Remarkably Reduced Expression of FoxO3a in Metaplastic Colorectum, Primary Colorectal Cancer and Liver Metastasis[*]

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Summary: The forkhead family members of transcription factors (FoxOs) are expected to be potential cancer-related drug targets and thus are being extremely studied recently. In the present study, FoxO3a, one major member of this family, was identified to be down-regulated in colorectal cancer through micro-array analysis, which was confirmed by RT-PCR and Western blot in 28 patients. Moreover, immunohistochemistry (IHC) showed that the expression levels of FoxO3a were remarkably reduced in 99 cases of primary colorectal cancer, liver metastasis, and even in metaplastic colorectal tissue. IHC also revealed an exclusion of FoxO3a from the nucleus of most cells of tumor-associated tissues. Silencing FoxO3a by siRNA led to elevation of G_2-M phase cells. We conclude that the downregulation of FoxO3a may greatly contribute to tumor development, and thus FoxO3a may represent a novel therapeutic target in colorectal cancer.
Key words: FoxO3a; colorectal cancer; transcription factor; cell cycle arrest; target therapy

Colorectal cancer is a most common malignant tumor of alimentary system with an increasing incidence which was anticipated to reach 9 million per year of the whole world after the year 2020[1]. Early detection and prevention are key factors in controlling and curing this disease. Colorectal cancer develops as the result of a series of genetic and epigenetic alterations that lead to transformation of normal colorectal epithelium to colorectal adenocarcinoma[2]. Identifying the key alterations of genes is critical for our understanding of this disease, as well as our ability to correctly treat it. In this study, we performed gene micro-array on primary colorectal cancer tissues and the metastatic tumor tissues, and find that many genes were down-regulated in the tumor tissues,.

FoxO3a belongs to the forkhead family of transcription factors which are intersections among pathways regulating various cellular functions, such as metabolism, differentiation, proliferation, and survival[3]. FoxO3a is determined to function as a trigger for cell cycle arrest and apoptosis through expression of genes necessary for cell death. Since cancer can be considered as a result of perturbation of the critical balance between cell proliferation and cell death, it is proposed that disrupting a gene with roles in apoptosis can contribute to survival of transformed cells and confer an advantage to oncogenesis[3]. Previous reports have demonstrated that FoxO loss of function plays a role in various forms of cancer, such as breast cancer, prostatic neoplasms and chronic lymphocytic leukemia[4]. There is growing evidence that FoxO genes may be classified as tumor suppressors, and that inactivation of FoxO is a pivotal event in tumorigenesis[4]. Chemical molecules that act to restore the function of a defective tumor suppressor gene are now anticipated to be prospective cancer-related drugs[5]. To reveal the potential significance of FoxO3a in treatment of colorectal cancer, its expression pattern in colorectal cancer needs to be elucidated. Furthermore, given that colorectal cancer is a gradually advancing disease, understanding of how the expression of FoxO3a alters during the tumor progress would be an important step toward developing associated target treatments of early primary tumor or early metastasis.

In this study, we disclosed that FoxO3a was significantly downregulated in primary colorectal cancer at transcription level as well as at translation level. Based on exploration of FoxO3a expression in 99 patients with colorectal cancer, we further addressed a remarkably reduced expression of FoxO3a in primary tumor and liver metastasis tumor, and even in metaplastic colorectal tissue, an acknowledged precancerous change. We also described that FoxO3a was excluded from the nucleus and relocated in cytoplasm in tumor tissues. Finally, we demonstrated that silencing FoxO3a in a colorectal cell line could reduce the ability of FoxO3a to induce cell

cycle arrest, which may contribute to tumor progression.

1 MATERIALS AND METHODS

1.1 Patients and Specimens

All samples in this study were collected from the in patients admitted on Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology (HUST, China). The diagnoses of colorectal cancer were confirmed histopathologically. The informed consents were obtained from the patients. This research was conducted under the approval of Institutional Review Board. Tissue samples were quickly frozen in liquid nitrogen within 30 min after abscission, and were stored in liquid nitrogen for later use. For immunohistochemistry staining, paraffin-embedded tissue blocks of patients with colorectal cancer were collected from the Pathological Center of Tongji Hospital, Tongji Medical College, HUST.

