Published in final edited form as:

Clin Cancer Res. 2011 April 15; 17(8): 2120–2129. doi:10.1158/1078-0432.CCR-10-2668.

Aberrant Methylation of the X-linked Ribosomal S6 Kinase RPS6KA6 (RSK4) in Endometrial Cancers

Summer B. Dewdney¹, BJ Rimel¹, Premal H. Thaker¹, Dominic M. Thompson Jr.², Amy Schmidt², Phyllis Huettner³, David G. Mutch¹, Feng Gao⁴, and Paul J. Goodfellow^{1,2}

¹Division of Gynecologic Oncology, Department of Obstetrics and Gynecology, Washington University School of Medicine and Siteman Cancer Center, St Louis, MO

²Division of Endocrine and Oncologic Surgery, Department of Surgery, Washington University School of Medicine and Siteman Cancer Center, St Louis, MO

³Department of Pathology and Immunology, Washington University School of Medicine and Siteman Cancer Center, St Louis, MO

⁴Division of Biostatistics, Washington University School of Medicine and Siteman Cancer Center, St Louis, MO

Abstract

Purpose—Effective treatments for advanced endometrial cancer are lacking. Novel therapies that target specific pathways hold promise for better treatment outcomes with less toxicity. Mutation activation of the FGFR2/RAS/ERK pathway is important in endometrial tumorigenesis. RPS6KA6 (Rsk4) is a putative tumor suppressor gene and is a target of the ERK signaling pathway. We explored the role of RSK4 in endometrial cancer.

Experimental design—We showed that RSK4 is expressed in normal endometrial tissue and is absent or much reduced in endometrial cancer. Based on previously reports on methylation in other cancers we hypothesized that the absence of RSK4 transcript is associated with epigenetic silencing rather than mutation. We determined the methylation and expression status of RSK4 in primary endometrial cancers and cell lines and the effects of treatment with a demethylating agent. The relationship between RSK4 methylation and clinicopathologic was assessed.

Results—RSK4 is frequently hypermethylated in endometrial cancer cells lines and in primary endometrial cancer compared to normal endometrial tissue. RSK4 methylation was significantly associated with tumor grade, with higher grade tumors having lower levels of methylation ($p=0.03$). RSK4 methylation levels were not associated with other clinical variables. We did find that RSK4 methylation was significantly correlated with expression in primary endometrial tumors and in cell lines. Re-activation of RSK4 by 5-azacytidine was successfully performed showing 8 to >1,200 fold increases in transcript levels.

Conclusion—RSK4 appears to be epigenetically silenced in endometrial cancer as evidenced by hypermethylation. Its role as suppressor in endometrial cancer, however, remains uncertain.

Keywords

Endometrial cancer; methylation; RPS6KA6; RSK4; epigenetics

Introduction

In the United States, endometrial cancer is the most common malignancy of the female genital tract. It is estimated that 42,160 women were diagnosed with this disease in 2009, resulting in 7,780 deaths (1). Fortunately, the majority of endometrial cancer is diagnosed at an early stage due to abnormal uterine bleeding, and most of these women are cured with surgery. However, the prognosis for patient with advanced stage and recurrent endometrial cancers is poor with an approximate 12 month median overall survival (2). Effective treatments for these advanced cancers are lacking, and the chemotherapy regimens most commonly used have high toxicities.

Targeted therapies that come from study of cancer biology hold promise for more specific and effective treatments and less toxicity than is seen with conventional cytotoxic chemotherapy. The FGFR2/RAS/ERK signaling pathway is frequently activated in endometrial cancers (3, 4). A number of components of the signaling cascade (FGFR2, ras, and MEK) have been or are currently being evaluated as targets for therapy.

RPS6KA6 (RSK4) is an ERK substrate (5). RSK4 has been identified as a modulator of p53-dependent proliferation arrest in human cells and has been shown to inhibit transcriptional activation of specific targets of receptor tyrosine kinase (RTK) signaling as well as activation of ERK (6). It also has been implicated as a tumor suppressor gene, showing tumor suppressor activities in breast, colon and renal carcinomas (7-9). RSK4 overexpressing mammary tumors in transgenic mice are noninvasive and do not metastasize (10). Furthermore, in breast cancer cell lines, RSK4 overexpression decreased proliferation and invasive ability (10). Finally, down regulation of RSK4 has been seen in primary human colon cancer (11).

RSK4 is located on the X-chromosome (Xq21.1) and subject to X-inactivation. It is part of the p90 ribosomal S6 kinase family, which includes both activating and inhibitory isoforms RSK1, RSK2, RSK3, RSK4. RSK4 play a role in the in regulation of cellular division, survival, and differentiation via substrate phosphorylation. It appears to be constitutively activated in cells, may function to suppress FGFR2-RAS-ERK signal transduction and cell proliferation. Its expression may be one mechanism to restrict cell growth (12).

We found that RSK4 is expressed in normal uterine tissue and is absent or much reduced in endometrial cancer (cell lines and primary tumors). Given the high frequency of the so-called CpG island methylator phenotype in endometrial cancers and that RSK4 is an X-linked gene and normally subject to methylation and epigenetic silencing, we sought to determine if the aberrant CpG methylation could explain loss of expression. We hypothesized that the absence of RSK4 transcript is associated with epigenetic silencing in endometrial cancers.

