



The hypermethylation of MUC2 promoter associated with mRNA and metastasis in esophageal squamous cell carcinoma

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Background: We evaluated the association between MUC2 methylation status and mRNA level in esophageal squamous cell carcinoma (ESCC) patients.

Methods: The methylation index was examined by methylation-specific PCR and MUC2 mRNA was examined in paired tissue samples of 310 ESCC patients by real-time PCR.

Results: The mean of MUC2 methylation index was 0.68 in ESCCs, and only 0.45 in non-tumor samples. There was a significant association between MUC2 methylation and lymph node metastasis, distant metastasis in ESCC. The lower level of MUC2 mRNA were in ESCC tissues (Mean_{-ΔCt} = -6.65), and higher in Non-tumor tissues (Mean_{-ΔCt} = -4.11). The lower MUC2 mRNA ($-\Delta\Delta C_{t_{MUC2}} \leq -2.54$) was 112/185 (60.54%) in ESCC with AJCC stage III-IV, 105/162 (64.81%) with N2-3 Lymph node metastasis, and 99/142 (69.72%) with differentiation G2. Meanwhile, the level of MUC2 mRNA were lower in ESCC patients with methylated promoter (Mean_{-ΔΔCt} = -3.58) than those with hypomethylation (Mean_{-ΔΔCt} = -0.32). And the ESCC patients with MUC2 silencing and hypermethylation in promoter had an unfavorable outcome.

Conclusions: The results implied that the hypermethylation could be an important factor for silencing of MUC2 mRNA and a potential biomarker for poor survival in ESCC.

Keywords: MUC2; esophageal squamous cell carcinoma (ESCC); methylation

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Introduction

Esophageal cancer is divided into two main types: squamous cell carcinoma and adenocarcinoma. Esophageal squamous cell carcinoma (ESCC) in China is a type of high incidence of esophageal cancer (1). Despite improvements in clinical treatments, the 5-year survival are still very poor regardless of race and gender and are not significantly improved over the past several decades (2,3).

Epithelial surfaces are protected by mucins, which are secreted by the gastrointestinal tract. As a secretory mucin gene, MUC2 is high expressed in the cells of human colon

and small intestine, by which to protect gastro-intestinal tract from damaging by several risk factors (4-6). The abnormal level of MUC2 mRNA and protein has been found in many types of cancers (7). The results found that MUC2 and MUC5AC were decreased in endometrial and cervical tissues (8). Genetic variations in MUC2 could be an important factor for gastric cancer with *H. pylori* infected patients (9). The expression of MUC2 is decreased in gastric signet-ring cell carcinoma (10). The MUC2 expression was increased induced Bile acids in colon carcinoma cells through involving of AP-1 (11). Galectin-3 could up-regulate of MUC2

transcription through AP-1 activation in colon cancer (12).

The level of the MUC2 mRNA could be controlled by the promoter methylation in tumorigenesis. The expression of MUC2 mRNA is associated with promoter methylation status in mucinous gastric carcinoma (13). The promoter of MUC2 gene was highly average 87% methylated in PANC1 cell line, while average 43% methylated in the BxPC3 cell line and average 33% methylated in normal colon crypts (14). Yokoyama *et al.* found MUC2-negative expression in pancreatic ductal adenocarcinomas, MUC2(+) expression in intestinal-type intraductal papillary mucinous neoplasms, and MUC2(-) expression in gastric-type intraductal papillary mucinous neoplasms. The promoter methylation of MUC1, MUC2 and MUC4 was significant associated with mucin expression in pancreatic neoplasms (15). Meanwhile, the CpG island methylation MUC2, MUC5AC and MUC6 had a positive association with expression and other clinicopathologic variables (16). The results have found that the mRNA level of MUC2 gene silenced by promoter hypermethylation is a risk factor for a unfavorable clinical outcome in HCC (17). These results indicated that the promoter methylation of MUC2 gene could be an important marker in ESCC.

However, we have no plenty information in the regulation of MUC2 gene expression in ESCC, especially in DNA methylation. The aim is to examine the promoter methylation associated with MUC2 mRNA expression in 310 ESCC patients in our study.