1.2 Cell Culture and shRNA Transfection

The human colon adenocarcinoma cell line, SW480, was obtained from the American Type Culture Collection (ATCC, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (Hyclone, USA) at 37°C in a 5% CO_2 humidified atmosphere.

In order to silence FoxO3a expression, shRNA targeting FoxO3a sequence (nucleotides 987—1009) was synthesized and inserted into pSilencer2.1-U6 hygromycin vector (Ambion, USA), and a shRNA-plasmid was generated. SW480 cells were plated in a 6-well plate, and when they reached 60%—70% confluence, cells were transfected with the shRNA-plasmids at a concentration of 5 μg per well using Lipofectamine 2000 (Invitrogen, CA) according to the manufacturer's instructions.

1.3 Oligonucleotide Micro-array

The Human Genome Array (Capitalbio Corporation, Beijing, China) was used in the current study, which contains probe sets representing 21 522 human transcripts. Total RNA was extracted from the tissues using Trizol reagent (Invitrogen, USA), and the samples were delivered to the bio-chip company after they had met the quantity and quality criteria. The following steps were carried out by the bio-chip company. Briefly, 5 μg of purified total RNA was used as template to synthesize double-strand DNA with T7-(dT)15 primer in RNase-free condition. After second-strand DNA synthesis, *in vitro* transcription was performed, followed by the second reverse transcription using random-primers. Then KLENOW enzyme (Takara, Japan) was applied to produce biotin-labeled cDNA. Then the labeled cDNA was submitted to the Human Genome Array and the hybridization was carried out at 42°C for 16 h followed by twice rinses. Normal colon tissue samples were used as control and hybridized to the micro-array prior to the tumor associated tissue samples. Finally, the arrays were scanned by LuxScan 10KA double passage laser scanner (Capitalbio Corporation, China). Expression differences were determined by the value of each gene provided by the company.

1.4 RNA Extraction and Semi-quantitative RT-PCR

Total RNA was extracted from frozen tissue samples. RNA extraction and semi-quantitative reverse-transcription polymerase chain reaction (RT-PCR) were performed according to the standard procedures[6]. A 1182-bp-long FoxO3a specific PCR product was amplified using FoxO3a specific primers: 5'-TGGCAA-GCACAGAGTTGG-3' (sense), and 5'-GGCGTGGGAT-TCACAAAG-3' (antisense). PCR procedures were as follows: 95°C for 3 min, 28 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 80 s, followed by 72°C for 10 min. Beta-actin specific sense primer 5'-TGACCC-AGATCATGTTTGAGA-3' and antisense primer 5'-GA-CTCGTCATACTCCTGCTTG-3' were used to obtain a 739-bp-long product as a standard control. PCR procedures were as follows: 95°C for 3 min, 28 cycles of 94°C for 30 s, 53°C for 30 s, and 72°C for 1 min, followed by 72°C for 10 min. The PCR products were visualized by ethidium bromide staining after 1% agarose gel electrophoresis.

1.5 Western Blot

Western blot was performed as previously described[7]. Briefly, whole-cell lysate was generated from cells or from patient samples by lysis with HEPES buffer (25 mmol/L HEPES, pH 7.4; 150 mmol/L NaCl; 1% Nonidet P-40; 0.25% sodium deoxycholate; 1 mmol/L EGTA; 1 mmol/L DTT; 50 μg/mL trypsin inhibitor; 1 mmol/L PMSF; and 10 μg/mL aprotinin). Lysates were subjected to 12% SDS-polyacrylamide gel for protein electrophoretic separation and then the proteins were transferred onto a nitrocellulose membrane. Thereafter, the membrane was blocked with 5% nonfat dry milk and 0.1% Tween 20 for 2 h at room temperature and then probed with primary antibodies for incubation at 4°C overnight. Immune complexes were detected with HRP-conjugated secondary antibody and visualized by ECL detection system (Pierce, USA). Rabbit-anti-human FoxO3a polyclonal antibody (Cell Signaling, USA) and mouse-anti-human beta-actin monoclonal antibody (Sigma, USA) were used as primary antibodies.

