Original Article

Methylation Status of TUSC3 Is a Prognostic Factor in Ovarian Cancer

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BACKGROUND. Current prognostic information in ovarian cancer is based on tumor stage, tumor grade, and postoperative tumor size. Reliable molecular prognostic markers are scarce. In this article, the authors describe epigenetic events in a frequently deleted region on chromosome 8p22 that influence the expression of tumor suppressor candidate 3 (*TUSC3*), a putative tumor suppressor gene in ovarian cancer. **METHODS.** Messenger RNA expression and promoter hypermethylation of *TUSC3* were studied in ovarian cancer cell lines and in tumor samples from 2 large, independent ovarian cancer cohorts using polymerase chain reaction-based methods. **RESULTS.** The results indicated that *TUSC3* expression is decreased significantly because of promoter methylation in malignant ovarian tumors compared with benign controls. Almost 33% of ovarian cancer samples had detectable *TUSC3* promoter methylation. Furthermore, methylation status of the *TUSC3* promoter had a significant and independent influence on progression-free and overall survival. **CONCLUSIONS.** *TUSC3* hypermethylation predicted progression-free and overall survival in ovarian cancer. The current observations suggested a role for N-glycosylating events in ovarian cancer pathogenesis in general and identified the epigenetic silencing of *TUSC3* as a prognostic factor in this disease. ***Cancer* 2013;119:946-54.** © *2012 American Cancer Society.*

KEYWORDS: *TUSC3*, methylation, ovarian cancer, glycosylation, progression-free survival, overall survival, biomarker..

INTRODUCTION

Epithelial ovarian cancer is the most lethal gynecologic malignancy and the fourth most frequent cause of cancer-related death among women in industrialized countries.[1] Because >75% of women are diagnosed with advanced disease (International Federation of Gynecology and Obstetrics [FIGO] stages III and IV), an early diagnosis presents 1 of the challenges of this disease. Patients with advanced ovarian cancer undergo intensive multimodal therapy consisting of cytoreductive surgery and (neo)adjuvant platinum-based and taxane-based chemotherapy. Regardless of the progress made in surgical and medical therapies over recent decades, the outcome for women with advanced ovarian cancer remains grim. Innovative, hypothesis-driven approaches to biomarker development remain essential even in the era of high-throughput sequencing and various omics. Studies of promoter methylation patterns in tumor suppressor genes have yielded several promising methylated biomarker candidates.[2–4] In contrast to a gene or protein expression analysis, methylation can easily be detected using polymerase chain reaction (PCR)-based methods in both tumor material and body fluids.[5–7]

TUSC3 (tumor suppressor candidate 3), originally named N33, was identified as a potential tumor suppressor gene in prostate cancer[8,9] and is located on chromosome band 8p22. Known homozygous deletions of this region in pancreatic cell lines[10,11] and prostate cancer cell lines[12,13] contain no other cancer-related genes except *TUSC3*, although mutations of the *TUSC3* coding sequence have not been observed. Through a systematic screening of this region on 8p22, we recently observed that *TUSC3* is significantly down-regulated in high-grade ovarian cancer and may have prognostic significance.[14] *TUSC3* deletions or mutations reportedly are frequently associated with mental retardation.[15,16] TUSC3 shares high sequence homology with Ost3p, a subunit of the oligosaccharyltransferase (OST) complex involved in N-glycosylation of proteins in *Saccharomyces cerevisiae*,[17–19] suggesting an analogous function in mammalian cells. Alterations of protein N-glycosylation have been associated with some carcinogenic traits, such as invasiveness and metastatic potential.[20–22] Deregulated enzymatic activities of proteins directly involved in N-glycosylation or the availability of potential

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glycosylation sites determined by the branching of N-glycans are considered to be crucial for these effects.[23–26] In the current report, we describe the epigenetic regulation of *TUSC3* expression through promoter methylation in ovarian cancer and define *TUSC3* as an independent prognostic factor in 2 large, independent cohorts of women with ovarian cancer.

MATERIALS AND METHODS

Patient Characteristics

A written informed consent form for the scientific use of biologic material was signed by all participating patients according to the requirements of the Institutional Ethics Committee of the Medical University of Vienna and the Ethics Committee of the Charite Hospital of Berlin. All patient-associated information was anonymized for further analysis.