Methods

Patients and tissue samples

The 310 patients were randomly selected from Changzhou Cancer Hospital and Nanyang Center Hospital between 2001 and 2014 in China. The tissues included tumors tissues and the corresponding non-tumor tissues were obtained from surgical resection after operation. The all of the relevant medical recorded were reviewed carefully by three pathologists individually. The patients ageing from 36 to 82 years included 180 men and 130 women. The study was agreed with the ethics standards of the committee on Human Experimentation of the Soochow University.

Methylation analysis of MUC2

We detected the promoter methylation of MUC2 gene by methylation-specific PCR assay as previously described (17). The first was to obtained DNA from ESCC tissues, then

the DNA was treated by bisulfite modification according to our previous study (17). MUC2 methylation was measured using primers: forward primer of unmethylated MUC2 gene, 5'-GTTGTTTTATTTTGAAGAAGGTTGTG-3', reverse primer, 5'-TAACAAAAACAATATAAATTACACCCAAA-3'; forward primer of methylated MUC2 gene, 5'-GTTGTTTTATTTTGAAGAAGGTTGC-3', reverse primer, 5'-CGATATAAATTACGCCCGAA-3'. The MSP products were separated by 2% agarose gel electrophoresis and visualized by BIO-RAD Gel Doc XR + (Bio-Rad Laboratories, Inc.). Gels intensity profiles analysis was measured by Quantity One (Bio-Rad Laboratories, Inc.). Quantitative comparison of the band between samples was made for the same gel. We calculated the promoter methylation index (MI) of MUC2 gene by the following formula: $100 \times \text{methylated reaction} / (\text{unmethylated reaction} + \text{methylated reaction})$. ΔMI defined as $\text{MI}_{\text{ESCC}} - \text{MI}_{\text{Non-tumor}}$.

MUC2 mRNA expression by QPCR

Total RNA was isolated from 310 ESCC and non-tumor tissues. The first-strand cDNA was synthesized from 2 μg of total RNA. The level of MUC2 mRNA were detected by primers: forward primer (5'-CTTCGACGGACTCTACTACAGC-3') and reverse primer (5'-CTTTGGTGTTGTTGCCAAAC-3') (17). qPCR was carried out by the Mx3000P QPCR System (Stratagene, USA). The qPCR for MUC2 mRNA carried out in following conditions: 95 °C, 30 s; 60 °C, 30 s; and 72 °C, 1 min by 40 cycles. Meanwhile, β -actin mRNA was detected as an internal control. All results were normalized to β -actin.

Statistical analysis

Fisher's exact test, Chi-square test, and two-sample *t*-test were used to evaluate the statistical differences among the groups with different clinicopathological data. The survival data were analyzed using the Kaplan-Meier method, differences were determined using the log-rank test. All data was performed using SPSS software 18.0 (SPSS Incorporated, Chicago, IL, USA). $P < 0.05$ was considered significant.

Results

The methylation index (MI) MUC2 gene in ESCC patients

We analyzed the promoter methylation index of MUC2 gene by MSP in ESCC patients. The MI means the methylation index. Our results indicated that the mean MI of MUC2

