Tissue Protein Extraction Reagent (Thermo Fisher Scientific, Rockford, IL). Forty microgram (40 μg) of denatured proteins was subjected to SDS-PAGE, transferred onto nitrocellulose membrane and blotted using Pierce Fast Western Blot Kit, ECL Substrate (Thermo Fisher Scientific, Rockford, IL) based on manufacturer's protocol. The primary antibody used was monoclonal anti-SOS1 (Thermo Fisher Scientific, Rockford, IL). Normalization was made against *β*-actin expression. Bands were quantified using a densitometric image analysis software (UVP, Upland, CA).

C. Total RNA Isolation

The tissue specimens were immersed in RNA *later* solution (Ambion, Austin, TX) immediately after resection. Total RNA was isolated using Qiagen miRNeasy Mini Kit (Qiagen, Valencia, CA) according to manufacturer's instructions. RNA concentration was determined using NanoDrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE) and RNA integrity was assessed using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Samples with RNA integrity number (*RIN*) $\geq$ 7.0 were used for downstream experiments [20].

D. MiRNA Microarray and MiRNA Target Prediction

MiRNA profiling was conducted using GeneChip miRNA 2.0 Array (miRase version 15.0) (Affymetrix, Santa Clara, CA). Ten samples from each TNM stage were pooled in this assay. One microgram (1 μg) of total RNA was biotin-labeled using 3DNA Array Detection Flashtag Biotin HSR RNA Labeling Kit (Genisphere LLC, Hatfield, PA) prior to a 16-hour hybridization. The chips were washed and stained using Affymetrix Fluidics Station 450 and scanned with GeneChip Scanner 3000 7G. Cell intensity files were generated and subjected to expression analysis using GeneSpring GX 12.0 software (Agilent Technologies, Santa Clara, CA). Robust multichip averaging algorithm was applied for data normalization [21]. The normalized data were analyzed using t-test/ANOVA analysis with p value computations done asymptotically at $p < 0.05$. Subsequently, the gene lists were filtered at a fold change cut-off of 2.0. Hierarchical clustering was performed with average linkage and Euclidean distance metric. MiRNA target prediction software, Targetscan (www.targetscan.org) was used to analyze the miRNAs that could possibly target SOS1 mRNA [22].

E. Reverse Transcription and Quantitative Real-Time PCR

Reverse transcription was performed using TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA) according to manufacturer's protocol. Quantitative real-time PCR was conducted using Taqman Stem-Loop MicroRNA Assays and SOS1 gene assay on StepOnePlus Real Time PCR system (Applied Biosystems, Foster City, CA). RNU48 (a small nucleolar RNA) and β-actin were chosen as the endogenous controls in the miRNA and mRNA validation studies, respectively. All assays were performed in triplicate. Relative expressions were determined using comparative CT ($2^{-\Delta\Delta CT}$) method [23].

F. Statistical Analysis

The demographics were reported as mean ± standard deviation (SD) or frequencies and percentages for continuous and categorical variables, respectively. Statistical analysis was performed using IBM SPSS version 16.0 software (IBM Corporation, Armonk, NY). P-value < 0.05 was considered statistically significant.

III. RESULTS AND DISCUSSION

A. Overexpression of SOS1 in Colon Cancer Tissues

SOS1 has been reported to be overexpressed in several cancers and is known to contribute to increased invasiveness, angiogenesis and metastasis of cancerous cells [12]-[14]. In this study, the levels of SOS1 protein and mRNA in colon cancer tissues were evaluated in 60 paired tissues. SOS1 was shown to be generally overexpressed in the cancerous tissues in relative to the non-cancerous counterparts (Fig. 1 a) and

b)). The expression profile for the non-cancerous tissues was normalized to one. SOS1 protein/mRNA ratio was calculated and an increasing trend of expression was observed as the cancer progressed ($p < 0.05$) (Fig. 1 c)). The findings have highlighted the possible implication of SOS1 expression in colon cancer progression.