Materials and Methods

Patient samples

Primary endometrial tumor tissues, normal endometrium and normal blood specimens were obtained at time of hysterectomy, snap frozen and stored at -75°C. All patients consented to molecular and follow-up studies as part of ongoing Washington University Human Research Protection Office–approved research protocols (protocols 91-507 and 93-0828). Pathology reports including histologic subtype, grade, and stage were obtained along with clinical characteristics including body mass index (BMI), age, race, follow-up and adjuvant treatment.

Representative portions of tumors were formalin fixed, paraffin embedded and histologically evaluated. DNA and RNA were prepared from tumor tissues with $\geq 70\%$ neoplastic cellularity. Tumor DNA was prepared using proteinase K and phenol extraction or with the DNeasy Tissue Kit (Qiagen Inc, Valencia, CA). DNA was extracted from peripheral-blood leukocytes as previously described (13). Total RNA was extracted from cell lines and primary tissues using Trizol[®] (Invitrogen, Carlsbad, CA).

Combined bisulfite restriction analysis (COBRA) and bisulfite sequencing

Bisulfite conversion of cell line and primary tissue DNAs was performed using EZ DNA Methylation-Gold[™] Kit reagents (Zymo Research, Orange, CA). RSK4 sequences of interest were amplified using two rounds of PCR amplification (nested PCR). Primers, amplicon sizes, and restriction enzymes used are presented in Table 1.

Following digestion with the appropriate enzyme, restriction fragments were resolved on 10% polyacrylamide gels, stained with ethidium bromide, and photoimaged with a UV camera (ImageStore 7500 Version 7.12, White/UV Transilluminator; UVP, Inc., Upland, CA). Band intensities were quantified using ImageJ (National Institutes of Health, Bethesda, MD) to estimate the percent methylation/digestion for a given restriction enzyme cut site.

Cloning and sequencing of bisulfite converted tumor and normal DNAs was performed using standard methods (14). PCR products were cloned using the PCR-2.1TOPO TA vector (Invitrogen, Carlsbad, CA) and a minimum of seven clones for each cloning experiment sequenced using ABI Prism BigDye[®] Terminator chemistry v1.1 (Applied Biosystems, Foster City, CA).

RSK4 expression studies

cDNAs were prepared from total RNAs using the QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA) and RSK4 transcripts detected using conventional RT-PCR and quantitative real time PCR methods. qRT-PCR was performed using SYBR[®] Green (Bio-Rad) and the $\Delta\Delta CT$ method (15). GAPDH was used as the reference gene. The primers used for qRT-PCR were:

RSK4 Forward 5'-TGCTCAAGGTTCTTGGTCAG-3' in exon 3

RSK4 Reverse 5'-TTTGTCCGAAGTCTGTCTCG-3'; in exon 5

GAPDH Forward 5'-TGCACCACCAACTGCTTAGC-3';

GAPDH Reverse 5'-GGCATGGACTGTGGTCATGAG-3'

RSK4 reactivation studies

Endometrial cancer cell lines, AN3CA, SKUT1B, RL952, KLE, and HEC1A were obtained from American Type Culture Collection (Rockville, MD). SPEC-2 and Ark1 were provided by Anil Sood (M.D. Anderson Cancer Center) and Shi-Wen Jiang (Mercer University School of Medicine), respectively. All cell lines were tested for mycoplasma (Division of Comparative Medicine, WUSM). AN3CA, SKUT1B, RL952, KLE, and HEC1A were tested for microsatellite instability (NCI consensus panel) and expression for MSH2 and MLH1 by Western blot to confirm DNA mismatch repair status; this was consistent with previously published findings. PTEN and KRAS2 mutation status was similarly confirmed by direct sequencing of targeted exons as appropriate. The SPEC-2 and Ark1 cell lines were evaluated less extensively. Microsatellite repeat marker evaluation (NCI MSI consensus panel and two additional X chromosome repeats) however did not reveal any patterns of allelism suggestive of cell contamination (allelism consistent with diploid/heterozygous state).

These cell lines were then treated with 5-aza-deoxycytidine (5-Aza-2'deoxycytidine, Sigma Chemical, St. Louis, MO) essentially as described by Deng et al. (16). 1×10^5 cells were plated in 10cm dishes and 5-aza-C (5 or 10 uM) added 24 hours later. Cells were grown in the presence of 5-aza-C for 48 hours, after which the cell culture medium was changed to no 5-aza-C. Cells were harvested for RNA and DNA preparations 72 hours later.

RSK4 loss of heterozygosity (LOH) studies

Two microsatellite repeats in RSK4 were used to test for LOH in primary tumors. A CA_n repeat in intron 6 (ChrX:83,393,729-83,393,879 MSI 2 F 5'CCTACCCAAATTTCCCTTCC3', R 5'TCAGCCATTCTTCTACCACA3' and a GT_n/AG_n repeat in intron 1 (ChrX:83,436,967-83,437,129 MSI 1 F 5'AACAGGTCCTGCTGTAGTTTGG3' and R 5'CCATCTCAAATGCTTGGTAAAA3') and the flanking markers DXS1196 and DX2990 using ABI PRIMS Linkage Mapping Set v2.5 (product # 4329191) were amplified with fluorescently labeled primers and analyzed using capillary electrophoresis on an ABI 3130 Genetic Analyzer and GeneMapper Analysis software (Applied Biosystems, Foster City, CA).