Samples of ovarian tumors for establishing the test set were obtained between the years 2000 and 2003 from patients who underwent radical surgery with the primary objective of maximal tumor reduction at the Charite Hospital of Berlin. Normal ovaries (microdissected ovarian epithelium) and benign cyst samples came from patients who were diagnosed without malignant disease and underwent oophorectomy at the Medical University of Vienna. In total, 20 benign ovarian samples and 102 primary tumor samples were assessed, microdissected, and enriched for epithelial tissue (the clinicopathologic characteristics are provided in Table 1). The validation set included 97 ovarian tumor samples that were obtained between the years 2005 and 2008 (Table 2). The majority of patients in the test set (89%) and the validation set (92%) received a platinum-based first-line chemotherapy regimen (Tables 1 and 2). The study design adhered to Reporting Recommendations for Tumor Marker (REMARK) criteria, as applicable.[27]

Cell Culture and Cloning

The ovarian cancer cell lines A2780, A2780 ADR, MDAH-2774, ES2, OVCAR-3, Caov-3, MIA PaCa-2, and SKOV3 were obtained from the European Collection of Cell Cultures (Health Protection Agency Culture Collections, Porton Down, United Kingdom) or the American Type Culture Collection (Manassas, Va). Cells were cultivated in medium (Caov-3 cells, Dulbecco modified Eagle medium; MIA PaCa-2 and OV-CAR-3 cells, α-minimal essential medium; SKOV3 cells, McCoy medium; A2780, A2780 ADR, MDAH-2774, and ES2 cells, RPMI medium; H134 cells, Dulbecco modified Eagle medium with 25 mM HEPES, pH 8.0) enriched with

TABLE 1. Clinicopathologic Patient Characteristics: Test Set

Characteristic	No. of Patients (%)
Benign ovarian samples with corresponding mRNA samples	20 (20)
Age at operation: Mean±SD, y	49.9 ± 11.8
7 Normal ovaries: Mean age, y	51.6
13 Benign cysts: Mean age, y	49.0
Tumor samples (corresponding mRNA samples)	102 (99)
Age at diagnosis: Mean±SD, y	58.3 ± 11.0
Histology	
Serous	79 (77.4)[a]
Endometrioid	10 (9.8)
Mucinous	5 (4.9)
Clear cell	2 (2)
Other	5 (4.9)
Missing	1 (1)
FIGO stage	
I	17 (16.7)
II	10 (9.8)
III	51 (50)
IV	22 (21.6)
Missing	2 (2)
Tumor grade	
1	2 (2)
2	48 (46.1)
3	51 (50)
Missing	1 (1)
Residual disease after initial surgery	
≤1 cm	86 (84.3)
>1 cm	13 (12.7)
Missing	3 (2.9)
First-line chemotherapy	
Carboplatin and paclitaxel	66 (64.7)
Carboplatin, paclitaxel, and gemcitabine	17 (16.7)
Carboplatin and other	8 (7.8)
Other	3 (2.9)
None	5 (4.9)
Missing	3 (2.9)
Response to first-line chemotherapy	
NED[b]	6 (5.9)
NC[c]	3 (2.9)
PD[c]	12 (11.8)
PR[c]	5 (4.9)
CR[b]	43 (42.2)
Missing	16 (15.7)
Duration of response, mo	
0-6[b]	6 (5.9)
6-12[b]	5 (4.9)
>12[b]	6 (5.9)
Remissions/PD	
Without remission	49 (48)
With remission	33 (32.4)
PD	17 (16.7)
Missing	3 (2.9)

Abbreviations: CR, complete response; FIGO, International Federation of Gynecology and Obstetrics; NC, no change; NED, no evidence of disease; PD, progressive disease;
PR, partial response; SD, standard deviation.
[a] Percentages for histology were based on 102 tumor samples, because 1 patient (1%) was missing information on histology.
[b] These patients were responders to first-line chemotherapy.
[c] These patients were nonresponders to first-line chemotherapy.

TABLE 2. Clinicopathologic Patient Characteristics: Validation Set

Characteristic	No. of Patients (%)
No. of tumor samples	97
Age at diagnosis: Mean±SD, y	57.7 ± 10.7
Histology	
Serous	89 (91.7)[a]
Endometrioid	2 (2.1)
Mixed epithelial tumor	2 (2.1)
Other	4 (4.1)
FIGO stage	
II	2 (2.1)
III	82 (84.5)
IV	13 (13.4)
Tumor grade	
1	4 (4.1)
2	15 (15.5)
3	72 (74.2)
Missing	6 (6.2)
Residual disease after initial surgery	
≤1 cm	60 (61.9)
>1 cm	35 (36.1)
Missing	2 (2)
First-line chemotherapy	
Carboplatin and paclitaxel and other	78 (80.4)
Carboplatin and docetaxel	5 (5.2)
Cisplatin and paclitaxel/docetaxel	6 (6.2)
Other	4 (4.1)
Missing	4 (4.1)

Abbreviations: FIGO, International Federation of Gynecology and Obstetrics; SD, standard deviation.
[a] Percentages for histology were based on 97 tumor samples.