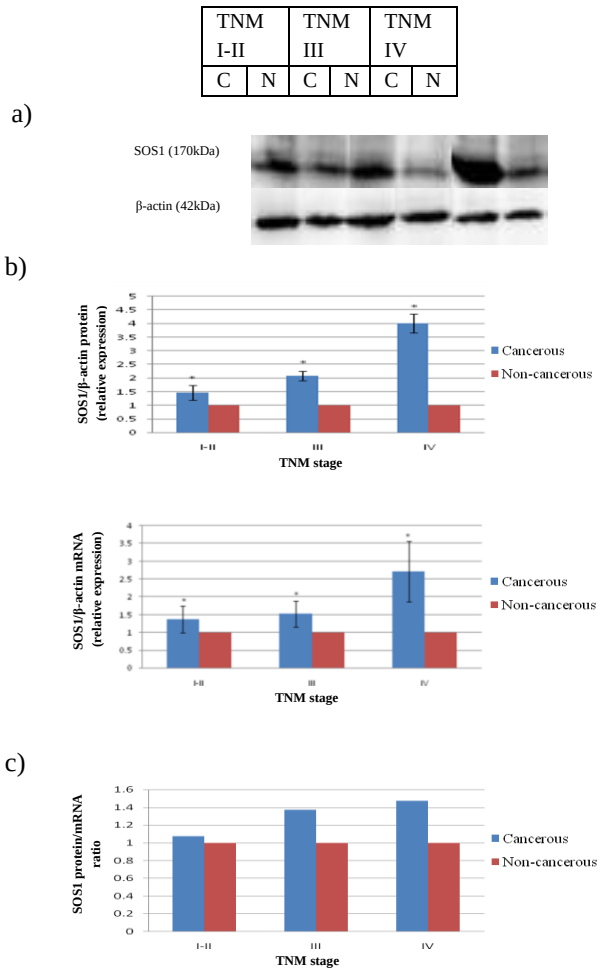

Fig. 1. SOS1 expression in colon cancer tissues versus adjacent non-cancerous tissues. a) SOS1 protein expression, normalized against β-actin protein, b) SOS1 mRNA expression, normalized against β-actin mRNA, c) SOS1 protein/mRNA ratio. *$p < 0.05$.

B. MiRNA Profiling of Colon Cancer Tissues

MiRNA profiling using 30 pairs of cancerous and non-cancerous colon tissue samples ($n = 10$ in each group) was illustrated in Fig. 2. A total of 47 hsa-miRNAs ($p < 0.05$) were detected. Twenty six miRNAs (miR-105, -1246, -1247, -1308, -146b-3p, -182, -183, -183*, -18a, -18a*, -18b, -203, -21*, -224, -424*, -429, -493*, -503, -509-3p, -550*, -552, -584, -622, -663b, -767-5p, -941) were found to be upregulated while 21 miRNAs (miR-124, -138, -139-3p, -139-5p, -149, -195, -215, -23b*, -28-3p, -29b-2*, -30a, -30a*, -30c-1*, -30c-2*, -378, -378c, -378*, -383, -422a, -497, -887) were found to downregulated. Since SOS1 expression has been determined to be overexpressed in this study, we have refined our *in silico* search to only the downregulated miRNAs. Notably, eight miRNAs (miR-124, -195, -28-3p, -30a, -378, -378c, -422a, -497) were predicted to target the 3'UTR region on SOS1 mRNA using the Targetscan software. The downregulations of these miRNAs have been previously reported in various independent colon cancer researches [24]-[29].

