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Infrequent methylation of the DUSP6 phosphatase in endometrial cancer

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Abstract

Objective—Dual-specificity phosphatase six (DUSP6, MKP3, or PYST1) dephosphorylates phosphotyrosine and phosphothreonine residues on ERK-2 (MAPK1) to inactivate the ERK-2 kinase. DUSP6 is a critical regulator of the ERK signaling cascade and has been implicated as a tumor suppressor. DNA methylation in the first intron of DUSP6 abrogates expression in a subset of pancreatic cancers. We sought to determine whether DUSP6 was similarly silenced by methylation in endometrial cancer, a tumor type in which there is frequent activation of the ERK pathway.

Methods—109 endometrial cancers were analyzed for DUSP6 methylation using combined bisulfite restriction analysis (COBRA). The cohort included 70 primary endometrioid endometrial cancers, 21 primary endometrial tumors of adverse histological types, and 18 endometrial cancer cell lines. Primary tumors, cell lines, and normal endometrial tissues were analyzed for DUSP6 mRNA levels using quantitative RT-PCR and pERK levels by Western blots and/ or immunohistochemistry.

Results—Methylation of the first intron of the DUSP6 gene was seen in 1/91 primary endometrial cancers investigated. The methylated tumor was also methylated at the more 5' regulatory region of DUSP6. Q-RT-PCR revealed that DUSP6 transcript levels varied widely in primary endometrial tumors. DUSP6 mRNA levels did not correlate with pERK status in primary tumors, consistent with the existence of negative feedback loops activated by pERK that result in transcription of DUSP6.

Conclusion—DUSP6 methylation is a rare event in endometrial cancer. Silencing of the DUSP6 phosphatase is unlikely to contribute to constitutive activation of the ERK kinase cascade in endometrial cancer.

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Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

Introduction

Endometrial cancer is the most common gynecological malignancy in the United States, with 42,160 new cases and 7,780 deaths predicted in 2009 [1]. Although most women present with early stage disease and are cured with a hysterectomy, approximately 15% of patients suffer from recurrent or persistent disease that is often fatal [2]. Discovery of the molecular lesions that contribute to endometrial tumorigenesis will provide opportunities for targeted therapies for endometrial cancer.

Endometrioid endometrial carcinomas comprise about 80% of uterine cancers. Several key genetic events associated with the development of endometrioid endometrial cancer have been described. Inactivating mutations in the PTEN tumor suppressor and gain-of-function CTNNB1 mutations are seen in 26-80% and 25-38% of tumors respectively [3]. Gain-of-function mutations in the ERK kinase cascade (FGFR2 or KRAS2), leading to ERK activation, are seen in 20-30% of tumors [4]. However, FGFR2 and KRAS2 mutations do not explain ERK-2 activation in all cases. ERK activation (pERK) is seen in over 60% of endometrial cancers ([5], and our unpublished data). The ERK kinase cascade is normally initiated by the binding of growth factors (ligands such as EGF and FGF) to cell-surface receptor tyrosine kinases, resulting in autophosphorylation of the tyrosine kinase domains of the intracellular protein of the receptor. This in turn triggers G-protein-mediated activation of the RAS kinase, which phosphorylates the RAF effector, which phosphorylates ERK-2 (MAPK1). ERK-2 has many phospho-targets involved in transcriptional regulation, translational regulation, and control of the cell cycle. Mutations in genes in the ERK kinase pathway contribute to the development of a variety of cancers. In endometrioid endometrial cancer, activating FGFR2 mutations are identified in 10-16% of endometrioid tumors and activating KRAS2 mutations in 10-30% of endometrioid tumors [4,6]. These mutations occur exclusively of one another [4]. In addition to mutational activation of the ERK cascade, increased ERK activation can result from silencing of the DUSP6 phosphatase that normally serves to inactivate ERK-2 [7].