1.6 Immunohistochemistry

For each paraffin-embedded block, three 3-μm-thick serial sections were made, one for hematoxylin and eosin (HE) staining and the other two for immunohistochemistry staining (IHC). For IHC, rabbit-anti-human FoxO3a polyclonal antibody (Cell Signaling, USA) was used as primary antibody, and purified normal rabbit IgG was used as a negative control. Slices were deparaffinized with xylene and hydrated through graded alcohols into water, and incubated with peroxidase blocking solution (DAKO, Carpinteria, CA) for 30 min, and then were immersed into citric acid (1 mmol/L) solution for antigen retrieval for 20 min at 98°C and allowed to cool down to room temperature. After that, sections were incubated with primary antibody at a concentration of 1 μg/mL overnight at 4°C. Then, slices were rinsed in excess PBS, and incubated with the goat-anti-rabbit peroxidase conjugated secondary antibody at a 1:500 dilution for 30 min at room temperature. After another rinse in excess PBS, the slices were incubated with horseradish peroxidase for 30 min at room temperature. Then the sections were rinsed again with PBS and the color reaction was done using the DAB+System (DAKO Cytomation, Denmark) according to the manufacturer's protocol. After washing with tap water, the slides were counterstained with hematoxylin solution.

For each section, the percentage of nuclear positive cells was estimated. Each section was scored according to the positive percentage as follows: 0, negative; 1, 1%–5%; 2, 5%–25%; 3, 25%–50%; 4, 50%–75%; 5, 75%–100% positive cells. Scoring was performed by two observers[8].

1.7 Cell Cycle Analysis

Flow cytometry was performed according to previously described procedures[9]. Briefly, harvested cells were fixed in ice-cold 80% ethanol at −20°C for 24 h and then were washed twice with PBS, followed by a resuspension in povidone iodine solution (50 μg/mL) with RNase A (5 mg/mL) for 30 min at room temperature. Cellular fluorescence was measured using a FACSVantage flow cytometer (Becton Dickinson, USA).

1.8 Statistical Analysis

The results of RT-PCR and Western blot were analyzed with the Quantity One 4.6 software. In short, the intensities of DNA bands or protein bands of expected size were quantitated, and for each specimen, the ratio of FoxO3a/Beta-actin intensity was determined. For specimens of one patient, if the ratio of the tumor tissue was lower than that of the normal tissue, down-regulated expression of FoxO3a was considered. Statistical analyses were performed with SPSS 13.0 software. The comparison of sample rate was performed by means of Pearson Chi-square test, with the significant level set at $P<0.05$.

2 RESULTS

2.1 Micro-array Analyses Identified Down-regulation of FoxO3a in Colorectal Cancer

In order to identify gene alterations of colorectal cancer, we compared gene expression profile of the entire genome of tumor tissues to normal colon tissue. Four kinds of tissue of 2 patients were enrolled in the micro-array study, including primary tumor lesion, the liver metastasis lesion, tumor-adjacent tissue (the colorectal tissue about 10 cm away from the primary tumor), and the dissected farthest colorectal tissue from the primary tumor lesions which were determined to be normal colon tissue pathologically. Reduced expression of FoxO3a was identified in one case of primary tumor, two cases of liver metastasis and two cases of tumor-adjacent tissue in the micro-array study (fig. 1).

2.2 Confirmatory RT-PCR and Western Blot

To confirm micro-array results, we checked FoxO3a expression at transcription level as well as at translation level in 28 patients with colorectal cancer. For each patient, two kinds of tissue samples were obtained, including the tumor tissue and the dissected farthest colorectal tissue from the tumor lesion. RT-PCR demonstrated that FoxO3a was down-regulated in 11 of 28 tumor samples (39.3%), while Western blot showed 19 of 28 tumor samples (67.9%) presented decreased expression of FoxO3a. Coincident expression alterations determined by RT-PCR and Western blot were observed in 15 of 28 cases (53.6%, fig. 2 and table 1).

