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Short communication

DIFFERENTIAL FUCOSYLTRANSFERASE IV EXPRESSION IN SQUAMOUS CARCINOMA CELLS IS REGULATED BY PROMOTER METHYLATION

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Abstract: Enhanced fucosyltransferase IV (*FUT4*) expression correlates with increased tumor malignancy in many carcinomas. However, little is known about the regulation of *FUT4* expression, and whether *FUT4* expression is influenced by the methylation status of the *FUT4* promoter is unclear. In this study, we demonstrated that *FUT4* expression is negatively correlated with the methylation degree of a CpG island in the *FUT4* promoter, suggesting that the methylation status of *FUT4* promoter regulates the expression of *FUT4*. The results indicate that manipulating the methylation status of the *FUT4* promoter to regulate *FUT4* expression may be a novel approach in the treatment of malignant tumors.

Key words: Fucosyltransferase, *FUT4* promoter, Methylation, A431 cells, SCC12 cells

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Abbreviations used: BSP – bisulfite sequencing PCR; FUT4 – fucosyltransferase IV; MSP – methylation-specific PCR; SCC – squamous carcinoma cell; 5-aza-dC – 5-aza-2-deoxycytidine

INTRODUCTION

Fucosyltransferases (FUTs) are the key enzymes that regulate the synthesis of fucosylated oligosaccharides, such as Lewis a (Lea), sialyl Lewis a (sLea), Lewis b (Leb), Lewis X (LeX), sialyl Lewis X (sLeX) and Lewis Y (LeY), which are bound to cell surface glycoproteins or glycolipids [1]. FUTs are classified as α-1, 2 FUTs (FUT1 and FUT2), α-1, 3/4 FUTs (FUT3, FUT4, FUT5, FUT6, FUT7 and FUT9) and α-1, 6 FUT (FUT8) based on their acceptor specificity [2-3]. FUTs play important roles in cancer biology, as increased fucosylation levels of glycoproteins and glycolipids have been reported in a number of cancers [4-6].

FUT4, a key enzyme for the synthesis of α 1, 3-fucosylated oligosaccharides such as LeY, catalyzes the transfer of fucose (Fuc) residues from GDP-Fuc to [Fucα1→2Galβ1→4GlcNAcβ1→R] in α-1, 3 linkage. Elevated *FUT4* expression has been found to correlate with tumor progression [7-9]. Although the impact of *FUT4* expression on cell proliferation has been studied [10-11], little information is available on how *FUT4* expression is regulated. Accumulating evidence suggests that tumor progression is related to the abnormal hypomethylation of growth regulating genes [12]. These genes, such as *synuclein-γ* (*SNCG*), and *paired-box gene 2* (*PAX2*), are methylated in normal tissues, but become hypomethylated and highly expressed in cancers [12-13]. Moreover, glycogene expression regulation is involved in promoter methylation status. Incomplete synthesis of carbohydrate determinants, such as sLea and sLeX, occurs through the silencing of glycogenes by DNA methylation in early stage cancers [14]. The promoter region of the human *B4GALNT2* gene is heavily hypermethylated in many gastrointestinal cancer cell lines and leads to decreased expression of Sd(a) carbohydrate [15]. However, it is unclear whether *FUT4* expression is directly influenced by the methylation status of its promoter.

Using two SCC cell lines, A431 and SCC12 cells, we found that a lower methylation level of *FUT4* promoter correlated with a higher *FUT4* expression in A431 cells compared with that in SCC12 cells. Furthermore, we demonstrated that treatment with 5-aza-2-deoxycytidine (5-aza-dC), a common methyltransferase inhibitor, significantly decreased the methylation of *FUT4* promoter and increased *FUT4* expression in SCC12 cells, but did not significantly affect the already low methylation level of *FUT4* promoter and high level of *FUT4* expression in A431 cells. To our knowledge, this is the first report to evaluate the role of *FUT4* promoter methylation status in *FUT4* expression.

MATERIALS AND METHODS

Cell culture

The human A431 cell line was obtained from the American Tissue Culture Collection (Manassas, VA), and the SCC12 cell line was provided by Dr. Rheinwald, Harvard University (Boston, MA). Both A431 and SCC12 cells were maintained in DMEM/F12 (1:1, Invitrogen) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 50 μg/ml streptomycin at 37°C under 5% CO_2 in humidified air.