A number of dual-specificity phosphatases regulate specific kinases in normal mammalian cells. DUSP1, DUSP2, and DUSP4 localize to the nucleus and target JNK, p38, and ERK; DUSP5, DUSP6, DUSP7, and DUSP9 localize to the cytoplasm and target ERK. All of the phosphatases are expressed in normal human uterine tissue [8]. The mouse knockout of DUSP6 shows no gross abnormalities, but has significantly increased phospho-ERK [9]. RNAi-mediated knockdowns of DUSP6 result in increased phospho-ERK, showing a direct relationship between the level of this phosphatase and pERK [10,11].

DUSP6 has been identified as a tumor suppressor gene and is inactivated in several different types of cancer. A recent study showed that ~18% of primary lung cancers exhibit loss of heterozygosity at the DUSP6 locus. DUSP6 expression shows an inverse correlation with grade in lung cancer [12] and DUSP6 has been implicated as a tumor suppressor gene in non-small-cell lung cancer [13]. The accumulation of reactive oxygen species in ovarian cancer causes ubiquitination and proteasomal degradation of DUSP6, leading to increased ERK-2 activity and cell proliferation [10]. A third mechanism of inactivation, DNA methylation, has been observed in pancreatic cancer cell lines and primary tumors [7,14]. Pancreatic cancers, like endometrial cancers, show frequent mutational activation of KRAS2 [15], which leads to increased pERK levels. Methylation of intron 1 of DUSP6 is associated with reduced expression of DUSP6 [7]. The region of intron 1 methylated in pancreatic cancer has promoter activity and includes a binding site for the ETS2 transcription factor. ETS2 is a target of ERK-2 and ERK-2 and DUSP6 are involved in a negative feedback loop. As phosphorylated (activated) ERK-2 accumulates in the cell, it phosphorylates ETS2, which in turn transcriptionally activates DUSP6, which functions by removing phosphate groups to

inactivate ERK-2 [16,17]. DUSP6 has also been shown to be upregulated through negative feedback by high levels of fibroblast growth factor (FGF) and KRAS2 [9]. DUSP6 expression is higher in Stage I than Stage II endometrial cancers [18]. Given the high frequency with which mutational activation of the ERK signaling pathway is seen in endometrial cancers, we hypothesized that methylation of the DUSP6 gene leading to low expression of DUSP6 might also contribute to constitutive activation of the ERK kinase cascade. We evaluated DUSP6 methylation in a large cohort of endometrial cancers representative of all grades, stages and histologic types.

Materials and Methods

Preparation of Nucleic Acids

All primary endometrial tumors and normal endometrium specimens analyzed were collected as part of IRB-approved studies (Washington University Medical Center Human Research Protection Office protocols HRPO-91-507, -93-0828 and -92-242). Histologic grading and typing were performed by gynecologic pathologists. Staging was determined using 1988 criteria from the International Federation of Gynecology and Obstetrics. Tissue specimens and blood were obtained at the time of surgery and stored at -70°C until nucleic acids were extracted (all primary tumors evaluated had $\geq 70\%$ neoplastic cellularity). Genomic DNA from tumor tissues, normal endometrium, and cell lines was extracted using the DNeasy Tissue kit (Qiagen, Valencia, CA). Total cellular RNA was extracted from tumors and cell lines using the Trizol reagent (Invitrogen).

DNA from eighteen endometrial cancer cell lines and one pancreatic cell line was also investigated. The cell lines were AN3CA, HEC1A, HEC59, HHUA, HOVA, Ishikawa, KLE, MDA H2774, MFE280, MFE296, MFE319, RL952, Sawano, TEN, UAC1053, UACC210, UACC297, and MiaPaCa-2.

Bisulfite Conversion

DNA bisulfite conversion was performed using a commercially available kit (EZ DNA Methylation Gold™ Kit, Zymo Research, Orange, CA).