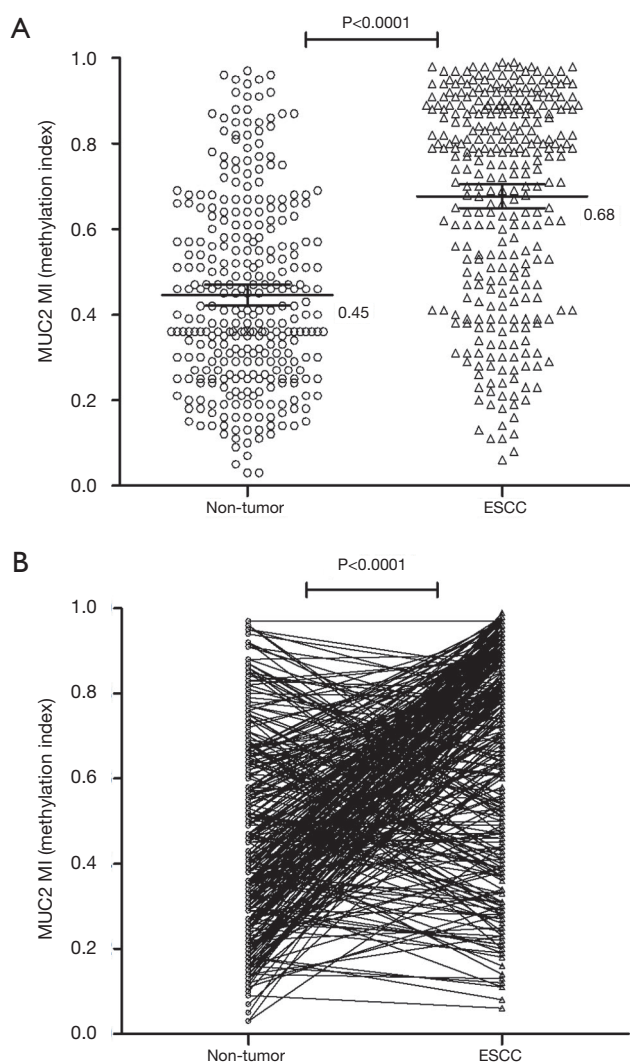


Figure 1 The methylation index of MUC2 promoter in ESCC patients. (A) The mean methylation index of MUC2 promoter in tumor and non-tumor tissues with ESCC patients; (B) the methylation index of MUC2 was decreased in ESCC than in non-tumor tissues. *t*-test was used for statistical differences.

promoter was 0.68 (95% CI, 0.65–0.70) in tumor tissues, and 0.45 (95% CI, 0.42–0.47) in Non-tumor tissues. This results implied that MI of MUC2 gene was significantly difference between the ESCC tissues and non-tumor tissues ($P < 0.0001$) (Figure 1). The MI of MUC2 promoter was elevated ($\Delta MI > 0$) in 211 (68.06%) of the 310 ESCC patients but only decreased ($\Delta MI \leq 0$) in 99 (31.94%) of the patients. It could indicate the hypermethylation of MUC2 gene is an important factor for the development of ESCC.

The methylation level of MUC2 gene is associated with and clinical characteristics

The ESCC patients were divided into two groups: MUC2 hypermethylation group ($\Delta MI > 0$), 211 cases; MUC2 hypomethylation group ($\Delta MI \leq 0$), 99 cases, respectively. We compared the related clinical characteristics in two groups of ESCC patients (Table 1). We found a statistical difference between $\Delta MI \leq 0$ and $\Delta MI > 0$ groups in Lymph node metastasis and Distant metastasis; $P < 0.0001$, $P = 0.026$, respectively), by which implied that MUC2 gene hypermethylation could be more in ESCC patients with metastasis cases.

For the next, we want to know the relation between the promoter MI of MUC2 gene and outcomes after surgery, we analyzed the survival by the Kaplan-Meier method in different groups (Figure 2). We found the median cumulative survival was 36 months in hypermethylation cases, but was for 41 months in hypomethylation group (log-rank $P = 0.0004$; HR = 1.778; 95% CI, 1.29–2.45). The cutoff value 0.68 was set according to the mean MI of MUC2 in tumor tissues. Meanwhile, we did not find difference in ESCC patient with $MI > 0.68$ (37 months) compared with 39 months in those with $MI \leq 0.68$ group (log-rank $P = 0.130$; HR = 1.288; 95% CI, 0.93–1.79). These results suggested that ESCC with demethylation from non-tumor to tumor could have a poorer prognostic.