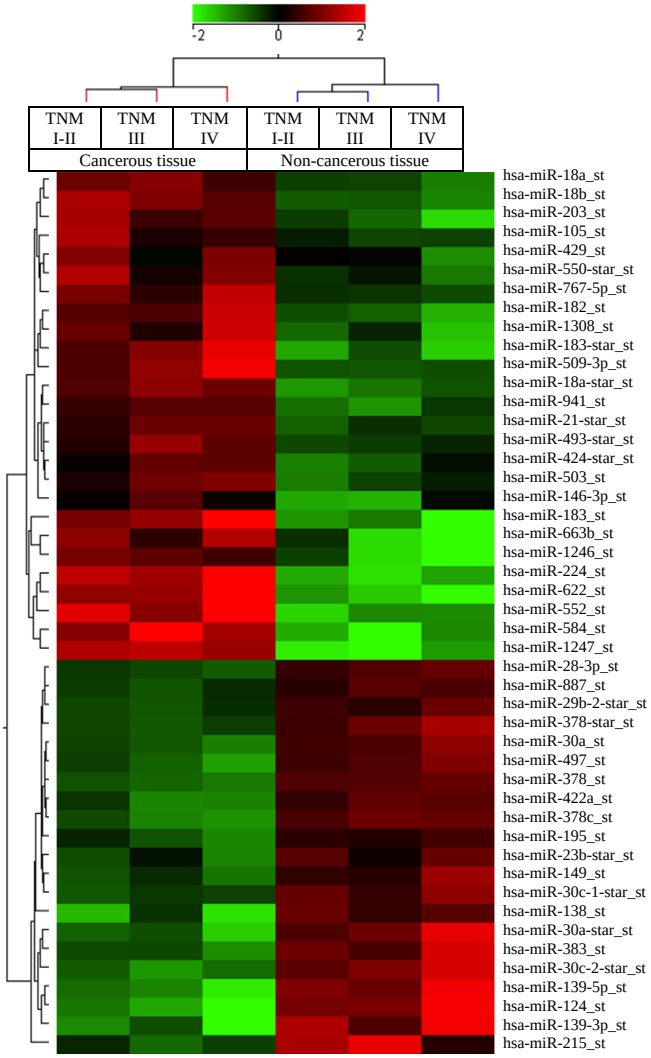

Fig. 2. Hierarchical clustering of colon cancer patients. Green denotes downregulation whereas red denotes upregulation.

C. Validation of Potential MiRNAs

Validation of miRNA microarray results via quantitative real-time PCR was performed using the 60 paired tissues. Among the eight miRNAs, only miR-195 revealed a significant level of downregulation ($p < 0.05$) (Fig. 3). The miRNA expression was normalized against RNU48 and the expression profile for the non-cancerous tissues was set as one. Mir-195 has been suggested to possess tumor suppressive function in several cancers. First report on the downregulation of miR-195 in colon cancer was by Liu *et al.* [25]. Our findings conformed to their results whereby significant downregulation of miR-195 was observed in colon cancer patients. Liu *et al.* [25] also reported that the restoration of miR-195 level in both *in vitro* and *in vivo* experiments could reduce cell viability, increase cell apoptosis and suppress tumorigenicity. Moreover, miR-195 was found to inhibit cell cycle progression and cell proliferation in liver [30], gastric [31] and bone cancers [32].

From our *in silico* analysis, the pairing region between the seed sequence of miR-195 and SOS1 3'UTR was obtained and shown in Fig. 4. However, miR-195 shares the same SOS1 3'UTR with several miRNAs, including miR-497

which was also found to be downregulated in our study. The main reason is that a single mRNA could be regulated by multiple miRNAs [33].

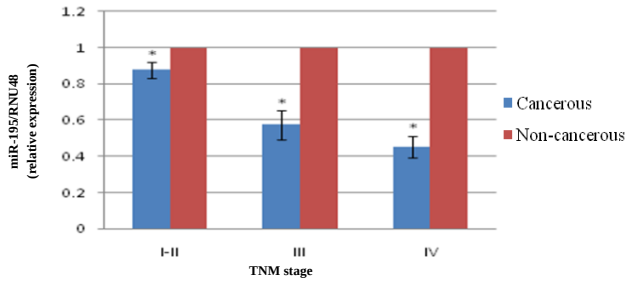

Fig. 3. MiR-195 expression, normalized against RNU48. *$p < 0.05$.

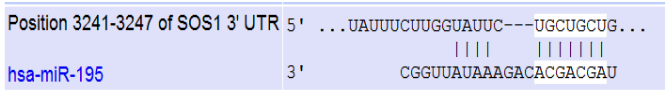

Fig. 4. Predicted pairing region between SOS1 mRNA and miR-195 using Targetscan software. The binding site consisted of seven base pairs and was highlighted in white.

IV. Conclusion

The analyses of SOS1 protein-mRNA and miR-195 expression in primary colon cancer patients have shown that SOS1 overexpression was coupled to miR-195 downregulation. Future work via miRNA modulation would be useful in elucidating the association between miR-195 and SOS1 in colon cancer development and progression.

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