Statistical analysis

For the analysis of primary tumors, RSK4 promoter methylation was categorized into 3 groups according to the % ZraI site methylation: <50%, 50-75%, and >75%. The associations between RSK4 methylation status and other demographic/clinical characteristics were assessed using Fisher exact test, analysis of variance (ANOVA) or Kruskal-Wallis rank-sum test as appropriate. The effect of RSK4 methylation on overall survival (OS) or disease-free survival (DFS) was described by Kaplan-Meier product limit method and compared using log-rank test. OS was defined as the time from the date of surgery to the date of death due to any causes, and survivors were censored at the date of last contact. DFS was defined as the time from the date of surgery to the date of recurrence or death, whichever occurred first. All analyses were two-sided and significance was set at a p-value of 0.05. Statistical analyses were performed using statistical package SAS (SAS Institutes, Cary, NC).

Results

Methylation status of the promoter region of RSK4 (RPS6KA6)

Normal DNAs—We analyzed the ~ 1000bp RSK4 CpG island for methylation using combined bisulfite restriction analysis (COBRA). Three overlapping assays 5' of exon 1 and two from intron 1 were evaluated in DNAs prepared from normal blood and endometrium to determine the patterns of methylation seen in noncancerous tissues. A ZraI restriction site (GACGTC) shared by assays #9 and #10 showed patterns of methylation expected for an X-linked gene. Normal male DNA was unmethylated (single active X chromosome) and normal female DNA showed approximately 50% methylation (consistent with one unmethylated X chromosome and one methylated inactive X) (Figure 1A). The cytosines evaluated with the ZraI restriction digests map 414 and 417 bp 5' of the exon 1. More 5' sequences (assay #1) and the two assays located in intron 1 showed variable methylation in male and/or female DNAs. Assay #1 revealed methylation in males and the intron 1 sequences were unmethylated in a subset of normal female DNAs (data not shown).

Analysis of an expanded cohort of normal male (N=12) and female (N=34) DNAs assessing methylation at the ZraI site (GACGTC), using the optimized assay #10, gave a mean % methylation of 0.7% and 40%, respectively (range 0.2 to 2% for males and 25-59% for females).

Cell lines and primary tumor DNAs—COBRA assay #10 (ZraI digestion) was used to evaluate RSK4 promoter methylation in eight endometrial cancer cell lines (endometrioid endometrial cancer cell lines: AN3CA, KLE, Ishikawa, SKUT1B, HEC1A, and RL952, and the serous cancer cell lines: SPEC2 and Ark1). Five of the six endometrioid endometrial cancer cell lines and one of the two serous cell lines were fully methylated. HEC1A, showed ~50% methylation whereas Ark1 was unmethylated (Figure 1B).

A survey of primary endometrioid endometrial tumors revealed the majority of cancers were heavily methylated with an apparent trend towards low level methylation in high grade (G3) tumors. Serous cancers on the other hand were largely unmethylated (Figure 1B). The matched normal DNAs (both endometrioid and serous cancer cases) showed the expected ~50% methylation (data not shown).

To ensure the results of ZraI restriction digests (recognition site GACGTC) were representative of methylation of the region assayed (14 CpGs in the assay # 10 156 bp amplicon), we cloned and sequenced bisulfite converted normal male, normal female and endometrial cancer tumor DNAs. For all three DNAs the percent methylation for the entire region based on bisulfite sequencing was similar to the fraction of molecules methylated at the ZraI site as determined by COBRA (Figure 2). The three most proximal CpG pairs (referred as sites 12-14) were, however, significantly less methylated in both normal female blood DNA and tumors compared to the more 5' CpG pairs. Given the proximal CpGs in the region may not be methylated on the inactive X chromosome we focused our DNA methylation on the ZraI digestions. We recognize that a more detailed analysis of methylation could reveal that there are other CpG dinucleotides that show a different methylation pattern. However, it has been our experience that in general, the methylation in a CpG Island is highly correlated with variation across the region.

RSK4 promoter methylation in primary tumors

Our initial exploratory studies on RSK4 methylation in a primary tumors revealed methylation levels were lower in higher grade tumors (grade 3 endometrioid cancers and uterine serous cancers). To better determine if RSK4 methylation is associated with clinicopathologic features we evaluated a total of 158 primary tumors (146 endometrioid cancers, 11 uterine papillary serous carcinomas (UPSC) and 1 mixed endometrioid/serous cancer). The clinical and molecular characteristics of the endometrioid patient population can be seen in Table 2. The endometrioid primary tumors were selected for approximate equal distribution of grades to allow us to follow-up on the initial observation that high grade tumors appeared less methylated. The average methylation of the RSK4 promoter in primary endometrioid tumors as assessed by ZraI COBRA was 63% (range 3 to 96%). Serous cancers on the other hand were largely unmethylated (Figure 1B) with an average methylation of 18% (range 8 to 34%), less than expected for a normal female tissues with one active and one inactive X chromosome.

RSK4 methylation was significantly associated with tumor grade. Higher grade endometrioid tumors have lower levels of methylation ($p=0.03$). The mean % methylation for grade 1, 2 and 3 tumors was 71, 63 and 55 respectively. Methylation was not associated with patient age, race, BMI, stage, or adjuvant treatment. There was no association with overall survival or progression free survival and the mean follow up time was 52 months. RSK4 methylation was, however, significantly associated with MLH1 methylation ($p<0.0008$) (Table 2). In addition, we did find that 40% of tumors with a high level of RSK4 methylation had either a KRAS2 or FGFR2 mutation but there was not a statistically significant association between methylation RSK4 and mutations in either gene or both combined.