DUSP6 COBRA Assays

COBRA (Combined Bisulfite Restriction Analysis) was performed as previously described [19]. We used two rounds of amplification (nested PCR). Three assays were designed at the DUSP6 5' upstream region, 5' UTR, and intron 1. The primers used in nested PCR, amplicon sizes, and restriction digestions used are presented in Table 1.

Restriction fragments were resolved on 10% polyacrylamide gels, stained with ethidium bromide, and photoimaged with a UV camera (ImageStore 500 Version 7.12, White/UV Transilluminator; UVP, Inc., Upland, CA). Band intensities were captured and quantified using the program ImageJ (National Institutes of Health, Bethesda, MD).

cDNA preparation and quantitative RT-PCR

Total RNA preparation was used as a template to generate first-strand cDNA synthesis using the QuantiTect Reverse Transcription Kit (Qiagen). Quantitative gene expression was performed using SYBR® Green (BioRad) methods [20] and relative expression was calculated using the $\Delta\Delta\text{CT}$ method. Quantitative RT-PCR primers were: Forward 5' CCCCTTCCAACCAGAATGTA 3', Reverse TGCCAAGAGAACTGCTGAA 3'. GAPDH was used as the reference gene, PCR primers were: Forward 5' TGCACCACCAACTGCTTAGC 3', Reverse 5' GGCATGGACTGTGGTCATGAG 3'.

Immunohistochemistry

Immunohistochemistry was performed for a subset of primary tumors investigated by COBRA. Five micrometer sections of paraffin-embedded, formalin-fixed tissues were obtained of eight endometrioid tumors. MKP-3 staining was performed with anti-MKP3 antibody (sc-8598, goat anti-human, polyclonal Santa Cruz Biotechnology, Inc, Santa Cruz, CA) at 1:100 dilution followed by a biotinylated secondary antibody at 1:500 dilution (Donkey anti-goat, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) using VECTASTAIN® Elite ABC reagent (Vector Laboratories, Burlingame, CA). Signals were developed with the 3,3'-diaminobenzidine (DAB) Substrate Kit for Peroxidase (Vector Laboratories, Burlingame, CA). ERK and phospho-ERK staining was performed using anti-ERK1/2 (#9012, rabbit anti-human, Cell Signaling Technology, Inc., Danvers, MA) and anti-phospho-ERK (Thr 202/Tyr 204, #9101S, rabbit anti-human, Cell Signaling Technology, Inc., Danvers, MA) at 1:100 dilution. Signals were developed with the 3,3'-diaminobenzidine (DAB) Substrate Kit for Peroxidase (BioCare Medical, Concord, CA).

Western Blots

Protein was extracted using lysis buffer containing a mixture of protease and phosphatase inhibitors. ERK and phospho-ERK were detected using the same antibodies used for IHC (1:1000 dilutions). Goat anti-Rabbit IgG-HRP (sc-2030, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used as a secondary antibody at a concentration of 1:1250.

Results

Three COBRA assays were used to evaluate DUSP6 methylation (Figure 1A). Assay 3, located in intron 1, includes the region previously shown to be methylated in pancreatic cancers. Methylation of this region prevents transcription factor binding and is associated with loss of DUSP6 expression [7, 16, 17]. The 5' upstream region, as well as exon 1 and intron 1, are CpG rich. Because the CpG methylation that is associated with gene silencing most often involves promoter regions upstream of the transcription start site [21], we further evaluated more 5' regions of DUSP6 for methylation, using additional COBRA Assays 1 and 2.

*Bst*UI (CGCG) and *Mbo*I (GATC) restriction digests were used to evaluate methylation in intron 1 (Assay 3) in 91 primary uterine tumors, representing a diverse group of grades, stages, and histologies. A single endometrioid tumor (2070, stage IC, grade 2) showed methylation at both the *Mbo*I and *Bst*UI sites (Table 2, Figure 1B). None of the 18 endometrial cancer cell lines showed methylation in intron 1 of DUSP6. MiaPaCa-2, the pancreatic cancer cell line previously shown to have DUSP6 methylation and very low expression of DUSP6 [7], had approximately 40% methylation at the *Bst*UI and *Mbo*I sites by COBRA (Figure 1B).