10% fetal calf serum, 50 U/mL^{-1} penicillin G, and 50 μg/mL^{-1} streptomycin sulfate at 37°C in a humidified atmosphere with 5% CO_2. The 32 ovarian cancer cell lines that were used in our study were derived from patients and cultivated as described previously.[14] For the reconstitution of *TUSC3* expression, the coding DNA sequence of the IMAGE clone (BC010370 in pDNR-LIB; Takara Bio Europe/Clontech, St. Germain-en-Laye, France) was cloned into the expression vector pLP-IRES-neo (Takara Bio Europe/Clontech). For the TUSC3-FLAG-tag (TUSC3-FLAG) fusion protein, the FLAG peptide was cloned in frame behind the C-terminus of *TUSC3*. The empty vector pLP-IRESneo was used as a control. Transfections were performed using Lipofectamine 2000 (Invitrogen, Darmstadt, Germany), and stable clones were selected with G418 (Sigma-Aldrich, Munich, Germany) (H134 cells, 700 μg/mL^{-1}; MIA PaCa-2 cells, 800 μg/mL^{-1}) and subcultured with half of these concentrations. For the collagen I and bovine serum albumin adhesion assay, the cells were incubated on coated 96-well enzyme-linked immunosorbent assay plates for 30 minutes, washed, and the remaining cells were quantified with the Cell Titer-Blue cell-viability assay (Promega, Madison, Wis).

DNA and RNA Isolation

Genomic DNA from approximately 15 mg of frozen tissue or cell lines was extracted using the DNeasy Tissue Kit (Qiagen, Hilden, Germany) and quantified with the Pico-Green dsDNA Quantitation Kit (Molecular Probes, Darmstadt, Germany). Total RNA from frozen tissues was extracted using the Agilent Total RNA Isolation Mini Kit (Agilent Technologies, Waldbronn, Germany). Total RNA from cancer cell lines was prepared using the RNeasy Mini Kit (Qiagen) and was quality/quantity assessed on RNA Nano Chips (Lab-on-a-Chip, Agilent Technologies).

Methylation-Specific Polymerase Chain Reaction and 5-Aza-2′-Deoxycytidine Treatment

Bisulfite treatment with 1 μg genomic DNA was done as described previously.[28] Methylation-specific PCR (MSP) was performed with 25 ng of bisulfite-treated DNA using previously published primers[29] for the test cohort and primers of our own design (methylated sequence: sense, 5′-GGTCGGGTAGGCGTGGTGCG-3′; antisense, 5′-CCGCCCCGCTTACCTACGACGT-3′; with amplification of a 122-base-pair product; unmethylated sequence: sense, 5′-GAGGTTGGTTGGGTA GGTGTGGTGG-3′; antisense, 5′-CACAACCACCACCCCACTTACCTA-CAACAT-3′; with amplification of a 136-base-pair product) for the validation cohort with AmpliTaq Gold DNA polymerase (Applied Biosystems, Carlsbad, Calif). Bisulfite sequencing was performed directly with the PCR products purified with the QIAquick PCR Purification Kit (Qiagen). PCR products for the validation cohort were quantified using ALFexpress II (GE Healthcare, Madison, Wis). Because this method yielded continuous data in contrast to the binary results from the original MSP, we dichotomized it at the 29.4% level to match the distribution of *TUSC3* methylation negative and positive samples in the test cohort. DNA demethylation of the MZ6 cancer cell line was performed using a 72-hour treatment with 1 μM or 2.5 μM 5-aza-2′-deoxycytidine (Sigma-Aldrich) in cell culture medium, and relative *TUSC3* expression was evaluated by quantitative real-time reverse transcriptase (RT)-PCR.

cDNA Synthesis and Quantitative Real-Time Reverse Transcriptase-Polymerase Chain Reaction

cDNA was synthesized from 1 μg DNase I-digested total RNA using the DuraScript RT-PCR Kit (Sigma-Aldrich). Expression was relatively quantified using TaqMan probes specific for *TUSC3* Hs00185147_m1 and β2-

microglobulin Hs99999907_m1 (Applied Biosystems), as described elsewhere,[14,30] and was expressed in relative ratio units. All PCR reactions were performed in at least 3 independent experiments, and RT-negative and template-negative controls were included.