MUC2 mRNA expression in tumor and non-tumor tissues

To quantify relatively MUC2 mRNA levels in tumor and non-tumor tissues by a real-time PCR assay. The expression of MUC2 mRNA is lower in ESCC samples (mean_{- ΔCt} = -6.65; 95% CI, -6.95 to -6.35) than that in non-tumor tissues (mean_{- ΔCt} = -4.11; 95% CI, -4.39 to -3.82). There was a significant difference in MUC2 mRNA between different tissues ($P < 0.0001$, Figure 3). And, we found 104 (33.55%) of the ESCC patients with MUC2 mRNA elevated ($-\Delta \Delta Ct > 0$), 206 (66.45%) of the patients with MUC2 mRNA decreased ($-\Delta \Delta Ct \leq 0$), by which implied MUC2 mRNA silenced could be an important factor in ESCC.

MUC2 mRNA expression associated with clinicopathologic data in ESCC

The expression of MUC2 mRNA in 310 ESCC patients related with clinicopathological variables is summarized in Table 2. There was a statistical difference between MUC2 mRNA with AJCC stage, lymph node metastasis, and

Table 1 The methylation index of MUC2 gene correlated with clinicopathologic variables in ESCC

Variables	No.	$\Delta MI \leq 0$ (N=99)	$\Delta MI > 0$ (N=211)	Odds ratio (95% CI)	P value*
Gender				0.81 (0.5–1.31)	0.390
Male	180	54	126		
Female	130	45	85		
Age				1.33 (0.82–2.16)	0.249
≥ 60 years	170	59	111		
< 60 years	140	40	100		
Size				1.36 (0.84–2.21)	0.214
< 3 cm	172	60	112		
≥ 3 cm	138	39	99		
Tobacco				1.56 (0.96–2.54)	0.072
Yes	168	61	107		
No	142	38	104		
Alcohol				0.79 (0.49–1.28)	0.336
Yes	147	43	104		
No	163	56	107		
Depth of invasion				0.77 (0.46–1.31)	0.336
T ₁₋₂	224	68	156		
T ₃₋₄	86	31	55		
AJCC stage				1.14 (0.70–1.85)	0.605
I–II	125	42	83		
III–IV	185	57	128		
Lymph node metastasis				2.77 (1.68–4.54)	<0.0001
N ₀₋₁	148	64	84		
N ₂₋₃	162	35	127		
Distant metastasis				0.52 (0.29–0.93)	0.026
M ₀	251	73	178		
M ₁	59	26	33		
Differentiation				–	0.561
G ₁	88	28	60		
G ₂	142	49	93		
G ₃	80	22	58		

*, comparison was done with Pearson's Chi-square test.