2.3 Expression Pattern of FoxO3a Determined by IHC

We further studied the protein expression pattern of FoxO3a in a larger amount of patients with colorectal cancer by using IHC. Totally, paraffin-embedded tissue blocks of 99 patients with colorectal cancer were collected. Likewise, for each patient, tissue blocks of the primary cancer lesion and the dissected farthest colorectal tissue from cancer lesion were collected. Pathologically, the farthest colorectal tissues from the cancer lesion of 88 patients were normal, while the rest 11 had intestinal metaplasia which was commonly regarded as precancerous change. Among the former 88 patients, 9 had developed liver metastasis, and the tissue blocks of metastasis lesion were also collected.
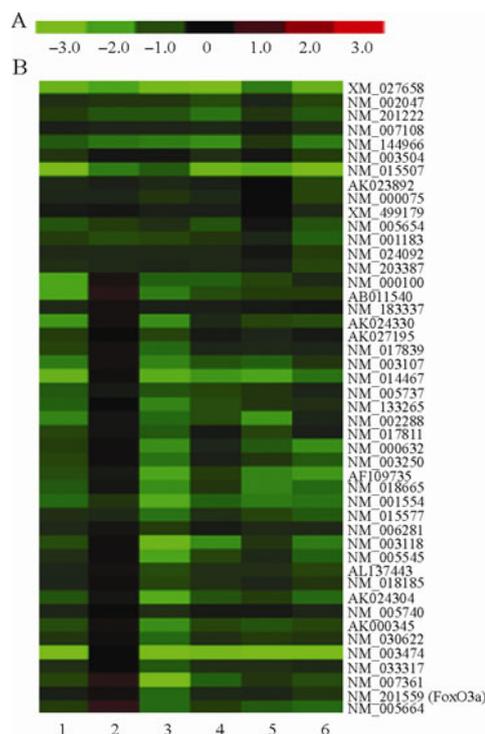
Fig. 1 Down-regulation of FoxO3a in colorectal cancer identified by oligonucleotide micro-array analysis

Four kinds of tissues of 2 patients with colorectal cancer were enrolled in the micro-array study, including primary tumor tissue, tumor-adjacent tissue (the colorectal tissue about 10 cm away from the primary tumor), liver metastasis tissue, and the dissected farthest colorectal tissues from the primary tumor lesion. RNA extracted from normal colon tissues of the two patients were mixed and used as control. Then RNA isolated from the primary tumor, the metastatic tumor, and tumor-adja- cent tissues were respectively hybridized to 6 micro-arrays. In the cluster analysis, red color represented up-regulated genes, while green color represented down-regulated genes (B). The standard bar showed relations between the colors and the values of the genes which were provided by the bio-company (A). Briefly, genes with values between -1 to 1 were considered to be equally expressed, and those with values out of the fraction were considered to be differentially expressed significantly.

1 and 5: tumor- adjacent tissue; 2 and 6: primary tumor; 3 and 4: liver metastasis
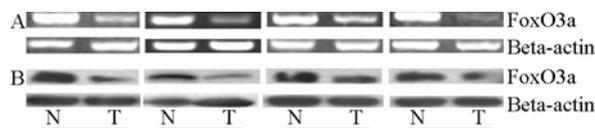
Fig. 2 Expression of FoxO3a in primary tumor tissues and normal colorectal tissues of patients with colorectal cancer

Primary tumor tissues and normal colorectal tissues were obtained from 28 patients with colorectal cancer, and were examined for FoxO3a transcripts and protein. Here presented four typical examples of markedly reduced expression of FoxO3a in primary colorectal cancer identified by RT-PCR (A) and Western Blot (B). T and N represented primary tumor tissue, and normal colorectal tissue respecttively, and the underline represented tissues from the same patient. Samples at homologous places respectively in A and B were from the same patient.

Since FoxO3a is a transcription factor which plays a role in the differential expression of genes by binding to their DNA regulatory elements[10], we studied its cyto-location which is essential for its functioning. Generally, in all the normal colorectal glandular epithelial cells, FoxO3a was strongly expressed in nucleus, and staining of cytoplasm was also positive (fig. 3C, and fig. 4A), while in tumor-associated tissues and metaplastic colorectum, most positive cells presented FoxO3a staining only in cytoplasm (fig. 3F and I, and fig. 4B).

To evaluate the nuclear expression of FoxO3a in normal colorectum and cancerous tissues, sections were compared according to their IHC scores. The nuclear expression of FoxO3a was considered to be low when the section got score of 0−3, while score of 4−5 was considered to represent high expression. First, the nuclear expression of FoxO3a of all the cancerous tissues, including primary tumor and liver metastasis, was significantly lower than that of normal colorectal tissues ($P<0.01$, table 2). Interestingly, the nuclear expression of FoxO3a in metaplastic colorectal tissues was also remarkably reduced as compared with normal colorectal tissues ($P<0.01$, fig. 4 and table 2). Second, in order to understand whether the expression of FoxO3a was altered during cancer progress, the expression patterns of FoxO3a were compared among metaplastic colorectum, primary cancer and the liver metastasis. However, no significant difference was detected. Finally, we tried to reveal whether the downregulation of FoxO3a was associated with liver metastasis, a key malignant behavior of colorectal cancer. It was found there was no significant difference between the primary cancer with and without liver metastasis (table 2).