Semi-quantitative RT-PCR and real-time PCR

Total RNA was extracted and purified from cells using the TRIzol® reagent (Invitrogen) in the presence of DNase. The first-strand cDNAs were synthesized by RT reaction using oligo-dT primer with M-MLV reverse transcriptase (Takara). Semi-quantitative PCR and real-time PCR were carried out following the conditions listed in the Supplemental Table in Supplementary material at http://dx.doi.org/10.2478/s11658-012-0003-x.

5-aza-dC treatment

After plating for 24 h, cells were treated with either 1-10 μM 5-aza-dC (Sigma) or vehicle (2.5-25 μl acetic acid/l medium) for 72 h. 5-aza-dC was replenished every 24 h.

Methylation-specific PCR (MSP) and bisulfite sequencing PCR (BSP) assays

Genomic DNA was extracted and purified from cells pretreated with or without 5-aza-dC as indicated elsewhere, then incubated with bisulfite using EZ DNA Methylation-Gold Kit per manufacturer's instruction (ZYMO REASCHER). The bisulfite-modified DNAs were used as templates for MSP and BSP. MSP and BSP reactions were conducted following the conditions listed in the Supplemental Table. The MSP products were analyzed using 1.5% agarose gel electrophoresis. The BSP products were cloned into pMD18-T vector (Takara) and sequenced.

Western blot and lectin blot analysis

Western blot and lectin blot were performed as described [2, 10]. Total proteins (5-20 μg/lane) from the whole cell lysate were separated by 10% SDS-PAGE mini-gels and transferred electrophoretically onto nitrocellulose membranes. After blocking with 5% dry milk-TBST for 1 h at room temperature or 1x carbo-free blocking buffer overnight at 4°C, each membrane was incubated with goat anti-FUT4 antibody (1:100) overnight at 4°C or biotin-*Aleuria aurantia* lectin (AAL, 1 μg/ml) for 1 h at room temperature. The specific antibody binding was detected using HRP or avidin-HRP-conjugated secondary antibody. Anti-β-actin antibody was used to confirm the equal loading. All antibodies used in Western blot were made by Santa Cruz (Santa Cruz, CA) and biotin-AAL was made by VECTOR laboratories (Burlingame, CA). An enhanced chemiluminescence

(ECL) detection system (Amersham) was used to determine the expression of *FUT4* and fucosylated proteins.

Flow cytometry assay
Suspended single cells ($1x10^6$) prepared as indicated were permeabilized in 0.1% Triton X-100-PBS (4°C, 10 min) before incubating with goat anti-FUT4 antibody (room temperature, 1 h). FITC-conjugated rabbit anti-goat IgG was used to label the FUT4-positive cells followed by FACScan flow cytometer detection.

Immunofluorescence staining
Cells plated on the coverslips were fixed in cold acetone (-20°C, 20 min). 3% BSA was used to block non-specific binding (37°C, 2 h). Cells on the coverslips were incubated with goat anti-FUT4 antibody (1:100) and mouse anti-Golgi marker antibody (AE-6) (1:100, Santa Cruz, CA) overnight at 4°C. TRITC-conjugated rabbit anti-goat secondary antibody (1:50, Santa Cruz, CA) and FITC-conjugated donkey anti-mouse secondary antibody (1:50, Protein Tech Group, Inc) were used to detect the specific antibody binding. Images were captured using an Olympus BX51 microscope (Japan).

Statistical analysis
All data presented were obtained from at least three independent experiments and expressed as means ± standard deviation. Data were analyzed statistically by Student's *t* test, with $p < 0.05$ considered to be significant.

RESULTS

Expression of *FUT4* in cells
By semi-quantitative RT-PCR (Fig. 1A), Western blot (Fig. 1B), flow cytometry assay (Fig. 1C) and immunofluorescence staining (Fig. 1D), we found that *FUT4* was expressed in both cell lines, but its expression level was greatly enhanced in A431 cells compared to that in SCC12 cells. As shown in Fig. 1D, FUT4 predominantly co-localized with AE-6 in Golgi apparatus.