Statistical Analysis

Continuous variables are expressed as means and standard deviations, and categorical variables are expressed as absolute and relative frequencies. To compare frequencies between 2 or more groups, we used Fisher exact tests or Fisher-Freeman-Halton tests, respectively. To compare *TUSC3* expression between 2 or more groups, a *t* test or a 1-way analysis of variance, respectively, was performed using the log-transformed expression as independent variable. Analogously, the potential influence of methylation on *TUSC3* expression was investigated using a *t* test on the logarithmic scale. The potential influence of *TUSC3* methylation on progression-free and overall survival is presented in plots of the corresponding Kaplan-Meier estimates and quantified using Cox proportional hazards regression models. Because of the restricted number of events, only *TUSC3* methylation and the most prominent predictor, FIGO stage, were forced into the model; whereas the remaining variables were selected in a stepwise manner. The date of documented first relapse or the first documentation of disease progression, starting from the time of the first diagnosis, was used as the time point for disease progression. Univariate Cox models were used to demonstrate the influence of known prognostic factors and the potential new prognostic factor. To externally validate our estimated Cox regression models, a risk score for progression-free and overall survival was calculated with the coefficients from the Cox regression models that we built from our test set and corresponding data from our validation set. A Cox regression model using this risk score as a single predictor was used to validate the model estimated from the test set. Coefficients close to 1.00 indicated satisfactory prediction based on the model. *P* values $\leq .05$ were considered statistically significant. All computations were performed using the SAS statistical software package (version 9.2; SAS Institute Inc., Cary, NC), and the graphics were produced using SPSS software (version 13.0; SPSS Inc., Chicago, Ill).

RESULTS

Loss of Expression and Increased TUSC3 Promoter Methylation in Ovarian Cancer

Analyses of patient samples indicated that *TUSC3* messenger RNA (mRNA) expression was decreased significantly in malignant ovarian tumors compared with benign ovarian tissues ($P < .001$) (Fig. 1A, Table 3). We did not observe any significant differences in *TUSC3* expression with regard to patient age, FIGO stage, or histologic grade.

To evaluate methylation status of the *TUSC3* promoter in ovarian cancer, we used methylation specific PCR. Hypermethylation of the *TUSC3* promoter was observed in 30 of 102 ovarian cancer tissues (29.4%), and it was not observed in controls ($P = .003$) (Fig. 1A, B). *TUSC3* promoter methylation in primary tumors was correlated significantly with decreased *TUSC3* mRNA expression ($P < .001$) (Fig. 1A), suggesting a mechanism of promoter methylation-dependent epigenetic silencing of *TUSC3* in ovarian tumors. The statistical analysis revealed no significant association between *TUSC3* promoter methylation and clinicopathologic parameters like tumor histology, tumor grade, FIGO stage, or patient age (Table 4). To validate the results from MSP-based methods, we determined the CpG methylation frequency within the *TUSC3* promoter region using bisulfite sequencing in 3 hypermethylated cell lines and 2 controls. In the hypermethylated cell lines, on average, 75% of CpG islands were methylated compared with 10% in controls (data now shown).

Next, we used a panel of 38 ovarian cancer cell lines that has been introduced and characterized previously.[14] Expression and methylation analyses of ovarian cancer cell lines confirmed a highly significant correlation between *TUSC3* mRNA expression and *TUSC3* promoter methylation ($P < .001$) (Fig. 1A, C). We used MZ6 ovarian cancer cells, which demonstrated *TUSC3* promoter hypermethylation (Fig. 1B), and treated the cell line with the demethylating agent 5-aza-2′-deoxycytidine for 72 hours. Genome-wide demethylation led to a consequent increase in *TUSC3* mRNA expression in this ovarian cancer cell line (Fig. 1D). These results further illustrate the role of DNA methylation in the regulation of *TUSC3* expression.