Table 1 Comparison of FoxO3a expression in colorectal cancer tissue and normal colorectal tissue

Case	RT-PCR		Western blot		Case	RT-PCR		Western blot	
	Down-regulated	Up-regulated	Down-regulated	Up-regulated		Down-regulated	Up-regulated	Down-regulated	Up-regulated
1	N	Y	N	Y	15	N	Y	Y	N
2	Y	N	Y	N	16	N	Y	U	U
3	Y	N	Y	N	17	U	U	N	Y
4	Y	N	Y	N	18	N	Y	Y	N
5	Y	N	Y	N	19	N	Y	N	Y
6	Y	N	Y	N	20	N	Y	U	U
7	Y	N	Y	N	21	U	U	U	U
8	N	Y	N	Y	22	N	Y	U	U
9	Y	N	Y	N	23	Y	N	Y	N
10	U	U	Y	N	24	U	U	Y	N
11	N	Y	U	U	25	N	Y	Y	N
12	Y	N	Y	N	26	U	U	Y	N
13	Y	N	Y	N	27	U	U	Y	N
14	U	U	Y	N	28	Y	N	Y	N

Down-regulated: FoxO3a expression was down-regulated in colorectal cancer tissue as compared with normal colorectal tissue; up-regulated: FoxO3a expression was up-regulated in colorectal cancer tissue as compared with normal colorectal tissue; N: no; Y: yes; U: undetectable
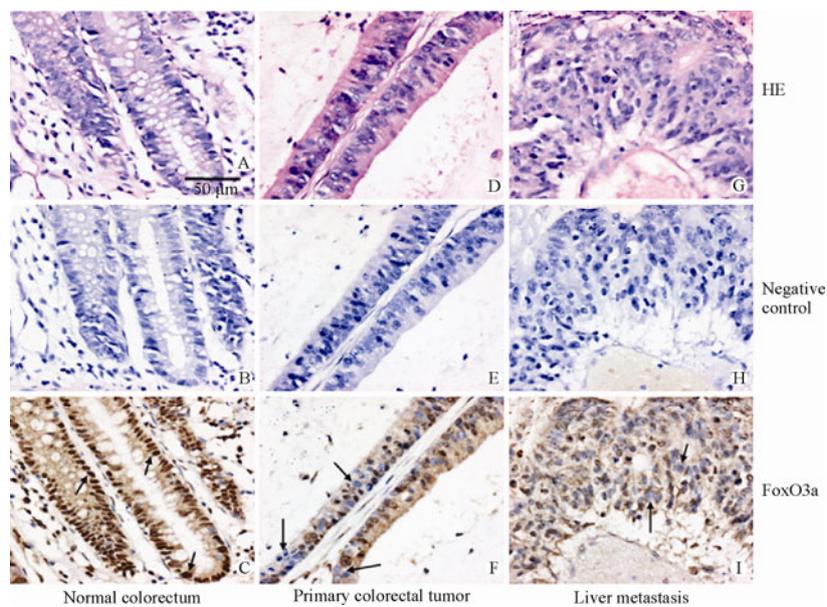
Fig. 3 Immunohistochemical staining of FoxO3a in a colorectal cancer patient with liver metastasis
Paraffin-embedded tissue blocks of normal colorectum, primary tumor and liver metastatic tumor of patients with colorectal cancer were collected. For each block, 3 3-μm-thick serial sections were made, one for hematoxylin and eosin (HE) staining and the other two for immunohistochemistry staining. In normal colon tissue (A, B, C), FoxO3a was highly expressed with strong staining of nearly all the glandular epithelial cells, especially in the nucleus (C). However, in primary tumor tissue (D, E, F) and liver metastasis tissue (G, H, I), the percentages of nuclear positive cells were lower and FoxO3a was mainly localized in the cytoplasm of most positive cells (F, I). Arrows indicated the expression pattern of FoxO3a in the nucleus.