Methylation status of the CpG island in the *FUT4* promoter
To analyze whether the expression of *FUT4* was regulated by the methylation status of the *FUT4* promoter, the CpG islands in *FUT4* promoter were predicted by the program MethPrimer (http//www.urogene.org//methprimer/indexl.html). The results indicated that two CpG islands were presented in the *FUT4* promoter from –29 to –725 bp, which are located upstream of the transcription initiation site, and fulfill the criteria for CpG islands [16]. We selected one of the two CpG islands (– 429 to – 671 bp), which contained 30 CpG sites (Fig. 2A), to evaluate the methylation status of *FUT4* promoter in A431 and SCC12 cells. By MSP assay, we found both methylated and unmethylated PCR products in these two cell lines. The primers to amplify the methylated fragments yielded more PCR products from SCC12 cells than from A431 cells (Fig. 2B, top row), whereas the

primers to amplify the unmethylated fragments yielded more PCR products from A431 cells than from SCC12 cells (Fig. 2B, bottom row). These results suggest that the CpG island in the *FUT4* promoter was more methylated in SCC12 cells than in A431 cells. The methylation status of the selected CpG island was also analyzed by BSP assay as described. At least 25 randomly selected clones per cell line were sequenced and the results from 5 representative clones were presented. We found that the 30 CpG sites of the selected CpG island in the *FUT4* promoter were rarely methylated in A431 cells (Fig. 2C), but highly methylated in all of the SCC12 clones tested (Fig. 2D).