TUSC3 Affects Cancer Cell Proliferation and Adhesion

We reconstituted TUSC3 expression in a cancer cell line model using H134 cells (an ovarian cancer cell line with methylated *TUSC3*) and MIA PaCa-2 cells (a pancreatic carcinoma cell line with homozygous deletion of *TUSC3*). *TUSC3* expression was stably reconstituted with DNA constructs that coded for the TUSC3-FLAG fusion protein or full-length *TUSC3* cDNA (TUSC3), respectively. Reconstitution was confirmed quantitatively by mRNA expression (Fig. 1E). We also assessed the

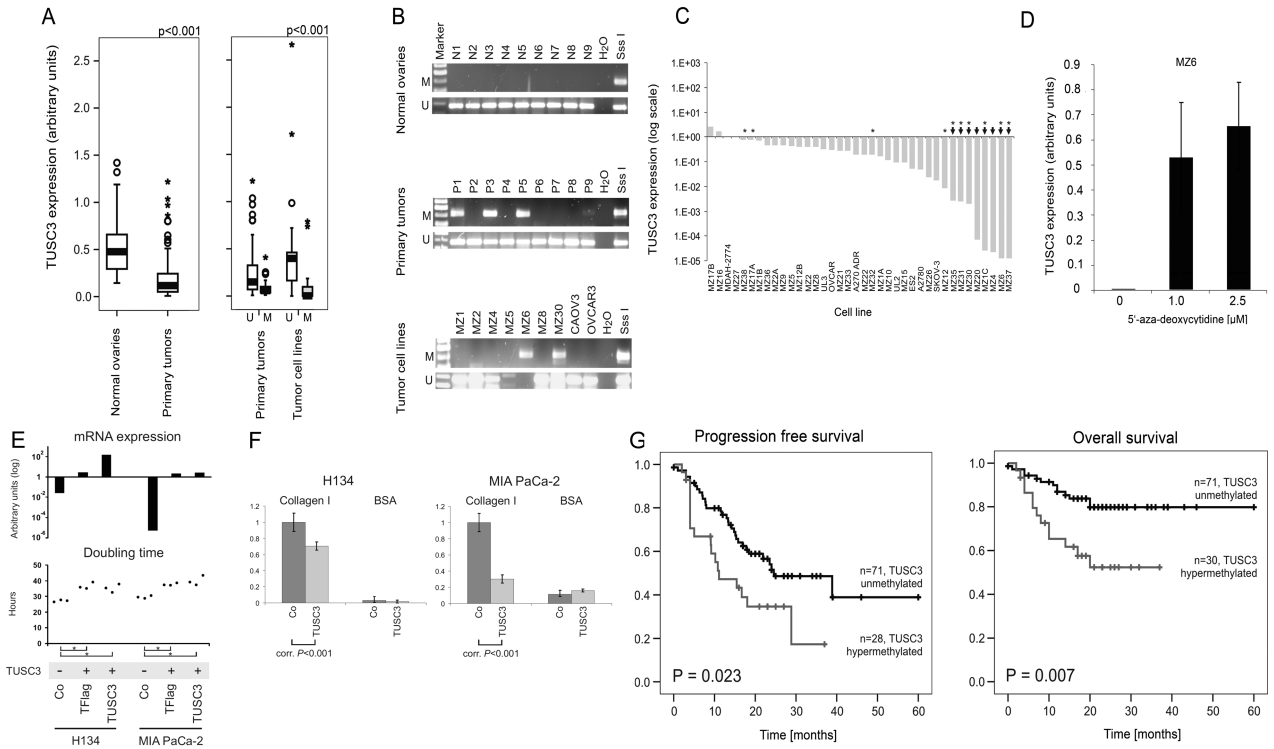

Figure 1. *TUSC3* expression in ovarian cancer and survival analysis. (A) These box plots illustrate (*Left*) median expression levels of the tumor suppressor candidate 3 (*TUSC3*) gene in benign ovarian tissue and ovarian tumors and (*Right*) the expression of *TUSC3* subdivided into unmethylated (U) and hypermethylated (M) fractions, including cell lines. (B) Representative ethidium bromide agarose gels reveal methylation-specific polymerase chain reaction (PCR) products from (*Top*) 9 benign ovarian tissue samples (N1-N9), (*Middle*) 9 ovarian tumor samples (P1-P9), and (*Bottom*) 9 ovarian cancer cell lines. *Sss* I (CpG methylase)-methylated human DNA was used as a positive control. (C) The expression of *TUSC3* is illustrated in 38 ovarian cancer cell lines (log scale). Arrows indicate cell lines that had silenced *TUSC3* expression, and asterisks indicate cell lines that had methylation-specific PCR products. The expression of *TUSC3* was determined by quantitative reverse transcriptase-PCR and was normalized to β-2 microglobulin. (D) The reconstitution of TUSC3 expression in the MZ6 cell line is illustrated. Cells either were cultivated in the presence of 1.0 M or 2.5 M of the demethylating agent 5-aza-2′-deoxycytidine or were left untreated for 48 hours. *TUSC3* expression was determined as indicated in C. (E) This chart illustrates the in vitro characterization of cell line models that had reconstituted *TUSC3* expression (H134 cells and MIA PaCa-2 cells). Messenger RNA (mRNA) expression is illustrated in arbitrary units, and doubling time is illustrated in hours. Co indicates control; TFlag, TUSC3-FLAG fusion protein; +, TUSC3 positive; − TUSC3 negative. (F) The adhesion of cells to 96-well plates that were coated with collagen I and bovine serum albumin (BSA) (as a control) is illustrated. Binding of the control cells to collagen I was set arbitrarily to 1. All experiments were performed in triplicate, and significance was calculated using *t* tests. (G) These Kaplan-Meier survival curves illustrate progression-free and overall survival for patients who had tumor samples with hypermethylated or unmethylated *TUSC3*. The date of either the first documented relapse or the first documented disease progression, starting from the time of the first diagnosis, was used as the time point to define progression. *P* values indicate the statistical significance determined using univariate Cox regression analysis.