We evaluated more 5' sequences for methylation using COBRA assays to determine whether the methylation seen in tumor 2070 and the MiaPaCa-2 cell line was restricted to intron 1 (Assay 3 in our studies, Figure 1A). Assays 1 and 2, in the 5' upstream region and 5' UTR respectively, were evaluated in tumor 2070 and MiaPaCa-2. In addition, seven endometrial cancer cell lines (AN3CA, HEC1A, Ishikawa, KLE, MFE296, RL952, and SKUT1B), 3 normal endometrial tissues, and 33 primary tumors from the cohort evaluated with Assay 3 were evaluated for methylation with Assays 1 and 2. None of the samples evaluated showed methylation at Region 1 (data not shown). Tumor 2070 and the MiaPaCa-2 cell line, however, showed methylation in Region 2 using *Bst*UI and *Hpy*CH4IV COBRA (data not shown).

Quantitative RT-PCR showed DUSP6 mRNA was reduced in the MiaPaCa-2 cell line compared to all cell lines, tumors, and normal tissues assessed (Figure 2A). DUSP6 expression varied widely in the normal endometrial tissues, endometrial cancer cell lines, and tumors

investigated (Figure 2A). DUSP6 transcript levels in normal endometrial tissues varied approximately two-fold (range 272 to 601 arbitrary expression units relative to the MiaPaCa-2 cell line). Expression in endometrial cancer cell lines ranged from 56 to 861 units and in primary endometrioid endometrial cancers from 55 to 889 units (Figure 2A). DUSP6 transcript levels were not correlated with the pERK levels as assessed by Western blots and IHC (Figure 2B,C). Tumor 2070, which has *DUSP6* methylation, did not show a substantial reduction in DUSP6 expression at the mRNA level. Samples with low DUSP6 expression at the mRNA level (2027T, 1570T, 1474T, 1655T, and others) did not show methylation at any region of the DUSP6 gene. Immunohistochemistry revealed DUSP6 expression in all tumors evaluated, including the specimen 2070 with 5' UTR and intron 1 methylation (data not shown).

Discussion

To the best of our knowledge this study is the first to assess DUSP6 methylation in a large cohort of endometrial cancers. We conclude that methylation of DUSP6 is an infrequent event in endometrial cancers based on our observation of a single methylated case among 91 tumors investigated. None of the eighteen endometrial cancer cell lines evaluated showed DUSP6 methylation, further supporting our conclusion that DUSP6 methylation is uncommon in endometrial cancers. In contrast to previous findings in pancreatic cancers [7], DUSP6 intron 1 methylation did not appear to affect mRNA or protein expression as assessed by quantitative RT-PCR and IHC. However, we had a single example of a primary tumor with DUSP6 methylation and it is difficult to speculate as to why the observed methylation was not associated with reduced expression. One possible explanation for the difference in DUSP6 expression in the endometrial cancer we observed and what has been described for pancreatic adenocarcinomas could be the extent of the methylation. Tumor 2070 had an estimated 20% methylation of DUSP6 at intron 1. The pancreatic adenocarcinomas with lowered DUSP6 expression were shown to have $\geq 40\%$ methylation at intron 1 of DUSP6 [7] and the 20% methylation observed in sample 2070 may not be sufficient to affect DUSP6 expression.

