High Phospho-Stathmin(Serine38) Expression Identifies Aggressive Endometrial Cancer and Suggests an Association with PI3K Inhibition

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Abstract

Purpose: High Stathmin expression has recently been associated with clinical progress in endometrial cancers. Stathmin protein activity is modulated by phosphorylation, and the Serine38 site is one of four Stathmin phospho-sites. The presence and significance of pStathmin(S38) is largely unknown in human cancers, and we here examined the associations between this marker and tumor cell proliferation, clinicopathologic phenotype, and survival impact in endometrial cancer. A relationship with possible treatment targets was explored by integrated analysis of transcriptional alterations.

Experimental Design: Primary endometrial cancers from two independent patient series (n = 518/ n = 286) were analyzed. Biomarkers were assessed by immunohistochemistry, FISH, flow cytometry, DNA oligonucleotide microarray, single-nucleotide polymorphism array, and Sanger sequencing, and related to clinicopathologic annotations and follow-up information.

Results: High pStathmin(S38) level was associated with poor prognosis, independent of other features, and correlated to increased tumor cell proliferation as well as high Stathmin levels. On the basis of transcriptional differences between high/low pStathmin(S38) tumors, phosphoinositide 3-kinase (PI3K)/mTOR/HSP90 were suggested as possible targets in pStathmin(S38)-high cases. High pStathmin(S38) was associated with several PI3K pathway alterations: amplification of the 3q26 region, increased PIK3CA copy number (FISH) and a PI3K activation score (all P < 0.05).

Conclusions: High pStathmin(S38) is a novel biomarker of increased tumor cell proliferation and impaired prognosis as reported here for independent cohorts of endometrial cancer and not previously shown in human cancer. Our data support a rationale for further studies exploring effects of drugs inhibiting the PI3K signaling pathway in pStathmin(S38)-high endometrial cancer, including a potential value of pStathmin(S38) in predicting response to PI3K/mTOR/HSP90 inhibitors.

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Introduction

Stathmin is a cytosolic phospho-protein known to be overexpressed in several malignancies (1). It is suggested to be a marker of PTEN loss (2) and to play a role in tumor progression (1, 3). Furthermore, it is considered important in signal transduction and involved in biologic processes such as cell-cycle progression, apoptosis, and cell migration (1). Microtubular destabilization by Stathmin is suggested to happen through promotion of microtubule catastrophe or by preventing tubulin incorporation in growing microtubules (4). Stathmin protein function is regulated at a posttranslational level by different mechanisms, of which phosphorylation is the most studied (1). Thus, Stathmin has 4 Serine phospho-sites (Ser16, -25, -38, and -63), and phosphorylation is shown to inactivate Stathmin's
Translational Relevance

The oncoprotein Stathmin has recently been shown to be a strong prognostic marker in endometrial cancer. In vitro studies have shown that phosphorylation of Stathmin inactivates the protein function. Both unphosphorylated and phosphorylated Stathmin are considered to be of importance for progress through mitosis and potentially for tumor proliferation. Here, we show how high levels of phospho-Stathmin (Serine38) are associated with aggressive endometrial cancer and reduced survival, also in multivariate survival analyses, in two independent patient cohorts (in total 804 patients). Through integrated molecular profiling, a link between pStathmin(S38) level and tumor cell proliferation is shown. PI3K/mTOR/ HSP90 are indicated as potential targets for therapy in pStathmin(S38) high cases. This suggests the need for further study of pStathmin(S38)'s potential to predict therapy response in clinical trials of PI3K/mTOR/HSP90 inhibitors in endometrial carcinoma.

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Phosphorylation of Stathmin (Serine38) is critical for proper formation of microtubules (5–7). Expression of Stathmin and the regulation of protein activity by phosphorylation are important for cell division; inactivation of Stathmin by phosphorylation is critical for proper formation of microtubules and the mitotic spindle, and thereby, for entry into mitosis (7), whereas Stathmin’s destabilizing effects on microtubules are important to disassemble the mitotic spindle as the cells move through late stages of mitosis (8). During M-phase progression, cyclin-dependent kinases (CDK) 1/2 phosphorylate Ser25 and -38 and precede phosphorylation of Ser16 and -63 by other kinases, allowing the mitotic spindle to be properly organized (1, 7). Ser38 is suggested to be phosphorylated as well by kinases belonging to the mitogen-activated protein kinase (MAPK) family as well as the phosphoinositide 3-kinase (PI3K) pathway (1, 9).