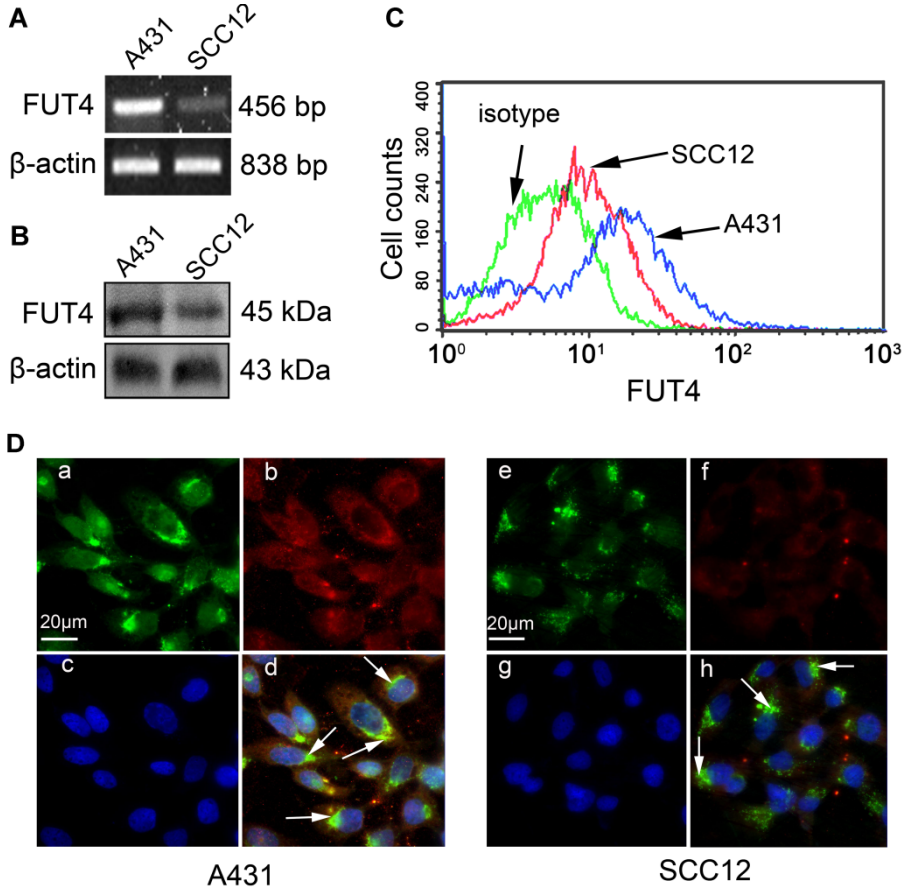

Fig. 1. The expression of *FUT4* in A431 and SCC12 cells. *FUT4* expression in A431 and SCC12 cells was detected by (A) semi-quantitative RT-PCR, (B) Western blot, (C) flow cytometry assay and (D) immunofluorescence co-staining of FUT4 with a Golgi marker, AE6 (see "Materials and methods"). The expression level of β-actin was used as the control for semi-quantitative RT-PCR and Western blot. Cells incubated with secondary antibody only served as an isotype control for flow cytometry assay. Golgi region was labeled as green, FUT4 red. Co-staining of Golgi region and FUT4 was indicated with arrows in "d" and "h" (yellow). DNA was stained with DAPI (blue). Scale bar = 20 μm.

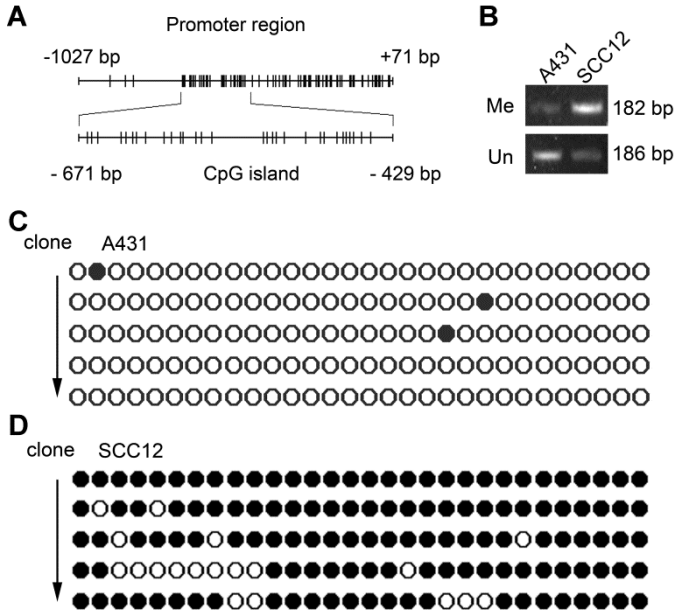

Fig. 2. Methylation status of a CpG island in *FUT4* promoter. A – Map of a CpG island in the *FUT4* gene promoter as predicted by MethPrimer software. CpG sites were presented with vertical bars. The CpG island (-429 to -671 bp) analyzed is highlighted. B – The products of PCR amplified with MSP primers were identified on 1.5% agarose gel. Me: methylation; Un: unmethylation. C, D – The products of PCR amplified with BSP primers were cloned. Five representative sequenced clones from each cell line were presented. Each circle represents one CpG site in the CpG island. ● : methylated CpG site; ○: unmethylated CpG site.

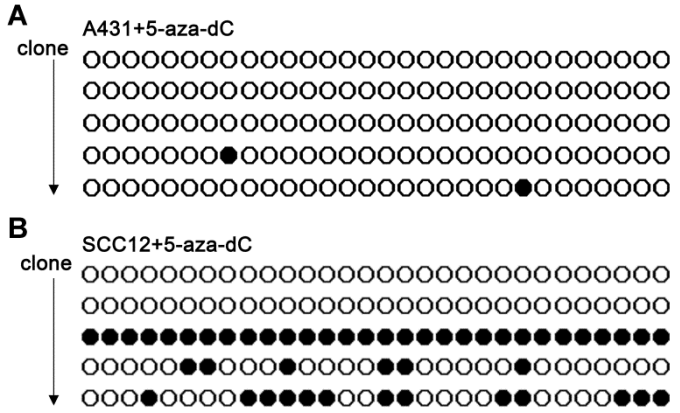

Fig. 3. Methylation status of a CpG island in the *FUT4* promoter in 5-aza-dC-treated cells. The products of PCR amplified with BSP primers were cloned. Five representative sequenced clones were presented. A – A431 cells treated by 5-aza-dC. B – SCC12 cells treated by 5-aza-dC. Each circle represents one CpG site in the CpG island. ● : methylated CpG site; ○: unmethylated CpG site.