Some primary tumors had less RSK4 methylation than the expected 50% for cells with one active X. All serous cancers investigated had low levels of RSK4 methylation (mean 18%, range 8-34). A subset of endometrioid tumor (N=22) also showed unexpectedly low methylation (< 1 SD deviation below mean for the 146 cases evaluated). The majority (64%) of the tumors with low methylation were grade 3 with only 32% and 4% grade 2 and 1, respectively. The low levels of methylation seen in those tumors could be associated with changes in copy number (loss of the inactive X or gain of active X chromosome) rather than a change in methylation of the inactive X *per se*. Using two intragenic microsatellite repeat polymorphisms and two flanking repeats to test for loss of heterozygosity or allelic imbalances we saw that only 4 of 19 (21%) informative tumors showed loss of heterozygosity at RSK4 and for the remaining 15 cases the pattern of alleles was consistent with the presence of two X chromosomes (an active and an inactive X). Two low methylators were MSI positive and as such non-informative for LOH analysis (data not shown). All twelve of serous cancers investigated (all with low level RSK4 methylation) were informative and 4 had LOH (33%). Together, 8 of 31 RSK4 “low methylators” (26%) had loss of an RSK4 allele. In addition to unexpectedly low levels of RSK4 methylation, we saw hypermethylation in a subset of tumors. Nineteen endometrioid tumors were heavily methylated (> 1 SD deviation above the mean, range 84 to 96%). Among these, 12 (3%) were MSI-positive cancers and showed MSI at one or more markers. All 7 of microsatellite stable cases were informative and none showed LOH.

Relationship between RSK4 methylation and expression in endometrial cancer cell lines and re-activation of RSK4 by 5-azacytidine

RSK4 expression in endometrial cancer cell lines was initially assessed by RT-PCR. The RT-PCR screen for expression was performed in three normal endometrial specimens, all of which expressed RSK4. Three primary tumors were analyzed (all endometrioid) and none of these expressed RSK4. In addition to the seven endometrial cancer cell lines, an immortalized normal endometrial epithelial line (EM-TERT) also expressed RSK4. Of the seven cell lines evaluated (AN3CA, SKUT1B, RL952, KLE, HEC1A, SPEC-2 and Ark1) only HEC1A expressed RSK4 (Figure 3). HEC1A shows ~50% methylation (Figure 1B) and as such the expression observed is consistent with its methylation status. AN3CA, SKUT1B, RL952 and KLE which did not express RSK4 are completely methylated (Figure 1B). Ark1 on the other hand did not express RSK4 but is completely unmethylated. Apart from the Ark1 cell lines the results for RT-PCR analyses were consistent with expression from the unmethylated RSK4 locus.

RSK4 expression was reactivated in fully methylated cell lines by 5-azacytidine (5-AzaC) treatment. RT-PCR revealed RSK4 transcripts in AN3CA, SKUT1B and RL952 following either 5 or 10uM 5-AzaC treatment (Figure 3). Transcripts were not detectable in the 5-AzaC treated KLE line. Quantitative real-time PCR demonstrated 8 to >1,200 fold increases in transcript levels (Figure 3). A replicate 5-AzaC treatment experiment for the RL952 and AN3CA lines showed comparable levels of reactivation. RSK4 protein was, however, undetectable by Western blot analysis (data not shown).

RSK4 methylation levels are correlated with expression in primary endometrioid endometrial tumors

Given the inverse correlation between RSK4 methylation levels and expression in cell lines, we went on to test expression in primary endometrioid endometrial tumors to determine if methylation and expression showed a similar relationship. Twenty-five primary tumors previously were evaluated by qRT-PCR focusing on representative cases with low levels of methylation (N=8), medium (N=9), and high level methylation (N=8). RSK4 expression was significantly associated with methylation ($p=0.0002$, Spearman rank correlation). The

tumors with high level methylation showed very low levels of expression, all less than the HEC1A cell line reference. Tumor classified as having moderate and low levels of methylation showed higher but variable levels of RSK4 transcripts (nine expressing less RSK4 than HEC1A and seven expressing more than HEC1A) (Figure 4).

Discussion

RSK4 is predominantly a cytosolic protein expressed at low levels in a broad range of human tissues (7, 11, 12, 17). Dummler and colleagues showed RSK4 is constitutively activated in serum starved cells. Their detailed functional characterization suggested activity results from constitutive phosphorylation, attributable in part to low basal levels of phosphoERK, and perhaps also by less well defined mechanisms that could include enhanced autophosphorylation (12). Dummler et al. speculated RSK4 may function to suppress ras-ERK signal transduction and cell proliferation (12). Studies from Lopez-Vicente and colleagues further implicated RSK4 as a tumor suppressor (7, 12). RSK4 mRNA levels were shown to be lower in human colon and renal tumor tissues compared to their normal counterparts and protein levels were similarly reduced based on Western blot analysis. RSK4 transduction of colon carcinoma and primary fibroblasts cell lines (IMR90) induced cell growth arrest and RSK4 inhibition immortalized IMR90 cells. Reduced RSK4 activity in the HCT116 colon cancer cell line was associated with a survival advantage when cells were exposed to DNA-damaging agents (7). RSK4 contributions to cancer phenotypes could be through regulation of cell cycle arrest and stress responses (7), tumor invasiveness and metastasis (8, 10).