Stathmin protein expression has recently been reported to be a prognostic marker in endometrial cancer (10) as well as in breast and urothelial carcinomas (11, 12), and it has also been suggested as a predictive marker for response to taxane treatment in cancer (13–15). Here, we hypothesized that immunohistochemically determined cellular levels of Stathmin phosphorylated at Serine38 [pStathmin(S38)] relates to tumor phenotype and survival in endometrial carcinoma. We also wanted to explore possible associations between high pStathmin(S38) and potential targets for therapy in pStathmin(S38)-high cases. By analyzing 2 independent patient series, we identify and validate for the first time that pStathmin(S38) adds independent prognostic information for patients with cancer, as shown here for endometrial carcinomas. A link between strong pStathmin(S38) tissue staining and high tumor cell proliferation as well as the PI3K pathway is supported by several measures, and transcriptional signatures associated with high pStathmin(S38) suggest drugs targeting PI3K/mTOR signaling and HSP90 as particularly relevant to test in clinical trials of endometrial carcinomas.

Materials and Methods

Patients and tumor samples

Formalin-fixed and paraffin-embedded (FFPE) as well as fresh frozen endometrial carcinoma tumor specimens were retrieved from the Bergen Gynecologic Cancer Biobank, and related to clinical and histopathologic data in 2 independent series: (i) the primary investigation set of fresh frozen and FFPE tumor tissue in parallel (n = 122/518, respectively), prospectively collected from May 2001 to December 2010; (ii) the retrospectively collected population-based validation series consisting of FFPE tumor tissue from 286 patients diagnosed from 1980 to 1990 (16). All patients were treated at the Section for Gynecological Cancer, Haukeland University Hospital (Bergen, Norway), a referral hospital for patients in the Western Health Region of Norway including Hordaland County, Bergen, Norway, as previously reported (17). Before extracting DNA and RNA from tumors in the primary investigation series, hematoxylin-stained frozen sections were evaluated to ensure high tumor purity in the available tissue (more than 80% tumor purity for the majority of cases). DNA and RNA were extracted from the sections immediately adjacent to the section investigated by frozen sections.

Primary investigation series (n = 518) The patients were prospectively enrolled from 2001 to 2010. Clinicopathologic data including age at diagnosis, International Federation of Gynecology and Obstetrics (FIGO) stage according to the 2009 criteria, histologic type, and histologic grade were obtained from the clinical records and routine histopathology reports. The nonendometrioid tumors include clear cell, serous, and undifferentiated carcinomas in both patient series. In the prospective investigation series, carcinosarcomas were also included. Ninety-four tumors were classified as nonendometrioid. Of these, 18 (19%) were clear cell carcinomas, 45 (48%) were serous carcinomas, 10 (11%) were undifferentiated carcinomas, and 21 were (22%) carcinosarcomas.

The median follow-up for survivors was 3.9 years (range, 0.1–8). Patients were followed from the date of primary surgery until June 15, 2011, or until death. The surgical treatment protocol was abdominal hysterectomy with bilateral salpingo-oophorectomy as primary treatment. The primary surgery also included pelvic lymphadenectomy as a staging procedure for the majority of patients (78%). Adjuvant therapy was recommended for patients with FIGO stage ≥ II and high-risk FIGO stage I patients, defined as nonendometrioid tumors or deeply infiltrating endometrioid grade 3 tumors. Adjuvant radio- and chemotherapy were given to 54 (11%) and 57 (12%) patients, respectively.

Validation series (n = 286) The patients were diagnosed with primary endometrial cancer in the period from 1981 to 1990. Clinicopathologic data, retrospectively obtained, included age at diagnosis, FIGO stage according to the 1988 criteria, and histologic type and grade based on
the results after histopathologic revision (I.M. Stefansson and L.A. Akslen; ref. 18). In the validation series, 31 tumors were classified as nonendometrioid, 15 (50%) as clear cell carcinomas, and 10 (40%) as serous carcinomas. The median follow-up period for the survivors was 18.5 years (range, 13.2–23.2). The last date of follow-up was June 30, 2004. The treatment protocol for this period was abdominal hysterectomy with bilateral salpingo-oophorectomy as the primary treatment. The pelvic and paraaortic lymph nodes were palpated and biopsied only if considered suspect, as previously reported (19). Radiotherapy and hormonal therapy were given as adjuvant therapy to 192 (71%) and 25 (9%) of the patients, respectively.