We observed methylation at the putative 5' regulatory region of DUSP6 in sample 2070 as well as MiaPaCa-2, a pancreatic cell line with low expression of DUSP6. Methylation at the more 5' region of the DUSP6 sequence has not been previously reported. The significance of this methylation is unknown; however, the importance of methylation at the 5' regions of genes has been well characterized [21,22]. Methylation in 5' regulatory regions can contribute to recruitment of repressive proteins, a closed chromatin structure, and gene silencing. The closed chromatin state may spread downstream from the 5' region [23,24]. Methylation could thus be initially targeted to either the 5' region or intron 1 of DUSP6, then spread to other regions, effectively silencing expression of the gene. It is presently unclear which region is methylated first *in vivo*.

The low rate of *DUSP6* methylation in endometrial cancers was somewhat unexpected given the fact many endometrial cancers have a CpG island methylator phenotype and, like pancreatic cancers, have frequent mutations in the ERK signaling pathway [25-27]. Hypermethylation of promoter regions and the resultant CpG island methylator phenotype (CIMP) as seen in endometrial cancers is a form of epigenetic deregulation [22,28]. The absence of methylation in the 5' region of DUSP6 in cancers that often show abnormal methylation of promoter regions could reflect strong selection for DUSP6 expression and regulation of ERK-2 phosphorylation in endometrial cancers and/or selection against tumor cells in which the DUSP6 promoter is methylated. Alternatively, the sequence or genomic context of DUSP6 could make it relatively resistant to methylation. Our methylation studies did not address the possibility of post-transcriptional or translational repression of DUSP6 expression; the variation in DUSP6 transcript levels could be explained by microRNA regulation or other post-transcriptional events.

Quantitative RT-PCR showed low DUSP6 mRNA levels in the MiaPaCa-2 cell line with DUSP6 methylation compared to the endometrial normal tissues, cancer cell lines, and primary cancers, consistent with transcriptional silencing by methylation. Endometrial cancer cell lines with low phospho-ERK (AN3CA, SKUT1B) exhibited high levels of DUSP6 mRNA. HEC1A, with high levels of pERK, had low DUSP6 mRNA expression, and Ishikawa and MFE296 had medium levels of both pERK and DUSP6, consistent with DUSP6 regulation of ERK phosphorylation. The level of DUSP6 expression we saw in the Ishikawa cell line is similar to what has been previously reported [29]. However, KLE and RL952 did not fit this expression pattern (Figure 2). While seventeen primary endometrial cancers assessed showed a large variation in DUSP6 transcript expression, there appears to be no relationship between DUSP6 mRNA and phospho-ERK status. Three normal endometrium tissues were also assessed and exhibited medium-high levels of DUSP6 mRNA (Figure 2A). An explanation for the lack of correlation between DUSP6 mRNA and phospho-ERK could be that other phosphatases are at work, such as DUSP5, DUSP7, or DUSP9 [9]. Feedback loops in place in response to activated ERK-2, FGF, and KRAS could also affect levels of DUSP6 when pERK levels are high.

Phosphorylated ERK-2 is seen in >60% of endometrioid endometrial cancer cases, including some that lack activating mutations upstream in the pathway ([5], and our unpublished data). KRAS2 and FGFR2 mutations are common in endometrioid endometrial cancers [4] but do not account for all of the cases with activated ERK. We hypothesized that aberrant hypermethylation of the DUSP6 gene and silencing of the DUSP6 ERK-2 phosphatase could be an additional mechanism of constitutive activation of the ERK kinase pathway in endometrial cancers. Given current interest in MEK inhibitors (MEK phosphorylates ERK) as biologic therapies for cancer, understanding how ERK activity is regulated is of increasing importance [30,31].

This study shows that DUSP6 methylation is uncommon in endometrial cancer. Further studies are required to determine whether the high rate of activated ERK seen in endometrial cancers is attributable to as yet unknown upstream activation events and whether DUSP6 activity is deregulated by other mechanisms in pERK-positive endometrial cancers.