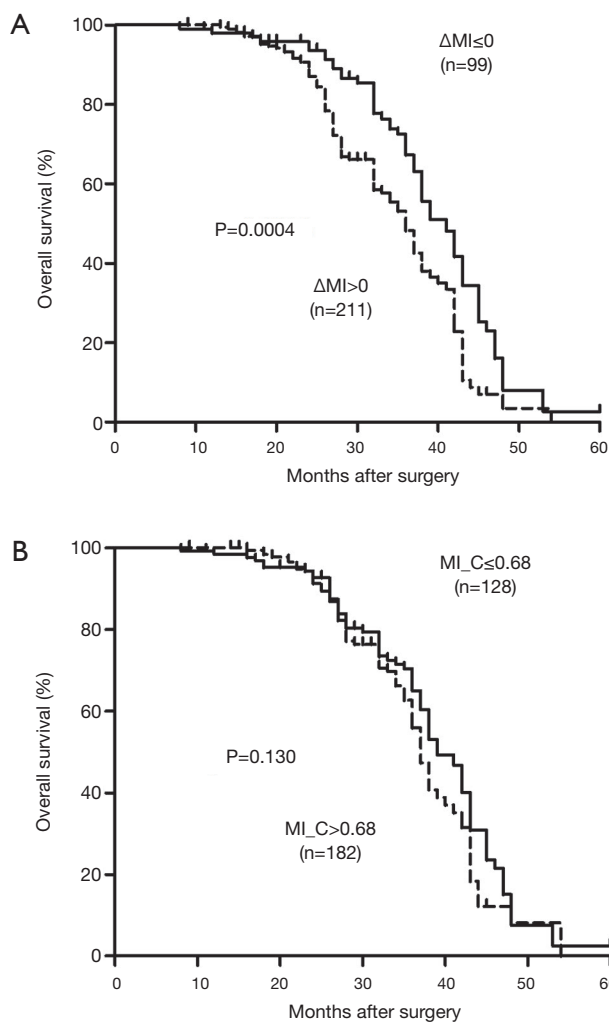


Figure 2 MUC2 promoter methylation with survival after surgery in ESCC patients. (A) The survival rate after operation by Kaplan-Meier analysis in different groups with MUC2 methylation index. The patients with hypermethylation of MUC2 ($\Delta MI > 0$) had a poor overall survival after surgery in ESCC patients; (B) the MUC2 MI was not significant associated with poor overall survival after operation in tumor tissues. ΔMI , $MI_{ESCC} - MI_{Non-tumor}$; MI_C , the methylation index of MUC2 in tumor tissues.

differentiation ($P=0.029$, $P=0.001$, $P<0.0001$, respectively). The lower MUC2 mRNA ($-\Delta\Delta Ct_{MUC2} \leq -2.54$) was 112/185 (60.54%) in ESCC with AJCC stage III-IV, 105/162 (64.81%) with N_{2-3} lymph node metastasis, and 99/142 (69.72%) with differentiation G2.

The next, we analyzed the association between MUC2 mRNA and survival of these patients (Figure 4). The

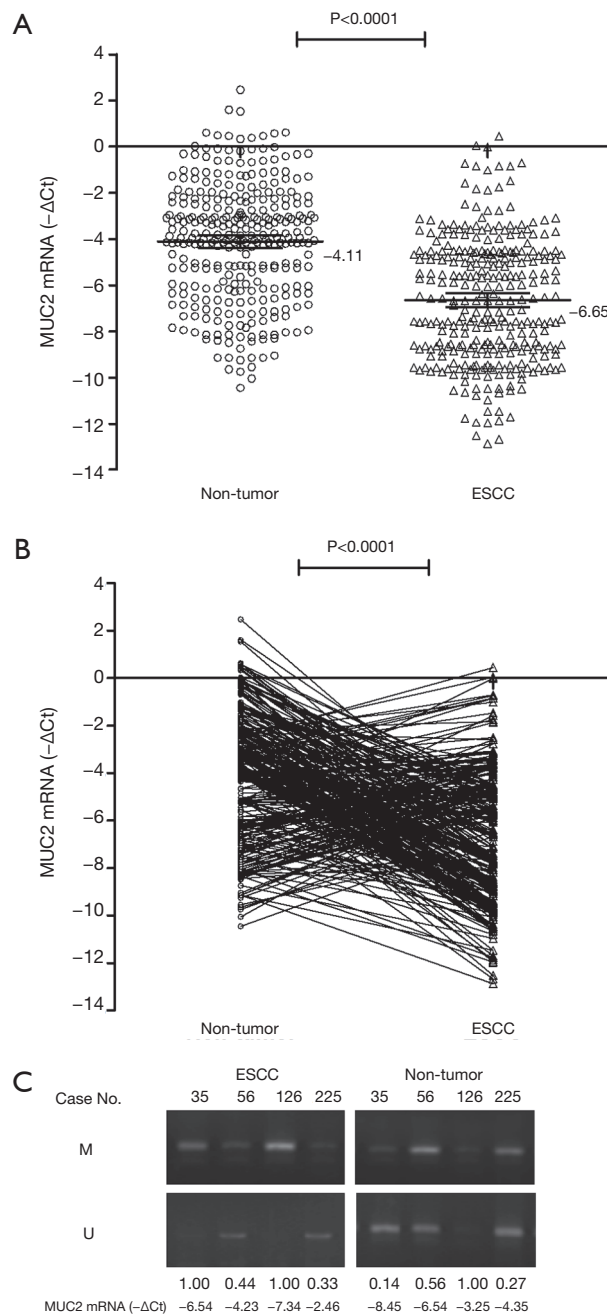


Figure 3 The mRNA expression of MUC2 gene in tissues. (A) The level of MUC2 mRNA was indicated by the Mean $_{-\Delta Ct}$ and 95% CI in different samples. (B) MUC2 mRNA was decreased in ESCC than in non-tumor tissues. *t*-test was used for statistical differences. (C) Representative patterns of MUC2 methylation. U, reaction specific for unmethylated DNA; M, reaction specific for methylated DNA. $-\Delta Ct$, $-(Ct_{MUC2} - Ct_{\beta-actin})$.