Tumor specimens were investigated for levels of pStathmin(S38) and Stathmin by immunohistochemistry (IHC; refs. 10, 20). mRNA expression was assessed by DNA oligonucleotide microarray for a subset of 122 freshly frozen tumor specimens from the primary investigation series.

Ethics statement. All parts of the study have been approved according to Norwegian legislation as well as international demands for ethical review. The study was approved by the Norwegian Data Inspectorate, Norwegian Social Sciences Data Services, and the Western Regional Committee for Medical and Health Research Ethics, REC West (NSD15501; REK 052.01). Patients were included in the study after written informed consent approved by the ethics committee (REC West).

Tissue microarray

Hematoxylin and eosin (H&E)–stained slides from tumors were evaluated to identify areas with high tumor purity for retrieval of 3 0.6-mm tissue cylinders to be mounted in a recipient paraffin block using a custom-made precision instrument (Beecher Instruments), as previously described (21, 22). Tissue microarray (TMA) sections of 5 μm were subsequently dewaxed with xylene/ethanol for immunohistochemical staining.

Immunohistochemistry

Details on the Stathmin staining have been previously reported (10). For pStathmin(S38), staining procedures were conducted using the Leica Microsystems Bond III Autostainer automated slide processing equipment. Heat-induced epitope retrieval was applied for 10 minutes in Bond Epitope Retrieval Solution 1 (citrate buffer and surfactant, pH 5.6–6.1; Leica Biosystems). Sections were blocked for peroxidase activity (Bond Refine Block; Leica Biosystems) and incubated for 15 minutes at room temperature with a rabbit monoclonal phospho-Stathmin antibody (clone D19H10; Cell Signaling Technologies, catalog #4191) diluted 1:200 in Bond Primary Diluent. The Bond Polymer Refine detection (Leica Biosystems) was added for 10 minutes at room temperature for antibody detection. Finally, slides were briefly counterstained with Leica SurgiPath SelecTech Hematoxylin (Leica Biosystems) for 5 minutes. Samples of normal tonsils known to yield positive staining for pStathmin(S38) were used as positive controls and by substituting the primary antibody with diluent only, as negative control.

Previously published PTEN IHC data for 2 separate antibodies (retrospective validation series; ref. 23) were included for assessment of the association between PTEN protein expression and pStathmin(S38) levels.

Proliferation markers. Assessments of Ki67, mitotic count, and S-phase fraction in the validation series have previously been described (18, 24). Briefly, Ki67 immunohistochemical staining was assessed in 5-μm full sections. After microwave epitope retrieval, the sections were incubated with a Ki67 polyclonal antibody (code no. A-047; Dako Cytomation Nordic Oslo). The percentage of positively stained nuclei was calculated from the area with most intense staining ("hot spot") by counting approximately 1,000 tumor cells at ×1,000 magnification.

The number of mitoses (e.g., "mitotic count") was counted in "hot spot" areas of highest histologic grade and highest mitotic activity, and counted in 10 high-power fields (×400).

Adjusted S-phase fraction, defined as the area between G1 and G2–M peaks, was calculated from DNA flowcytometric analyses from fresh, ethanol-fixed tissue and estimated according to the method described by Baisch and colleagues (25).

Evaluation of staining

The slides were evaluated by 2 of the authors who were blinded for patient characteristics and outcome (E. Wik and H.B. Salvesen) using a standard light microscope. A semiquantitative grading system incorporating staining intensity (score, 0–3) and area of tumor with positive staining (0, no staining; 1, <10%; 2, 10%–50%; and 3, >50% of tumor cells) was applied. Staining index (SI) was calculated as the product of staining intensity and area ranging from 0 to 9, as described in several publications (26, 27). If heterogeneity was seen for the 3 cylinders of each case, the 3 cylinders were given one overall averaged score in a manner similar to the approach applied for investigations of full sections for comparison (For cutoff defining methods, see "Statistical analyses"). A cutoff representing the upper quartile (SI > 4) was used to define high level of pStathmin(S38). For Stathmin, the upper quartile (SI = 9) defined high immunohistochemical expression, as previously reported (10).