Given the high frequency of activated ERK signaling in endometrial cancers (18-21) and RSK4's potential role in suppressing ERK signaling we hypothesized reduced RSK4 activity might contribute to endometrial tumorigenesis. RSK4 mutation is an infrequent event in cancer. Among 339 tumor specimens reported in the COSMIC database (<http://www.sanger.ac.uk/genetics/CGP/cosmic>), only two missense changes are described (22). Both mutations were seen in primary lung cancer (2 in 87 investigated) and involved the N terminal kinase. Given endometrial cancer frequently exhibit a CpG island methylator phenotype (23) we tested for increased methylation of the RSK4 CpG island in primary endometrial cancer and endometrial cancer cell lines. To the best of our knowledge there have been no prior studies on RSK4 methylation in endometrial cancers.

Because RSK4 is an X-linked gene subject to X-inactivation (24), our methylation analysis was focused on a region of the CpG island for which there was no methylation in normal male DNAs and ~50% methylation in normal female DNA. We observed both hyper- and hypomethylation of RSK4 promoter sequences in primary tumors and in endometrial cancer cell lines. In primary endometrial cancers, decreased RSK4 methylation was associated with deletion in 26% of the cases (8 of 31) investigated. Loss of the inactive X chromosome (methylated) in such tumors would explain the low level RSK4 methylation. Loss of the inactive X chromosome and replication of the active X has been described in basal like breast cancers (25). BRCA1 breast tumors (cancers with mutation in the BRCA1 tumor suppressor) have defects in X inactivation, genetic instability of the X chromosome and both gains and losses of the inactive X (26). The majority of primary endometrial tumors with low level RSK4 methylation (23 of 31, 74%), in our study did not appear to lose an X chromosome (or gain copies of the active X). We speculate that loss of methylation at the RSK4 locus on the inactive X chromosome has occurred in these cases. Whether the gain in methylation at RSK4 in endometrioid endometrial cancer is part of a more general abnormality in X chromosome DNA methylation and chromatin modification or is associated with the CpG methylator phenotype remains to be determined.

Our studies assessing the relationship between RSK4 methylation and expression suggest RSK4 CpG island methylation is associated with epigenetic silencing. The observation that higher levels of RSK4 methylation in primary tumors are associated with reduced mRNA expression combined with our demonstration that treatment of endometrial cancer cell lines that have extensive RSK4 methylation with a demethylating agent (5-azaC) results in increased RSK4 expression suggest a functional link between methylation and gene silencing. There may be selection to epigenetically silence RSK4 in endometrial cancers given its proposed role in suppressing ERK signal transduction (12). Endometrial cancers have frequent mutational activation of the ERK pathway (primarily FGFR2 and KRAS2). Immunohistochemical studies have revealed >60% of primary endometrial cancers have activation (pERK positivity) and that ERK activation is seen in the absence of KRAS2 and/or FGFR2 mutations (21) and our unpublished data. It is possible that reduced RSK4 activity could explain ERK activity in a fraction of cases for which no mutational activation of the signaling cascade is seen.

The relationship between RSK4 methylation and tumor grade we observed was unexpected. Reduced levels of methylation in poorly differentiated (grade 3) endometrioid tumors could reflect as yet unappreciated differences in RSK4 activity and/or ERK signaling in well-differentiated vs. poorly differentiated tumors. The RSK family members phosphorylate a range of substrates important in malignancies (27). Grade 3 tumors are less likely to express both estrogen and progesterone receptors than well or moderately differentiated tumors (28). Although RSK4's role in estrogen and progesterone receptor signaling has not been established, it is interesting to suggest RSK4 silencing in grade 3 tumors may reflect some interaction with the estrogen and progesterone receptor activities in endometrial cancer. High levels of RSK4 promoter methylation were associated with MSI but were not associated with survival, nor any of the other clinicopathologic variables we considered. The association between hypermethylation and MSI may be secondary to a global methylation, CpG island methylation that accounts for MLH1 silencing and the MSI phenotype. There is conflicting evidence as to whether MSI itself is a prognostic factor for endometrial cancer. Black and colleagues analyzed 473 tumors, including 93 MSI-positive cases and found that the presence of MSI was independently associated with a more favorable clinical outcome through a multivariate analysis (29). Our group evaluated 447 cases of endometrial cancer, identifying 147 MSI positive cases and found no association between survival and MSI status (30). The NCIC (National Cancer Institute-Canada) recently analyzed 163 endometrial cancers for MSI and found MSI positivity (N=32) was associated with a worse prognosis (31). Although RSK4 methylation is associated with the MSI phenotype the biologic relationship remains to be determined.

In conclusion, our studies suggest what may be a causal association between RSK4 methylation and transcriptional silencing. Epigenetic silencing of RSK4 could abrogate the gene's normal tumor suppressor functions. The inverse association between methylation and tumor grade was unexpected and may be indicative of an alternate mechanism for tumorigenesis in poorly differentiated cancers and an as yet unappreciated role for RSK4 in malignancies. Additional studies will be required to elucidate the biologic and clinical significance of RSK4 methylation in endometrial cancer.