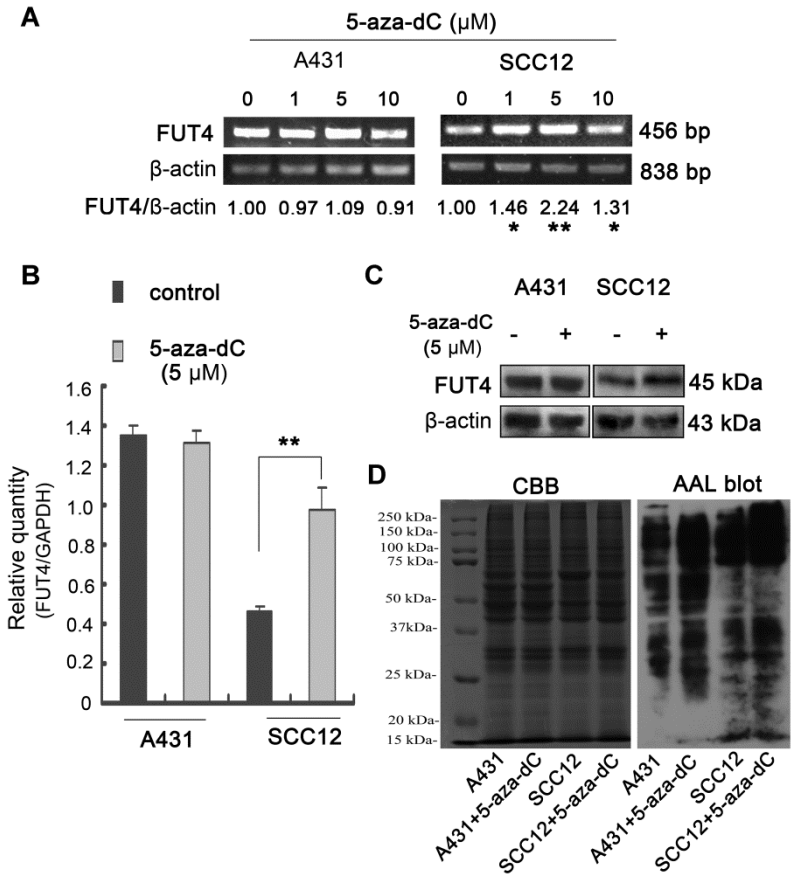

Fig. 4. FUT4 and fucosylated protein expression in 5-aza-dC-treated cells. By (A) semi-quantitative RT-PCR, (B) real-time PCR and (C) Western blot, *FUT4* expression was examined after treatment of cells without or with 1-10 μM 5-aza-dC. The expression level of β-actin or GAPDH was used as an internal control. The band density of PCR products in agarose gel was analyzed using NIH ImageJ program. All data presented are an average of three independent PCR reactions. * $p < 0.05$; ** $p < 0.01$. By (D) AAL blot, fucosylated protein expression was detected (right panel), and Coomassie Brilliant Blue (CBB) staining of gels showed comparable amounts of proteins in each lane (left panel).

Effect of 5-aza-dC on the methylation status of the CpG islands in the *FUT4* promoter

The methylation status of the CpG islands in the *FUT4* promoter was also examined after treatment with 5-aza-dC. Results acquired by BSP assay showed that treatment with 5-aza-dC did not significantly change the number of methylated CpG sites in any of the five randomly selected clones from A431 cells (Fig. 3A vs. 2C). However, the numbers of methylated CpG sites were significantly reduced in all of the SCC12 clones tested, with two clones completely free of methylation (Fig. 3B vs. 2D). Consistently, MSP assay also showed that treatment with 5-aza-dC only decreased the degree of methylation

in the *FUT4* promoter in SCC12 cells, but not in A431 cells (not shown). These results imply that 5-aza-dC preferentially demethylates the *FUT4* promoter in cells with a hypermethylated *FUT4* promoter.

Effect of 5-aza-dC on the expression of *FUT4* and fucosylated proteins

By semi-quantitative RT-PCR (Fig. 4A), real-time PCR (Fig. 4B) and Western blot (Fig. 4C), we found that the expression of *FUT4* was dramatically increased in SCC12 cells ($p < 0.01$) when cells were treated with 5 μM 5-aza-dC. In contrast, treatment with different doses of 5-aza-dC (1, 5, or 10 μM) did not further enhance the already high expression level of FUT4 in A431 cells, in which the *FUT4* promoter is hypomethylated. These results suggest that *FUT4* expression is regulated by its gene promoter methylation status. The effects of 5-aza-dC on the expression of fucosylated proteins in A431 and SCC12 cells were also detected by biotinylated AAL blot (Fig. 4D). The results show that the fucosylation of proteins was significantly increased in 5-aza-dC treated SCC12 cells.