FISH

PIK3CA copy number alterations were assessed by FISH for 66 cases: The area of highest tumor grade was identified on H&E-stained slides. TMAs were prepared as reported above, and TMA sections were treated at 56°C overnight before deparaffinization. Paraffin pretreatment of TMA sections was conducted according to the Reagent Kit protocol (Vysis) before hybridization. Dual color FISH was conducted by using a digoxigenated BAC probe (BAC RP11-245C23, German Science Centre for Genome Research, DE) harboring the PIK3CA gene and a commercially available Spectrum-Orange–labeled chromosome 3 centromeric
probe (CEP3, D3Z1; Abbott) as a reference. Hybridization and posthybridization washes were done according to the LSI procedure (Abbott). Visualization of the gene probe was carried out by using fluorescent isothiocyanate (FITC)-conjugated sheep anti-digoxigenin (Roche Diagnostics) as described previously (28). Slides were counterstained with 125 ng/ml 4',6-diamino-2-phenylindole in an anti-fade solution. Copy numbers of gene-specific and centromere signals were estimated for each tissue spot as previously described (29–31). A tumor was considered to have increased PIK3CA copy number if individual tumor cells on average had 3 or more gene signals, regardless of the gene/CEP signal copy number ratio.

SNP array analysis and DNA sequencing

In the primary investigation series, genomic DNA was extracted from surgically dissected, fresh frozen primary tumors. Single-nucleotide polymorphism (SNP) arrays interrogating 116,204 SNP loci were evaluated for 70 cases and Sanger sequencing of PIK3CA exon 9 and 20 for 245 cases, as previously described (32, 33). Previously published PTEN sequencing data from the retrospective validation series (34) were included to assess whether PTEN mutations were associated with pStathmin(S38) levels.

Oligonucleotide DNA microarray analyses

Extracted RNA was hybridized to Agilent Whole Human Genome Microarrays 44 k (cat no. G4112F) according to the manufacturer's instructions (www.agilent.com). Arrays were scanned using the Agilent Microarray Scanner Bundle. Microarray signal intensities were determined using J-Express (www.molmine.no). Median spot signal data were used as intensity measure. The expression data were quantile normalized. False-discovery rate (FDR) <0.1 was used as cutoff when identifying genes and pathways significantly differentially expressed between tumors with high versus low pStathmin(S38), using, respectively, significance analysis of microarrays (SAM; ref. 35) for single gene detection and gene set enrichment analysis (GSEA; ref. 36), based on gene sets available through MSigDB (www.broadinstitute.org/gsea/msigdb).

PI3K activation score. A PI3K activation score was calculated in the DNA microarray data based on a published PI3K signature (cell lines stably transfected with activated PIK3CA; ref. 37), subtracting the sum of the expression values of genes downregulated from genes upregulated in the transfected cell lines. Expression values of each gene were normalized by a common mean and scaled to the same SD.

Connectivity Map

The correlation between the global expression pattern and potential new therapeutics for patients with high tumor pStathmin(S38) was assessed in the primary investigation cohort. Associations between the pStathmin(S38) transcription signature and drug signatures in the Connectivity Map database (38) were explored. Genes differentially expressed (FDR < 0.1) between tumor subsets of low and high pStathmin(S38) levels were included in the signature as the basis for the analyses in the Connectivity Map.