Acknowledgments

Grand Support: NIH CA071754

References

1. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun MJ. Cancer statistics, 2009. *CA Cancer J Clin*. 2009; 59:225–249. [PubMed: 19474385]
2. Ray M, Fleming G. Management of advanced-stage and recurrent endometrial cancer. *Semin Oncol*. 2009; 36:145–154. [PubMed: 19332249]
3. Byron SA, Gartside MG, Wellens CL, Mallon MA, Keenan JB, Powell MA, Goodfellow PJ, Pollock PM. Inhibition of Activated Fibroblast Growth Factor Receptor 2 in Endometrial Cancer Cells Induces Cell Death Despite PTEN Abrogation. *Cancer Res*. 2008; 68:6902–6907. [PubMed: 18757403]
4. Byron SA, Pollock PM. FGFR2 as a molecular target in endometrial cancer. *Future Oncol*. 2009; 5:27–32. [PubMed: 19243295]
5. Frodin M, Gammeltoft S. Role and regulation of 90 kDa ribosomal S6 kinase (RSK) in signal transduction. *Mol Cell Endocrinol*. 1999; 151:65–77. [PubMed: 10411321]
6. Myers AP, Corson LB, Rossant J, Baker JC. Characterization of mouse Rsk4 as an inhibitor of fibroblast growth factor-RAS-extracellular signal-regulated kinase signaling. *Mol Cell Biol*. 2004; 24:4255–4266. [PubMed: 15121846]
7. Lopez-Vicente L, Armengol G, Pons B, Coch L, Argelaguet E, Lleona M, Hernandez-Losa J, de Torres I, Ramon y Cajal S. Regulation of replicative and stress-induced senescence by RSK4, which is down-regulated in human tumors. *Clin Cancer Res*. 2009; 15:4546–4553. [PubMed: 19584160]
8. Thakur A, Rahman KW, Wu J, Bollig A, Biliran H, Lin X, Nassar H, Grignon DJ, Sarkar FH, Liao JD. Aberrant expression of X-linked genes RbAp46, Rsk4, and Cldn2 in breast cancer. *Mol Cancer Res*. 2007; 5:171–181. [PubMed: 17314274]
9. Thakur A, Xu H, Wang Y, Bollig A, Biliran H, Liao JD. The role of X-linked genes in breast cancer. *Breast Cancer Res Treat*. 2005; 93:135–143. [PubMed: 16187233]
10. Thakur A, Sun Y, Bollig A, Wu J, Biliran H, Banerjee S, Sarkar FH, Liao DJ. Anti-invasive and antimetastatic activities of ribosomal protein S6 kinase 4 in breast cancer cells. *Clin Cancer Res*. 2008; 14:4427–4436. [PubMed: 18628456]
11. Lleona ME, Vidal F, Gallardo D, Diaz-Fuertes M, Rojo F, Cuatrecasas M, Lopez-Vicente L, Kondoh H, Blanco C, Carnero A, Ramon y Cajal S. New p53 related genes in human tumors: significant downregulation in colon and lung carcinomas. *Oncol Rep*. 2006; 16:603–608. [PubMed: 16865262]
12. Dummler BA, Hauge C, Silber J, Yntema HG, Kruse LS, Kofoed B, Hemmings BA, Alessi DR, Frodin M. Functional characterization of human RSK4, a new 90-kDa ribosomal S6 kinase, reveals constitutive activation in most cell types. *J Biol Chem*. 2005; 280:13304–13314. [PubMed: 15632195]
13. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Research*. 1988; 16:1215. [PubMed: 3344216]
14. Zigelboim I, Goodfellow PJ, Schmidt AP, Walls KC, Mallon MA, Mutch DG, Yan PS, Huang TH, Powell MA. Differential methylation hybridization array of endometrial cancers reveals two novel cancer-specific methylation markers. *Clin Cancer Res*. 2007; 13:2882–2889. [PubMed: 17504987]
15. Whitehead TD, Brownstein BH, Parry JJ, Thompson D, Cha BA, Moros EG, Rogers BE, Roti Roti JL. Expression of the proto-oncogene Fos after exposure to radiofrequency radiation relevant to wireless communications. *Radiat Res*. 2005; 164:420–430. [PubMed: 16187744]
16. Deng G, Chen LC, Schott DR, Thor A, Bhargava V, Jung BM, Chew K, Smith HS. Loss of heterozygosity and p53 gene mutations in breast cancer. *Cancer Research*. 1994; 54:499–505. [PubMed: 8275488]
17. Niehof M, Borlak J. RSK4 and PAK5 are novel candidate genes in diabetic rat kidney and brain. *Mol Pharmacol*. 2005; 67:604–611. [PubMed: 15615695]
18. Pollock PM, Gartside MG, Dejeza LC, Powell MA, Mallon MA, Davies H, Mohammadi M, Futreal PA, Stratton MR, Trent JM, Goodfellow PJ. Frequent activating FGFR2 mutations in endometrial carcinomas parallel germline mutations associated with craniosynostosis and skeletal dysplasia syndromes. *Oncogene*. 2007; 26:7158–7162. [PubMed: 17525745]