DISCUSSION

In this study, we demonstrated that the methylation of the CpG island in the *FUT4* promoter regulates *FUT4* expression. In comparison to the SCC12 cells, the degree of methylation of the CpG island in the *FUT4* promoter is significantly lower, and the expression level of *FUT4* is dramatically higher, in A431 cells. Treatment of the cells with 5-aza-dC preferentially decreases the methylation of the CpG island in the *FUT4* promoter and increases the expression of *FUT4* in SCC12 cells, and not in A431 cells. The expression of fucosylated proteins is also significantly elevated in SCC12 cells with 5-aza-dC treatment.

Increased *FUT4* expression is seen in carcinomas of the lung, stomach, melanoma, and acute myeloid leukemia [1, 7-9]. The mechanism of increased *FUT4* expression in cancers is still largely unknown. Hypomethylation in gene promoter regions is considered as one of the mechanisms for enhanced expression and activation of oncogenes or cancer-promoting genes during carcinogenesis [12]. For example, the expression of the *SNCG* gene is increased by hypomethylation in breast carcinoma, and elevated SNCG expression stimulates breast cancer proliferation and metastasis [12]. *FUT3* overexpression in gastric cells also depends on hypomethylation of its promoter [17]. A similar observation is reported in *FUT7* expression [18]. Although increases in the fucosylation level and *FUT4* expression were observed in MDA-MB-231 cells treated with a methyltransferase inhibitor, zebularine [19], it is unclear whether *FUT4* expression level is directly correlated with the methylation status of the *FUT4* promoter. Previous studies show that the *FUT4* promoter fits well with the CpG island model, and has a non-TATA box-dependent transcriptional start region [20]. Two CpG islands were predicted in *FUT4* promoter by the software, and we found that the positions of these two CpG islands are in the same region of the two *FUT4* promoter enhancers that were identified in myeloid and colon

adenocarcinoma cell lines [21]. Taken together, these studies indicate that the CpG islands in the *FUT4* promoter may play an important role in regulating *FUT4* expression, and this may be dependent on the methylation status of the CpG sites. By comparing the expression level of *FUT4* and the methylation status of a CpG island in the *FUT4* promoter, we discovered that *FUT4* expression level and the degree of methylation of the *FUT4* promoter region in A431 and SCC12 cells are negatively correlated.

We have previously found that the proliferation of A431 cells is increased by *FUT4* overexpression, and reduced by knocking down *FUT4* expression [10-11]. We have recently found that A431 cells have a relatively high proliferative ability, which correlates with a higher level of FUT4, while SCC12 cells had a relatively low proliferative capability, which correlates with a lower level of FUT4. Consistently, the proliferation of A431 and SCC12 cells is dramatically hindered by knocking down *FUT4* expression and significantly increased by *FUT4* overexpression (not shown). This evidence suggested that *FUT4* expression affects cancer proliferation. In addition, FUT4 is known to associate with other factors of malignancy such as metastasis [8]. Among FUT4 catalyzed fucosylated oligosaccharide antigens, LeY expression correlates with tumor proliferation, invasion and metastasis in carcinomas of breast, cervix, and ovary [4, 22-24]. Although a few studies have shown that sLeX and sLea act as selectin ligands that can mediate carcinoma metastasis [25-26], there is no direct evidence showing that the binding of LeY and selectins mediates a similar process. These studies indicate that regulation of *FUT4* promoter methylation to alter the expression of *FUT4* and the fucosylated tumor-associated antigens may be a potential approach to inhibit tumor malignancy.

In conclusion, different methylation levels of the *FUT4* promoter play a critical role to regulate *FUT4* expression, which in turn may affect the proliferative capabilities in squamous carcinoma cells. The degree of methylation in the *FUT4* promoter may serve as a promising biomarker for squamous cell carcinomas or a target for therapy.

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