Statistical analyses

Data were analyzed using SPSS (Statistical Package of Social Sciences), version 20.0 (SPSS, Inc.). A P value of less than 0.05 was considered statistically significant, except for the DNA microarray analyses. Mann–Whitney U test and the Spearman rank correlation were used for analyses of continuous variables between categories. Univariate survival analyses of time to death due to endometrial carcinoma (disease-specific survival) and time to recurrence for patients without metastases at time of diagnosis (recurrence-free survival) were conducted using the Kaplan–Meier method. Entry date was the date of primary surgery. Patients who died from other causes were censored at the date of death. Differences in survival between groups were estimated by 2 sided log-rank (Mantel–Cox) tests. Categories were compared using Pearson x² or Fisher exact test when appropriate. The Cox proportional hazards method was used for multivariate survival analyses. The variables were visually examined by a log-minus-log plot to check the assumptions about proportionality before incorporation into Cox multivariate proportional hazards regression models. Categorizing continuous variables without established cutoff values, cutoff points were based on quartile limits, with consideration also given to the frequency distribution plot for each marker. Quartile groups with similar survival in Kaplan–Meier (disease-specific) survival analyses were merged when dichotomizing the variables. Estimation of sample size was done by x² test using software East4, 2005 Cytel Software Corp. To reach 90% power detecting a 30% difference in 5-year survival (90% for patients with markers within normal range vs. 60% with pathologic markers) at a 5% level of significance, at least 65 patients were needed, assuming a ratio of 1:3 for positive versus negative markers.

Results

pStathmin(S38) expression associates with clinicopathologic phenotype and patient survival

pStathmin(S38) immunohistochemical staining was mainly cytoplasmic (Fig. 1A and B). High level of pStathmin(S38) was significantly associated with features of aggressive tumors, such as nonendometrioid histology, high histologic grade, and high FIGO stage, as well as with recurrent disease (Table 1). Also, high pStathmin(S38) predicted lymph node metastasis (OR = 3.3; P < 0.001). In the primary investigation series, a trend toward high pStathmin(S38) in serous and undifferentiated carcinomas and carcinosarcomas compared with clear cell carcinomas (P = 0.1) was seen. However, this finding was not present in the smaller validation series. High pStathmin(S38) was also associated with shorter disease-specific survival in both patient series studied (Fig. 1C and D). In the subsets of presumed low-risk endometrioid cases, grade 1/2...
endometrioid tumors, FIGO stages I/II grade 1/2 endometrioid tumors, and high pStathmin(S38) were still associated with significantly worse outcomes compared with low pStathmin(S38), although with borderline statistical significance ($P = 0.1$) for disease-specific survival for the subset of FIGO I/II, endometrioid grade 1/2, with only 8 events (Supplementary Fig. S1A–S1F). Among the FIGO I/II endometrioid, histologic grade 1 or 2 cases, 26 of 309 patients

### Table 1. Correlation between pStathmin(S38), clinicopathologic phenotype, and Stathmin expression in endometrial carcinomas

<table>
<thead>
<tr>
<th>Variables</th>
<th>Categories</th>
<th>Low n (%)</th>
<th>High n (%)</th>
<th>$P^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient age</td>
<td>$&lt;$65</td>
<td>187 (74)</td>
<td>65 (26)</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>$\geq 65$</td>
<td>182 (68)</td>
<td>84 (32)</td>
<td></td>
</tr>
<tr>
<td>Histologic subtype</td>
<td>Endometrioid</td>
<td>316 (75)</td>
<td>104 (25)</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td></td>
<td>Nonendometrioid</td>
<td>53 (54)</td>
<td>45 (46)</td>
<td></td>
</tr>
<tr>
<td>Histologic grade</td>
<td>Grade 1/2</td>
<td>272 (78)</td>
<td>77 (22)</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td></td>
<td>Grade 3</td>
<td>94 (57)</td>
<td>71 (43)</td>
<td></td>
</tr>
<tr>
<td>FIGO stage (2009)</td>
<td>I/II</td>
<td>322 (75)</td>
<td>110 (25)</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td></td>
<td>III/IV</td>
<td>47 (56)</td>
<td>39 (44)</td>
<td></td>
</tr>
<tr>
<td>Recurrence$^c$</td>
<td>No</td>
<td>305 (75)</td>
<td>101 (25)</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>46 (58)</td>
<td>33 (42)</td>
<td></td>
</tr>
<tr>
<td>Stathmin$^d$</td>
<td>Low</td>
<td>304 (81)</td>
<td>71 (19)</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>26 (33)</td>
<td>53 (67)</td>
<td></td>
</tr>
</tbody>
</table>

Missing cases: grade ($n = 4$), Stathmin ($n = 64$).

$^a$High pStathmin(S38) defined by score index $>4$.