2.4 Silencing FoxO3a Increased the G_2-M Phase Cells

To answer the possible role of the down-regulation of FoxO3a in colorectal cancer development, SW480 cells were transfected with the shRNA-plasmid targeting FoxO3a. Western blot showed that FoxO3a was successfully silenced after 48-h transfection (fig. 5C), and the percentage of G_2-M phase cells was increased from 8.17% to 14.17% after FoxO3a was silenced (fig. 5A and B). Similar results were got with three times of repeated experiments.
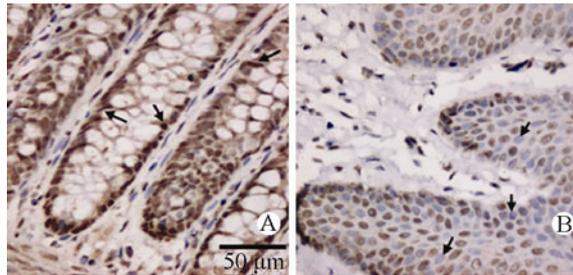
Fig. 4 Expression patterns of FoxO3a in metaplastic colorectal tissue

The expression of FoxO3a was evaluated by immunohistochemistry in metaplastic colorectal tissues. FoxO3a was highly expressed in nucleus of all the cells of normal colorectal tissues (A), while it was relocated in cytoplasm of many cells of metaplastic colorectum (B). Arrows indicated typical examples.

Table 2 Comparison of the expression of FoxO3a between colorectal tissues

Tissues	FoxO3a Low	High	P values
Normal colorectum[a]	2	77	<0.01
Metaplastic colorectum	4	7	
Normal colorectum[a]	2	77	<0.01
Primary colorectal tumors[b]	42	37	
Normal colorectum[c]	0	9	<0.01
Liver metastasis	6	3	
Metaplastic colorectum	4	7	>0.05
Primary colorectal tumors[d]	4	7	
Primary colorectal tumors[e]	4	5	>0.05
Liver metastasis	6	3	
Primary colorectal tumors with no metastasis	42	37	>0.05
Primary colorectal tumors with liver metastasis	4	5	

a: normal colorectal tissues from patients who developed colorectal cancer with no liver metastasis or intestinal metaplasia; b: primary colorectal tumors from patients who developed colorectal cancer with no liver metastasis or intestinal metaplasia; c: normal colorectal tissues from patients who developed colorectal cancer with liver metastasis; d: primary colorectal tumors from patients who developed colorectal cancer with intestinal metaplasia; e: primary colorectal tumors from patients who developed colorectal cancer with liver metastasis. Fisher exact test or χ^2 text was used for the P value test.
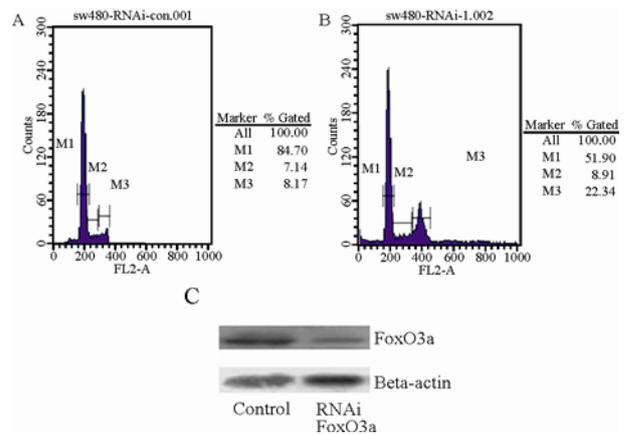
Fig. 5 Silencing FoxO3a increased the percentage of G_2-M phase cells

SW480 cells were transfected with shRNA-plasmids targeting FoxO3a or empty plasmids as a control. Western blot demonstrated that FoxO3a was successfully silenced after transfection (C). Cell cycles were analyzed by flow cytometry. Increased percentage of the G_2-M cells was identified after cells were transfected with FoxO3a-shRNA-plasmids (B), while cells in control group were transfected with empty plasmids (A).