influence of TUSC3 re-expression on cell proliferation and adhesion. In TUSC3-reconstituted H134 and MIA PaCa-2 cell lines, a significant decrease in cellular proliferation (expressed as increased doubling time) was observed (Fig. 1E). In addition to these findings, TUSC3 re-expression significantly decreased the adhesion of cancer cells to collagen I, an extracellular matrix component (Fig. 1F).

TUSC3 Promoter Methylation Predicts Survival in Patients With Ovarian Cancer

We previously observed that TUSC3 was down-regulated in high-grade ovarian cancer using a systematic approach to analyze putative tumor suppressor genes on the short arm of chromosome 8.[14] In the current analysis of a larger cohort, we observed that patients who had tumors with *TUSC3* promoter methylation had significantly shorter progression-free and overall survival rates, with a median progression-free survival of 11.1 months in the methylated group versus 24.6 months in the unmethylated group (Fig. 1G). In addition, a Cox proportional hazards analysis that included known risk factors (age, histologic subtype, FIGO stage, tumor grade, residual disease, and therapy response) and *TUSC3* methylation status revealed a significant association between *TUSC3* promoter

TABLE 3. *TUSC3* Expression in Ovarian Cancer and Survival Analysis

Clinicopathologic Characteristic	Expression of *TUSC3*: Mean (95% CI), Arbitrary Units	*P* for Comparison
Benign ovarian samples	0.465 (0.347–0.623)	
Tumor samples vs benign ovarian samples	0.105 (0.082–0.135)	< .001[a]
Age, y		
≤50	0.085	.299[a]
>50	0.114	
Histology		
Serous	0.121	.037[a]
Nonserous	0.064	
FIGO stage		
I	0.084	.764[b]
II	0.124	
III	0.104	
IV	0.126	
Tumor grade		
1	0.068	.188[b]
2	0.135	
3	0.086	

Abbreviations: CI, confidence interval; FIGO, International Federation of Gynecology and Obstetrics; *TUSC3*, tumor suppressor candidate 3.
[a] An analysis of variance was used to calculate *P* values after logarithmic transformation; means and CIs were calculated after back-transformation to the original scale.
[b] *T* tests were used to calculate *P* values after logarithmic transformation; means and CIs were calculated after back-transformation to the original scale.

TABLE 4. *TUSC3* Promoter Methylation and Clinicopathologic Characteristics

Clinicopathologic Characteristic	Methylation of *TUSC3*: No. of Patients/ Total No. (%)	*P* for Comparison[a]
Benign ovarian samples	0/20 (0)	
Primary tumor samples	30/102 (29.4)	.003
Age, y		.218
≤50	5/26 (19.2)	
>50	25/75 (33.3)	
Histology		.200
Serous	21/79 (26.6)	
Nonserous	9/22 (40.9)	
FIGO stage		.948
I	5/17 (29.4)	
II	2/10 (20)	
III	15/51 (29.4)	
IV	7/22 (31.8)	
Tumor grade		.102
1	2/2 (100)	
2	15/48 (31.2)	
3	13/51 (25.5)	

Abbreviations: FIGO, International Federation of Gynecology and Obstetrics; *TUSC3*, tumor suppressor candidate 3.
[a] The Fisher exact test and the Fisher-Freeman-Halton test were used to calculate *P* values.