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A. **B.** **Figure 1. COBRA Assays for the DUSP6 gene**

(A) Schematic representation of the DUSP6 gene. Black line on the left represents the 5' regulatory region (1200 bp), white rectangle represents the 440 bp 5' UTR of Exon 1, grey rectangle represents the coding sequence of Exon 1 (400 bp). Black line on the right represents Intron 1 (400 bp shown). Black rectangles represent CpG islands. Brackets indicate COBRA Assays 1, 2, and 3. (B) Representative COBRA; Assay 3 in intron 1. The universally methylated (UM) control shows the expected 158 and 194 bp bands for *Bst*UI and *Mbo*I digests, respectively. Tumor 2049 is unmethylated whereas tumor 2070 and Mia-PaCa-2 show methylation. B: *Bst*UI digestion, M: *Mbo*I digestion.

Figure 2. DUSP6 and pERK levels in endometrial cancer cell lines and primary tumors
(A) Level of DUSP6 mRNA in cancer cell lines assessed by Q-RT-PCR, shown as fold change relative to the MiaPa-Ca-2 cell line. All experiments were performed in triplicate and repeated at least once. Error bars indicate SEM. **(B)** Western blot to assess total ERK and pERK levels in endometrial cancer cell lines. M = MFE296, K= KLE, I = Ishikawa, H = HEC1A, S = SKUT1B, R = RL952, A= AN3CA. **(C)** Representative examples of very low (1316T), medium (1419T), and high (1655T) pERK expression in primary tumors, by immunohistochemistry.

Table 1

Primers and restriction digests used for COBRA assays.

Assay	Primers *	Amplicon Size	Restriction Digest Products
Region 1 (5' putative regulatory region)	Rd1 For 5'gaTatgTTtTTTtgTTaaTtgtaa 3' Rd1 Rev 5'aacaaactcttaAAtcaAtcc 3' Rd2 For 5'agTtTTtgTTTtagaatTattaa 3' Rd2 Rev 5'caAtccaAtActtttactAtattc 3'	147 bp	<i>Bst</i> UI (CGCG) 117 & 30 bp <i>Acc</i> II (CCGC) 62, 59 & 26 bp
Region 2 (5' regulatory region, 5' UTR)	Rd1 For 5'aagtTtTTtggtttatgtgTTTtg 3' Rd1 Rev 5'tctaactcctccctccaaAA 3' Rd2 For 5'ttgtgaatgaTaaaTtTattaaTa 3' Rd2 Rev 5'tttActatctcttAAactcaAcct 3'	196 bp	<i>Bst</i> UI 157 and 39 bp <i>Hpy</i> CH4IV (ACGT) 85, 74 & 37 bp
Region 3 (intron 1)	Rd1 For 5'tgTtgTtTaagaagTtTaagg 3' Rd1 Rev 5'tttAcatccccaacaatct 3' Rd2 For 5'ggattgaaaataTTtTgTtT 3' Rd2 Rev 5'tcctAcaaatcttaattcaaa 3'	254 bp	<i>Bst</i> UI 158, 45, 42, 7 & 2 bp <i>Mbo</i> II (GATC) 194 & 60 bp

* Capitalized letters indicate unmethylated C converted to T by bisulfite treatment.

Table 2

Clinical and molecular features of tumors analyzed for DUSP6 expression.

Breakdown of tumors analyzed for DUSP6 methylation	N (% methylated)
Primary uterine cancers (N)	91 (1.1 %)
<i>Stage</i>	
I	49 (2.0%)
II	7 (0%)
III	26 (0%)
IV	9 (0%)
<i>Histology</i>	
Endometrioid	70 (1.4%)
Grade 1 (33)	
Grade 2 (21)	
Grade 3 (16)	
Papillary serous	7 (0%)
Clear cell	7 (0%)
Carcinosarcoma	7 (0%)
<i>ERK Kinase Cascade Mutation Status</i>	
Wild type	55 (1.8%)
FGFR2 mutation	4 (0%)
KRAS2 mutation	8 (0%)
Unknown	24 (0%)

Grading and staging was performed according to FIGO staging.