3 DISCUSSION

While the FoxOs family has been studied extensively in signaling pathways and some neoplasms[11], little is known about its role in colorectal cancer. Here, we first reported the expression alteration of FoxO3a in colorectal cancer based on a survey of a large amount of clinical samples. Micro-array analysis identified reduced expression of FoxO3a in primary lesions as well as in the metastasis lesions of colorectal cancer. Confirmatory RT-PCR and Western Blot verified this finding by demonstrating that FoxO3a was down-regulated at the transcription level and the translation level in a moderate number of the primary tumor tissues examined. Immunohistochemistry analysis of 99 cases of colorectal cancer further uncovered that, compared to that in normal colorectal tissue, the nuclear expression of FoxO3a was remarkably reduced in the primary lesion as well as in the metastasis lesion. Moreover, it was found that the nuclear expression of FoxO3a was also down-regulated in the metaplastic colorectal tissue. It is therefore possible that the down-regulation of FoxO3a is associated with the early development of colorectal cancer, which would be important for early intervention of this disease. Besides, no significant difference was detected among precancerous lesions, primary lesions, and metastasis lesions. This may suggest that intervention of nuclear exclusion of FoxO3a might play a role in treatment of all the stages of colorectal cancer, including primary cancer, metastasis cancer, and even the metaplasia, a precancerous change.

Being a member of the forkhead family of transcription factors, FoxO3a is proposed to participate in several important pathways, such as IGF-1 signaling pathway[10], and mainly act as a hinge to kindle cell cycle arrest and apoptosis through regulation of expression of downstream effectors in response to upstream signals[12, 13]. Considering this, it is proposed that altering

the normal function of FoxO3a to inhibit cell cycle progression would tip the balance between cellular differentiation and neoplastic transformation, which would benefit tumor development[14]. Therefore, previous reports classified FoxO3a as a tumor suppressor and inferred that FoxO3a loss of function could play a critical role in oncogenesis[15]. FoxO3a aberration has been reported in several tumors, such as hematologic malignancies[16], breast cancers[17], and prostatic neoplasms[18], mainly focusing on its inactivation through phosphorylation and exclusion of the nucleus. Hence restoring normal function of FoxO3a was speculated to be a promising treatment of tumor[3]. Consistently, the data we presented here raise the potential utility of FoxO3a in development of therapeutic targets for cancer. However, it is also worth mentioning that our RT-PCR results demonstrated that, for more than a third of patients with colorectal cancer, the expression of FoxO3a was already decreased at the transcription level. This supports the notion that the way FoxO3a participates in tumorigenesis is not restricted to inactivation of this protein. Consequently, focusing only on the restoration of its function may not be enough for developing cancer-related drugs associated with the FoxO3a signaling pathway.

The immunohistochemistry also showed that FoxO3a was mainly localized in the cytoplasm of the tumor-associated tissues and the tissues with precancerous change, while the expression of FoxO3a was strong in the nucleus of the normal tissues. These data indicated that FoxO3a was excluded from the nucleus and relocated in the cytoplasm in colorectal cancer. Consistent with our finding, a previous report also demonstrated that FoxO3a was translocated from nucleus to cytosol of the tumor cells in breast cancer[15]. There is accumulating evidence supporting the speculation that FoxO3a transcriptional activity was regulated by altering its intracellular localization[11]. That is, nuclear exclusion of FoxO3a contributes to cell survival and thus has a pathological relationship with the development of cancer. Consequently, FoxO3a could be a new tool for a therapeutic intervention for tumor formation and progress in colorectal cancer. Kau et al had developed several small molecule chemical inhibitors of FoxO nuclear export that confined FoxO proteins in the nucleus where they functions in restraining cell proliferation and promoting apoptosis[5]. This finding may also be useful for treatment of colorectal cancer.

siRNA experiments further disclosed the possible role of downregulation of FoxO3a playing in the development of colorectal cancer. FoxO3a has been determined to mediate the expression of several genes, including Gadd45 and cyclin B1, which can modulate the G_2/M checkpoint[19, 20]. Thus FoxO3a could inhibit cell cycle progression and suppress tumor formation through modulation of the expression of downstream genes. In the present study, silencing FoxO3a greatly elevated the percentage of G_2/M phase SW480 cells. We propose the disability of FoxO3a function of cell cycle arrest contributed to this cell-cycle-change, and thus confer advantage to survival of tumor cells and tumor development.

In summary, this study demonstrated the close relation between the reduced expression of FoxO3a and the development of colorectal cancer. More importantly, the precancerous change and tumor metastasis were also determined to be associated with the decreased FoxO3a expression. These data may support the notion that FoxO3a could be a novel target for therapeutic intervention in colorectal cancer.