methylation and progression-free survival (relative risk [RR], 2.23; $P = .012$) as well as overall survival (RR, 5.14; $P = .001$). This association was independent of other risk factors, including FIGO stage and response to chemotherapy, which were the strongest predictive factors in our analysis (the interaction term was not significant) (Table 5). The hazard ratio of methylated versus unmethylated *TUSC3* promoter status for progression-free survival was significant (RR, 1.97; $P = .023$) and was only slightly inferior to an increase of 1 FIGO stage (RR, 2.82; $P < .001$) and to the presence residual disease after surgery (RR, 3.04; $P = .001$). Similarly, the significant influence of *TUSC3* methylation on overall survival (RR, 2.92; $P = .007$) was comparable to an increase of 1 FIGO stage (RR, 2.52; $P = .002$), to histologic subtype (serous vs nonserous: RR, 2.37; $P = .037$), and to residual disease after surgery (RR, 4.86; $P < .001$).

To validate our finding of TUSC3 hypermethylation in ovarian cancer and its prognostic significance, we determined TUSC3 methylation status in an independent cohort of 97 patients with late-stage ovarian cancer(FIGO stage ≥II) (Table 1) using a different MSP-based method. A second set of MSP primers targeting the same CpG island in the TUSC3 promoter was designed, and PCR products were quantified with a semiquantitative gel system and dichotomized at the 29.4% level, generating 29 positive samples (the rationale for this procedure is provided above; see Materials and Methods). The hazard ratio for *TUSC3* was estimated from a multiple Cox model using validation data in the same manner that was used for the test set data (disease-free survival: RR, 1.40; 95% confidence interval, 0.77–2.52; $P = .269$; overall survival: RR, 6.34; 95% confidence interval, 1.95–20.62; $P = .002$).

To confirm that the Cox regression models from our test and validation cohorts were similar, given the slightly different patient characteristics, risk scores for progression-free survival ($0.83 \times$ FIGO stage $+ 1.09 \times$ therapy response $+ 0.80 \times$ *TUSC3* methylation) and overall survival ($0.76 \times$ FIGO stage $+ 1.80 \times$ therapy response $+ 1.64 \times$ *TUSC3* methylation) were calculated with the coefficients from the Cox regression models we built from our test set (Table 5) and with the corresponding values (FIGO stage, response to chemotherapy, and *TUSC3* methylation status) from our validation set. Cox regression models using the respective risk score as a single predictor were used to validate the models estimated from the test set. The resulting regression coefficients were 1.05 (95% confidence interval, 0.67–1.42) for disease-free survival and 1.16 (95% confidence interval, 0.73–1.59) for overall survival, demonstrating a successful validation of

TABLE 5. Univariate and Multivariate Cox Proportional Hazards Analysis of Factors Affecting Progression-Free and Overall Survival in Ovarian Cancer

Variable	Univariate Analysis		Multivariate Analysis	
	RR (95% CI)	*P*	RR (95% CI)	*P*
Progression-free survival				
Age at diagnosis	1.02 (0.99–1.05)	.184	—[a]	
Histology: Nonserous vs serous[b]	1.16 (0.58–2.32)	.683	—[a]	
FIGO stage[c]	2.82 (1.87–4.26)[d]	< .001	2.30 (1.45–3.64)[d]	< .001
Tumor grade[c]	0.98 (0.58–1.65)	.933	—[a]	
Residual disease: >1 cm vs ≤1 cm[b]	3.04 (1.53–6.03)[d]	.001	—[a]	
Therapy response: No vs yes[b]	4.91 (2.64–9.10)[d]	< .001	2.97 (1.56–5.66)[d]	.001
Methylation of *TUSC3*: Yes vs no[b]	1.97 (1.10–3.53)[d]	.023	2.23 (1.19–4.17)[d]	fo.012
Overall survival				
Age at diagnosis	1.06 (1.02–1.10)[d]	.003	—[a]	
Histology: Nonserous vs serous[b]	2.37 (1.05–5.33)[d]	.037	—[a]	
FIGO stage[c]	2.52 (1.41–4.49)[d]	.002	2.14 (0.96–4.75)	.062
Tumor grade[c]	0.76 (0.38–1.53)	.446	—[a]	
Residual disease: >1 cm vs ≤1 cm[b]	4.86 (2.13–11.12)[d]	< .001	—[a]	
Therapy response: No vs yes[b]	9.56 (3.56–25.66)[d]	<.001	6.05 (2.06–17.73)[d]	.001
Methylation of *TUSC3*: Yes vs no[b]	2.92 (1.35–6.30)[d]	.007	5.14 (1.93–13.66)[d]	.001

Abbreviations: CI, confidence interval; FIGO, International Federation of Gynecology and Obstetrics; RR, relative risk; *TUSC3*, tumor suppressor candidate 3.
[a] This variable was not selected in the stepwise selection procedure.
[b] This was a categorical variable.
[c] This was an ordinal variable.
[d] This *P* value was statistically significant.

the regression model. Note that FIGO stage was incorporated into the risk score and, thus, was accounted for when transferring the coefficients estimated from the test set to the validation set.