Table 2 The level of MUC2 mRNA correlated with clinicopathologic variables in ESCC

Variables	No.	$-\Delta\Delta C_{MUC2}^t > -2.54$ (N=138)	$-\Delta\Delta C_{MUC2}^t \leq -2.54$ (N=172)	Odds ratio (95% CI)	P value*
Gender				0.85 (0.54–1.33)	0.469
Male	180	77	103		
Female	130	61	69		
Age				0.74 (0.47–1.16)	0.192
≥ 60 years	170	70	100		
< 60 years	140	68	72		
Size				1.81 (1.14–2.87)	0.921
< 3 cm	172	77	95		
≥ 3 cm	138	61	77		
Tobacco				0.78 (0.50–1.22)	0.272
Yes	168	70	98		
No	142	68	74		
Alcohol				0.68 (0.43–1.06)	0.089
Yes	147	58	89		
No	163	80	83		
Depth of invasion				1.74 (1.04–2.91)	0.034
T ₁₋₂	224	108	116		
T ₃₋₄	86	30	56		
AJCC stage				1.66 (1.05–2.63)	0.029
I–II	125	65	60		
III–IV	185	73	112		
Lymph node metastasis				2.23 (1.41–3.52)	0.001
N ₀₋₁	148	81	67		
N ₂₋₃	162	57	105		
Distant metastasis				0.62 (0.35–1.09)	0.095
M ₀	251	106	145		
M ₁	59	32	27		
Differentiation				–	<0.0001
G ₁	88	47	41		
G ₂	142	43	99		
G ₃	80	48	32		

*, comparison was done with Pearson's Chi-square test.