19. Dutt A, Salvesen HB, Chen TH, Ramos AH, Onofrio RC, Hatton C, Nicoletti R, Winckler W, Grewal R, Hanna M, Wyhs N, Ziaugra L, Richter DJ, Trovik J, Engelsens IB, Stefansson IM, Fennell T, Cibulskis K, Zody MC, Akslen LA, Gabriel S, Wong KK, Sellers WR, Meyerson M, Greulich H. Drug-sensitive FGFR2 mutations in endometrial carcinoma. *Proc Natl Acad Sci U S A*. 2008; 105:8713–8717. [PubMed: 18552176]
20. Hecht JL, Mutter GL. Molecular and pathologic aspects of endometrial carcinogenesis. *J Clin Oncol*. 2006; 24:4783–4791. [PubMed: 17028294]
21. Mizumoto Y, Kyo S, Mori N, Sakaguchi J, Ohno S, Maida Y, Hashimoto M, Takakura M, Inoue M. Activation of ERK1/2 occurs independently of KRAS or BRAF status in endometrial cancer and is associated with favorable prognosis. *Cancer Sci*. 2007; 98:652–658. [PubMed: 17388789]
22. Davies H, Hunter C, Smith R, Stephens P, Greenman C, Bignell G, Teague J, Butler A, Edkins S, Stevens C, Parker A, O'Meara S, Avis T, Barthorpe S, Brackenbury L, Buck G, Clements J, Cole J, Dicks E, Edwards K, Forbes S, Gorton M, Gray K, Halliday K, Harrison R, Hills K, Hinton J, Jones D, Kosmidou V, Laman R, Lugg R, Menzies A, Perry J, Petty R, Raine K, Shepherd R, Small A, Solomon H, Stephens Y, Tofts C, Varian J, Webb A, West S, Widaa S, Yates A, Brasseur F, Cooper CS, Flanagan AM, Green A, Knowles M, Leung SY, Looijenga LH, Malkowicz B, Pierotti MA, Teh BT, Yuen ST, Lakhani SR, Easton DF, Weber BL, Goldstraw P, Nicholson AG, Wooster R, Stratton MR, Futreal PA. Somatic mutations of the protein kinase gene family in human lung cancer. *Cancer Res*. 2005; 65:7591–7595. [PubMed: 16140923]
23. Whitcomb BP, Mutch DG, Herzog TJ, Rader JS, Gibb RK, Goodfellow PJ. Frequent HOXA11 and THBS2 promoter methylation, and a methylator phenotype in endometrial adenocarcinoma. *Clin Cancer Res*. 2003; 9:2277–2287. [PubMed: 12796396]
24. Carrel L, Willard HF. X-inactivation profile reveals extensive variability in X-linked gene expression in females. *Nature*. 2005; 434:400–404. [PubMed: 15772666]
25. Richardson AL, Wang ZC, De Nicolo A, Lu X, Brown M, Miron A, Liao X, Iglehart JD, Livingston DM, Ganesan S. X chromosomal abnormalities in basal-like human breast cancer. *Cancer Cell*. 2006; 9:121–132. [PubMed: 16473279]
26. Vincent-Salomon A, Gruel N, Lucchesi C, MacGrogan G, Dendale R, Sigal-Zafrani B, Longy M, Raynal V, Pierron G, de Mascarel I, Taris C, Stoppa-Lyonnet D, Pierga JY, Salmon R, Sastre-Garau X, Fourquet A, Delattre O, de Cremoux P, Aurias A. Identification of typical medullary breast carcinoma as a genomic sub-group of basal-like carcinomas, a heterogeneous new molecular entity. *Breast Cancer Res*. 2007; 9:R24. [PubMed: 17417968]
27. Carriere A, Ray H, Blenis J, Roux PP. The RSK factors of activating the Ras/MAPK signaling cascade. *Front Biosci*. 2008; 13:4258–4275. [PubMed: 18508509]
28. Shabani N, Kuhn C, Kunze S, Schulze S, Mayr D, Dian D, Gingelmaier A, Schindlbeck C, Willgeroth F, Sommer H, Jeschke U, Friese K, Mylonas I. Prognostic significance of oestrogen receptor alpha (ERalpha) and beta (ERbeta), progesterone receptor A (PR-A) and B (PR-B) in endometrial carcinomas. *Eur J Cancer*. 2007; 43:2434–2444. [PubMed: 17911007]
29. Black D, Soslow RA, Levine DA, Tornos C, Chen SC, Hummer AJ, Bogomolny F, Olvera N, Barakat RR, Boyd J. Clinicopathologic significance of defective DNA mismatch repair in endometrial carcinoma. *J Clin Oncol*. 2006; 24:1745–1753. [PubMed: 16549821]
30. Zigelboim I, Goodfellow PJ, Gao F, Gibb RK, Powell MA, Rader JS, Mutch DG. Microsatellite instability and epigenetic inactivation of MLH1 and outcome of patients with endometrial carcinomas of the endometrioid type. *J Clin Oncol*. 2007; 25:2042–2048. [PubMed: 17513808]
31. Mackay HJ, Gallinger S, Tsao MS, McLachlin CM, Tu D, Keiser K, Eisenhauer EA, Oza AM. Prognostic value of microsatellite instability (MSI) and PTEN expression in women with endometrial cancer: results from studies of the NCIC Clinical Trials Group (NCIC CTG). *Eur J Cancer*. 2010; 46:1365–1373. [PubMed: 20304627]

Translational Relevance

Novel therapies targeting specific pathways in endometrial cancer hold promise for better outcomes. Our work demonstrates the first known research of RSK4 in endometrial cancer. RSK4 is a putative tumor suppressor gene and its regulation has been demonstrated as a potential target in other cancers. Our study evaluated the methylation of RSK4 in a large set of well characterized endometrial cancer tissues, obtained from our clinical patient base. We found a correlation with methylation and tumor grade. As tumor grade is one of the most powerfully predictive clinical features of this disease, we sought to evaluate this molecular phenotype of methylation with a potential clinically used treatment of 5aza-cytadine. Our work shows a direct effect of demethylation on RSK4 which returns the expression to wild-type. This work could lead to further investigation of RSK4 as a potential target in the treatment of endometrial cancer.