REFERENCES

1 Eilstein D, Hedelin G, Schaffer P. Incidence of colorectal cancer in Bas-Rhin, trend and prediction in 2009. Bull Cancer, 2000,87(7-8):595-599

2 Grady WM, Markowitz SD. Genetic and epigenetic alterations in colon cancer. Annu Rev Genomics Hum Genet, 2002,3:101-128

3 Accili D, Arden KC. FoxOs at the crossroads of cellular metabolism, differentiation, and transformation. Cell, 2004,117(4):421-426

4 Ticchioni M, Essafi M, Jeandel PY, et al. Homeostatic chemokines increase survival of B-chronic lymphocytic leukemia cells through inactivation of transcription factor FOXO3a. Oncogene, 2007,26:7081-7091

5 Kau TR, Schroeder F, Ramaswamy S, et al. A chemical genetic screen identifies Inhibitors of regulated nuclear export of a Forkhead transcription factor in PTEN-deficient tumor cells. Cancer Cell, 2003,4(6):463-476

6 Radetzki S, Kohne CH, Von HC, et al. The apoptosis promoting Bcl-2 homologues Bak and Nbk/Bik overcome drug resistance in Mdr-1-negative and Mdr-1-overexpressing breast cancer cell lines. Oncogene, 2002,21(2): 227-238

7 Chen Q, Chai YC, Mazumder S, et al. The late increase in intracellular free radical oxygen species during apoptosis is associated with cytochrome c release, caspase activation, and mitochondrial dysfunction. Cell Death Differ, 2003,10:323-334

8 Santos NR, Torensma R, Vries T, et al. Heterogeneous expression of the SSX cancer/testis antigens in human melanoma lesions and cell lines. Cancer Res, 2000,60: 1654-1662

9 Wu J, Feng Y, Xie D, et al. Unscheduled CDK1 activity in G1 phase of the cell cycle triggers apoptosis in X-irradiated lymphocytic leukemia cells. Cell Mol Life Sci, 2006,63:2538-2545

10 Arden KC. FoxO: linking new signaling pathways. Mol Cell, 2004,14(4):416-418

11 Heide LP, Hoekman MF, Smidt MP. The ins and outs of FoxO shuttling: mechanisms of FoxO translocation and transcriptional regulation. Biochem J, 2004,380(2):297-309

12 Kops GJ, Medema RH, Glassford J, et al. Control of cell cycle exit and entry by protein kinase B-regulated forkhead transcription factors. Mol Cell Biol, 2002, 22(7):2025-2036

13 Scheijen B, Ngo HT, Kang H, et al. FLT3 receptors with internal tandem duplications promote cell viability and proliferation by signaling through Foxo proteins. Oncogene, 2004,23:3338-3349

14 Yuan M, Konstantopoulos N, Lee J, et al. Reversal of obesity- and diet-induced insulin resistance with salicylates or targeted disruption of Ikkbeta. Science, 2001,293

(5535):1673-1677

15 Hu MC, Lee DF, Xia W, *et al*. IkappaB kinase promotes tumorigenesis through inhibition of forkhead FOXO3a. Cell, 2004,117(4):225- 237

16 Gu TL, Tothova Z, Scheijen B, *et al*. NPM-ALK fusion kinase of anaplastic large-cell lymphoma regulates survival and proliferative signaling through modulation of FOXO3a. Blood, 2004,103(12):4622-4629

17 Belguise K, Sonenshein GE. PKCtheta promotes c-Rel-driven mammary tumorigenesis in mice and humans by repressing estrogen receptor alpha synthesis. J Clin Invest, 2007,117(12):4009-4021

18 Lynch RL, Konicek BW, McNulty AM, *et al*. The pro-gression of LNCaP human prostate cancer cells to androgen independence involves decreased FOXO3a expression and reduced p27KIP1 promoter transactivation. Mol Cancer Res, 2005,3(163):163-169

19 Tran H, Brunet A, Grenier JM, *et al*. DNA repair pathway stimulated by the Forkhead transcription factor FOXO3a through the Gadd45 protein. Science, 2002,296(5567): 530-534

20 Alvarez B, Martinez A, Burgering BM, *et al*. Forkhead transcription factors contribute to execution of the mitotic programme in mammals. Nature, 2001,413:744-747