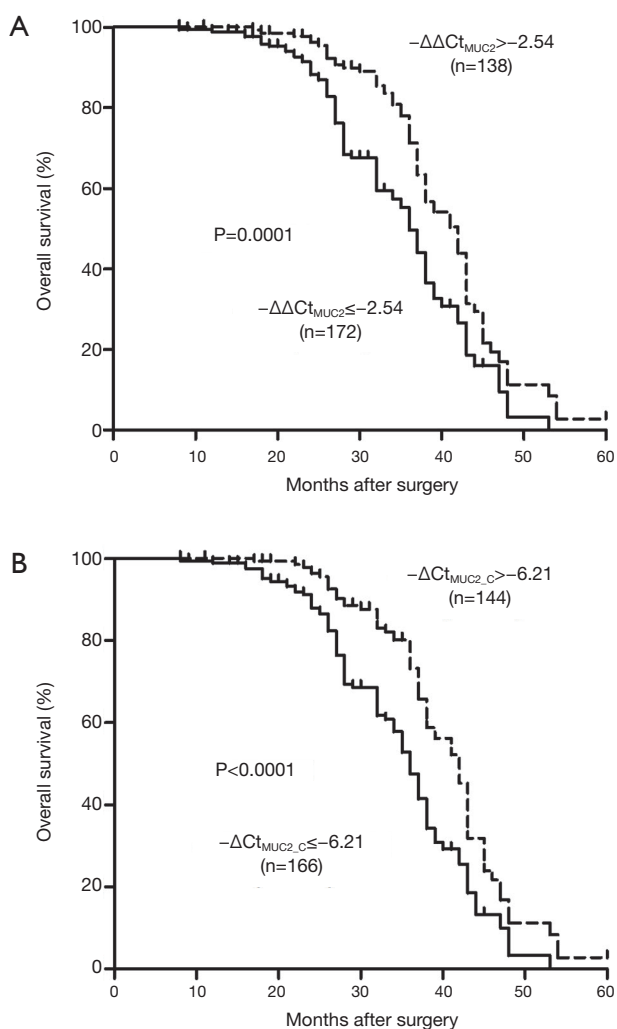


Figure 4 MUC2 mRNA associated with survival in ESCC. (A) The ESCC patients with decreased expression of MUC2 ($-\Delta\Delta Ct \leq -2.54$) had a poor OS after operation; (B) the lower level of MUC2 mRNA ($-\Delta Ct \leq -6.21$) were associated with poor survival in ESCC patients. $-\Delta\Delta Ct$, $-(\Delta Ct_{ESCC} - \Delta Ct_{Non-tumor})$; $-\Delta Ct$, $-(Ct_{ESCC} - Ct_{\beta-actin})$ in tumor tissues.

resulted indicated that those patients with decreased MUC2 mRNA ($-\Delta\Delta Ct < -2.54$) was significantly poor overall survival (36 *vs.* 42 months, $P=0.0001$; HR =0.526; 95% CI, 0.38–0.73). Meanwhile, we also found that ESCC patient with $-\Delta Ct_{MUC2} \leq -6.21$ had a shorter median cumulative survival (36 months) compared with 42 months in those with $-\Delta Ct_{MUC2} > -6.21$ group (log-rank $P < 0.0001$; HR =0.492; 95% CI, 0.35–0.69). The results gave us information that MUC2 silencing could be an important factor for poor survival in ESCC.

MUC2 mRNA silencing and promoter hypermethylation in ESCC

The next, we detected the relation between MUC2 MI and the level of MUC2 mRNA in ESCC tissues (Figure 5). The results indicated ESCC tissues with $\Delta MI > 0$ have a lower MUC2 mRNA level (Mean $_{-\Delta\Delta Ct} = -3.58$; 95% CI, -4.07 to -3.09), and ESCC tissues with $\Delta MI \leq 0$ have a higher MUC2 mRNA level (Mean $_{-\Delta\Delta Ct} = -0.32$; 95% CI, -1.03 to 0.39; $P < 0.0001$; Figure 5C). The decreased MUC2 expression was significant differently in different MI groups of MUC2 gene ($R^2 = 0.161$, $P < 0.0001$; Figure 5B; $R^2 = 0.181$, $P < 0.0001$; Figure 5D). But, there was no significant association between the demethylation status of MUC2 and mRNA expression in non-tumor tissues ($R^2 = 0.00013$; $P = 0.840$; Figure 5A). Thus, the DNA methylation of MUC2 could give us more information for loss of MUC2 mRNA.

Discussion

Mucins have a role for protecting the epithelial surfaces of the related tracts in the human body, which classified as an important factor for some putative transmembrane region (7). The abnormal MUC expression has been implicated in several cancers (18–20). However, Mucins had crucial roles in prognostic prediction and tumor invasion. The MUC2 (+) “intestinal” phenotype was associated with significantly worse prognosis in submucosal gastroesophageal adenocarcinomas, independent of node stage and other prognostic factors (21).

Our previous results indicated that the decreased mRNA expression of MUC2 gene was associated with MUC2 methylation in HCC (17). Research suggested that the DNA methylation could be a biomarker in carcinogenesis (22,23). We found that the mean MI of MUC2 promoter was 0.68 in ESCC, and 0.45 in Non-tumor samples. Also, it indicated a significantly association between the MUC2 MI and distant metastasis, Lymph node metastasis in ESCC patients. The ESCC patients with MUC2 hypomethylation tended to show better survival. Meanwhile, our results found more MUC2 silenced in ESCC tissues, and only 31.94% of ESCC patients with MUC2 mRNA elevated. And a statistical difference was found between MUC2 mRNA with AJCC stage, Lymph node metastasis, and differentiation. Another, ESCC patients with hypermethylation was associated with MUC2 silenced, by which implied that promoter methylation of MUC2 gene could play an important role in ESCC.