Figure 1. RSK4 5' region evaluated for methylation. **A.** RSK4 CpG island and location of COBRA assays used to assess methylation. Representative examples of COBRA for normal male and female DNAs with assay #10 showing absence of methylation in male and ~50% methylation in female. **B.** Assay #10 COBRA for endometrial cancer cell lines and primary endometrioid endometrial and serous cancer (ZraI digestion). The majority of cancer cell lines show extensive methylation. HEC1A is partially methylated, whereas Ark1 is completely unmethylated. Primary endometrioid endometrial cancers show high level methylation, whereas primary serous tumors show little or no evidence of methylation. Φ X174: HaeIII digested size marker; UM+: universally methylated positive control; Arrows indicate the 156 bp unrestricted PCR product and the 114 bp restriction fragment.

Figure 2.

Bisulfite sequencing for RSK4 amplicon #10. Representative cloning results for normal male and female blood leukocyte DNA and a heavily methylated endometrioid endometrial cancer. The estimated percent methylation of the ZraI site from COBRA analysis is given on the left and overall percent methylation for clones from each specimen are given. The box around cytosines at positions 2 and 3 indicates the location of the CpG pair assessed with the ZraI digestion (GACGTC).

Open circle: unmethylated CpG pair

Filled circle: methylated CpG pair

pbl: peripheral blood leukocyte

Figure 3.

Reactivation of RSK4 expression by 5-azacytidine. **A.** RT-PCR shows expression in HEC1A and testis RNA and absence of detectable transcripts in untreated RL952 and AN3CA. Treatment with 5-azacytidine (5 and 10 μ M) results in expression of RSK4. Transcripts are undetectable in the vehicle (DMSO) treated cells and evident with both 5 and 10 μ M 5-azacytidine treatment. **B.** Quantitative analysis of reactivation of RSK4 expression by 5-azacytidine treatments. The fold increase relative to the DMSO (control) is presented for each of the five cancer cell lines. Of the five cell lines evaluated, treatment of HEC1A with 5-azacytidine did not lead to a substantial increase in transcript levels and it is the only cell line that expresses as seen in Figure 3A. SKUT1B, RL952, and AN3CA all showed significant increases in RSK4 levels upon treatment. The KLE cell line failed to show reactivation of RSK4. Standard errors are given for the results for the independent 5 μ M treatments for RL952 and AN3CA.

The arrow indicates the expected 139 bp RT-PCR amplicon.

V: vehicle

AZA: 5-azacytidine

Figure 4.

RSK4 expression in primary endometrioid endometrial cancers. Transcript levels are expressed relative to HEC1A (y-axis). The percent Zral methylation for each sample is plotted along the x-axis. Tumor specimens are classified as having low (<40%), medium (40-70%), or high (>80%) Zral methylation. Relative expression was calculated using the $\Delta\Delta C_t$ method. The C_t and comparative $\Delta\Delta C_t$ were calculated for the expression of the *Rsk4* gene and then normalized to mean C_t value of GAPDH for each experimental sample. Sample was analyzed in triplicate and all experimental conditions were repeated for verification. The relative expression of RSK4 differed across the three groups ($p=0.0002$, Spearman rank correlation).

Table 1

Primers, amplicons and restriction digests for RSK4 COBRA.

Primers				
Assay	Forward	Reverse	Amplicon Size	Restriction Enzyme
#1	R1 5'tgggattgagagggttgTtg3'	5'tcaatA AaActtAAA AaAattcccc3'	210	BstUI and HpyCH4IV
	R2 5'gagggttgTtgTgagTatgtgga3'	5'AaAattccccaActtAAAAAaAaaAA3'		
#2	R1 5'agagtgtgtgtgaTtaagtTt3'	5'AcacaaatAtccccaAaAttac3'	186	HpyCH4IV and HhaI
	R2 5'aggggaaTgggggaTtg3'	5'tccccaAaAttacttaAtcacctt3'		
#5	R1 5'TTTTtTaagaTTtgTtg3'	5'aaacttaAtcacacaaactctAAC3'	158	BsrBI and AclI
	R2 5'tTaagaTTtgTtgTgggga3'	5'AAcccaAActAcactcactc3'		
#9	R1 5'TTaagTggggaatTtTatt3'	5'ctAtActAcctctccaaAaActAc3'	189	ZraI and BsrBI
	R2 5'agTggggaatTtTattga3'	5'ctcaccactAccaccacaa3'		
#10	R1 5'TTTTTTTTTTtTatTTTaTTaTT3'	5'AtAAAtAactcccccaacccc3'	156	ZraI
	R2 5'TtTaTTTTTaagTggggga3'	5'cccaacaccccaattctt3'		

Table 2

Clinicopathologic and molecular characteristics of endometrioid endometrial cancer patients investigated.

Clinical Characteristics		
Variable	Value	
	Number (%)	Mean (range)
Age (years)		65.3 (33-92)
BMI (kg/m ²)		32.8 (16-55)
Race		
Caucasian	125 (86%)	
African American	20 (14%)	
Other	1 (<1%)	
Stage *		
I	96 (67%)	
II	11 (8%)	
III/IV	36 (25%)	
Grade		
1	49 (34%)	
2	46 (31%)	
3	51 (35%)	
Adjuvant treatment		
Yes	97 (66%)	
No	49 (34%)	
Follow-up (months)		52.1 (0.2-162)
Molecular Characteristics		
RSK4 methylation (% Zra site methylation)		63 (3-96)
MSI status		
MSS/MSI-low	99 (68%)	
MSI-high	46 (32%)	
Total number of patients	146 *	

*
3 cases were missing stage data, and 1 case was missing MSI data.