DISCUSSION

Prognostic factors in ovarian cancer include disease stage, tumor grade, and size of the residual tumor after primary cytoreductive surgery.[31,32] By using publicly available gene-expression profiling data, we have systematically screened the region on 8p22 for differentially regulated genes and identified *TUSC3* as a candidate tumor suppressor gene in ovarian cancer.[14] In our current work, we validate our previous observations in a larger, completely independent patient population and demonstrate that the expression of *TUSC3* in ovarian cancer may be regulated by promoter methylation. We did not investigate other possible mechanisms for TUSC3 down-regulation in ovarian cancer, which may include micro-RNA–mediated silencing, transcriptional regulation, or homozygous deletions of 8p22, as demonstrated in prostate and pancreatic cancers.[11,13]

Some of our observations differ from the screening study, most probably because of the larger size of the cohorts. We can now observe a significant difference in *TUSC3* methylation and expression between ovarian cancer and controls, and we can confirm an impact of TUSC3 on ovarian cancer survival, although we do not observe differences between high-grade and low-grade tumors, as described previously.[14] Our cohorts did not explicitly exclude patients with FIGO stage I and II disease, but the stepwise multivariate analysis did include FIGO staging, hinting at a FIGO-independent prognostic effect of *TUSC3* methylation. A recent paradigm shift points toward distinct tissues of origin in ovarian cancer that are responsible for its various histologic subtypes.[33] Because our study was designed to be exploratory, we deliberately included different histologic subtypes of ovarian cancer, because we were acutely aware of the recent controversy regarding their differing origin and pathogenesis.[34,35] Nevertheless, the vast majority of our patients, particularly in the validation cohort, were diagnosed with advanced stage, high-grade, serous ovarian cancer. Hence, we propose the further evaluation of TUSC3 as a prognostic factor primarily in this subset of patients with ovarian cancer.

TUSC3 is the human homologue to *S. cerevisiae* Ost3p, a noncatalytic subunit of the oligosaccharyltransferase complex.[18,19] Analyses of the Ost3p and its yeast paralogue Ost6p (human MagT1/IAP) demonstrated their function in regulating glycosylation efficiency[36] and recently also uncovered their oxidoreductase activity as well as a possible role in magnesium transport.[37] Aberrant glycosylation of proteins can be observed in essentially all in vitro cancer models and human cancers, and many glycosylated epitopes constitute tumor-associated antigens,[38,39]

sustaining a long-standing debate regarding whether and how protein glycosylation is involved in tumorigenesis. Recently, global DNA methylation changes in ovarian cancer cells were linked to significant alterations of protein glycosylation.[40] One possible explanation for this effect is the re-expression of epigenetically silenced key glycosyltransferase enzymes. Treatment with 5-aza-2′-deoxycytidine restores sensitivity to carboplatin in patients with advanced ovarian cancer.[41] In addition, it has been demonstrated that defective multidrug resistance proteins lead to platinum resistance.[42] Defects in N-glycosylation also may affect tumor growth in cells with deregulated phosphatidylinositol 3-kinase/v-Akt murine thymoma viral oncogene homolog (PI3K-Akt) pathway.[26] We have observed that reconstitution of TUSC3 in vitro decreases proliferation as well as binding of cancer cells to the extracellular matrix. Consequently, we propose that loss of TUSC3 not only may lead to ovarian cancer growth but also may facilitate the adhesion of cancer cells to the extracellular matrix caused by differential glycosylation of yet unknown adhesion molecules. In ovarian cancer in particular, loss of TUSC3 may promote intraperitoneal dissemination, which is generally associated with a poor prognosis. In light if these data, our results further add to the picture and emphasize the role of methylation and N-glycosylation in ovarian cancer tumorigenesis.

A deeper understanding of the role of *TUSC3* promoter methylation in the development of ovarian cancer may offer additional possibilities for therapeutic interventions in the future. Regardless of the molecular function of TUSC3 protein, the frequency of *TUSC3* promoter methylation in ovarian cancer deserves prospective evaluation either on its own or as a part of a larger prognostic biomarker panel.

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CONFLICT OF INTEREST DISCLOSURES

The authors made no disclosures.

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