The ESCC patients with loss of MUC2 and promoter hypermethylation could be with an unfavorable outcome. There was a significant correlation found between MUC2

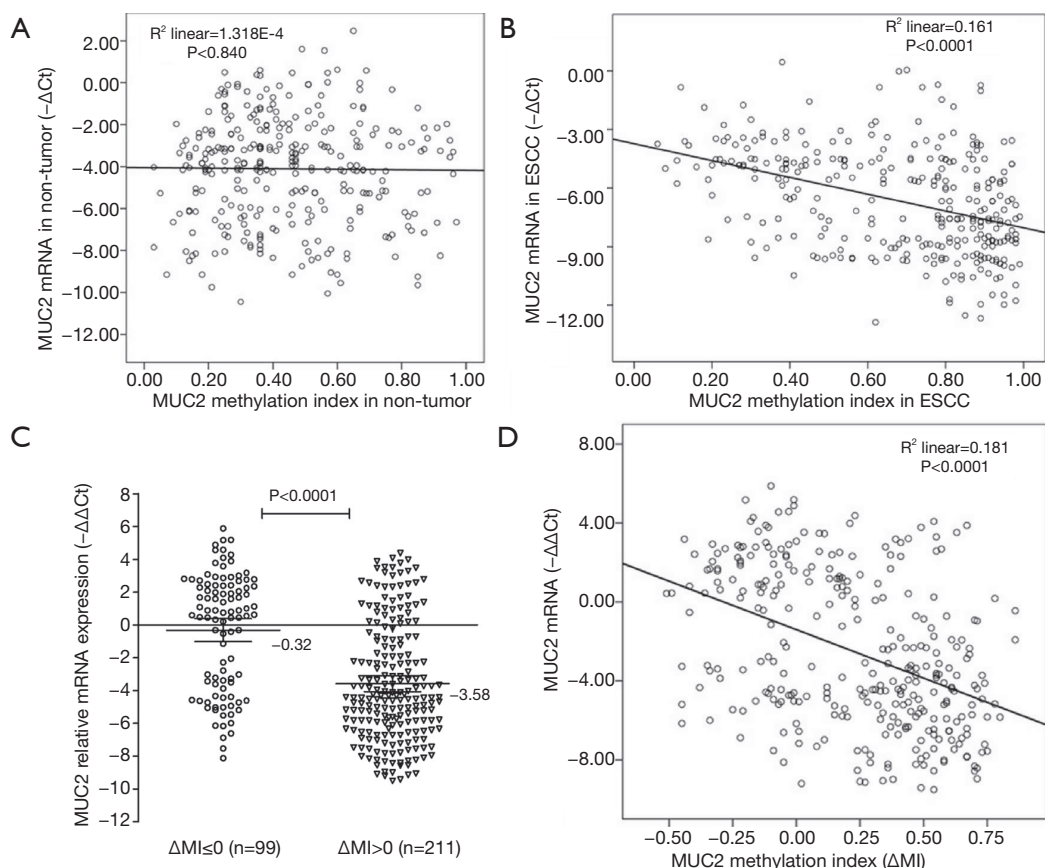


Figure 5 The correlation of MUC2 mRNA and MUC2 gene MI in ESCC patients. The level of MUC2 mRNA is associated with methylation index (MI) in scatter plots summarized in non-tumor tissues (A) and in ESCC tissues (B). *t*-test was used for statistical differences (C). The level of MUC2 mRNA ($-\Delta\Delta Ct$) and relative methylation (ΔMI) is summarized in ESCC patients by Pearson correlation test (D). $-\Delta\Delta Ct$, $-(\Delta Ct_{ESCC} - \Delta Ct_{Non-tumor})$; ΔMI , $MI_{ESCC} - MI_{Non-tumor}$.

mRNA and promoter methylation in ESCC. The key point to methylation alterations could be further investigation the related epigenetic molecular, which is critical for the epigenetic control of gene expression, such as DNA binding and protein-protein interactions in promoter key sites. The next step should give a detail description for these regulatory effects. DNA epigenetic modification could influence MUC2 gene expression in ESCC. It will be interesting to test whether methylation and protein alterations are reversible modifications in future.

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/tcr.2016.10.44>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This study had been approved by the Research and Ethical Committee of Changzhou Cancer

Hospital of Soochow University (No. 2014006) and written informed consent was obtained from all patients.

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