$^b$-$\chi$-square test.

$^c$Including only patients considered tumor free after primary surgery ($n = 485$).

$^d$High Stathmin defined by score index 9.
had recurrences. Fourteen of these were vaginal recurrences, with no survival impact if cured but with a likely impact on quality of life.

In multivariate survival analyses, high pStathmin(S38) independently predicted poor prognosis adjusted for histologic subtype, histologic grade, and myometrial infiltration among patients with tumors confined to the uterus (FIGO stage I/II; Table 2). This pattern of prognostic impact of high pStathmin(S38) expression was also seen in the validation series [HR, 2.2; 95% confidence interval (CI), 0.96–4.9; P = 0.07] when adjusted for the same histopathologic variables.

pStathmin(S38) immunohistochemical staining and scoring in TMAs were compared with whole section staining in 52 randomly selected cases (primary investigation series). Kappa value for reproducibility was 0.77, regarded as good strength of agreement, supporting that assessment in TMA sections is valid, also in line with previous studies for other tissue markers (18).

**pStathmin(S38) expression adds prognostic information to Stathmin**

pStathmin(S38) was significantly correlated to Stathmin expression (Table 1). As both pStathmin(S38) and Stathmin were shown to be independent prognostic markers in separate models, we further examined how pStathmin(S38) fared as a prognosticator compared with Stathmin. In a multivariate survival analysis of the primary investigation series, including both Stathmin and pStathmin(S38) expression and adjusting for histologic subtype, histologic grade, and myometrial infiltration, pStathmin(S38) maintained independent prognostic impact (HR, 1.8; 95% CI, 1.0–3.1; P = 0.05), whereas Stathmin did not (Supplementary Table S1). There was no significant interaction between Stathmin and pStathmin(S38) in this survival model (P = 0.2). Furthermore, in the validation series, pStathmin(S38) was superior to Stathmin, adjusting for the same histopathologic variables (Supplementary Table S2). When age was included in the model along with FIGO stage instead of myometrial infiltration, pStathmin(S38) was still significant in the validation series (HR, 1.8; P = 0.04) in contrast to Stathmin (P = n.s), whereas neither pStathmin(S38) nor Stathmin were significant in the primary investigation series, possibly reflecting more extensive and systematic lymph node sampling and staging in this cohort. pStathmin(S38) lost its independent association with survival when adjusting for adjuvant therapy (P = 0.15; both patient cohorts).

**Integrated analyses associate high pStathmin(S38) expression to tumor cell proliferation**

Unsupervised clustering of gene expression data defined 3 clusters of which 2 were enriched for cases with an aggressive phenotype. High pStathmin(S38) was more frequent (P = 0.03) in these 2 clusters also showing worse survival compared with the third cluster (Supplementary Fig. S2). Still, many of the cases segregating into the worse outcome clusters did not show a high pStathmin(S38) level, indicating that transcriptional alterations segregating endometrial cancer into phenotypic subtypes represent more complex alterations and no complete overlap with pStathmin(S38) level.

Transcriptional differences between tumors with high versus low levels of pStathmin(S38) were further explored by pathway analyses (GSEA) of DNA microarray data (primary investigation series). Gene sets comprising genes involved in cell-cycle progression and cell proliferation were highly enriched in tumors with high pStathmin

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**Table 2. Cox proportional hazard regression model used to estimate the prognostic value of pStathmin (S38) in endometrial carcinomas confined to the uterus in relation to histopathologic variables**

<table>
<thead>
<tr>
<th>Variable</th>
<th>N (%)</th>
<th>Unadjusted</th>
<th>Adjusted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HR*</td>
<td>95% CI</td>
</tr>
<tr>
<td>Histologic subtype</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endometrioid</td>
<td>355 (85)</td>
<td>4.1–20.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Nonendometrioid</td>
<td>61 (15)</td>
<td>9.3</td>
<td></td>
</tr>
<tr>
<td>Histologic grade</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade 1/2</td>
<td>308 (74)</td>
<td>2.9–16.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Grade 3</td>
<td>108 (26)</td>
<td>6.9</td>
<td></td>
</tr>
<tr>
<td>Myometrial infiltration</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;50%</td>
<td>300 (72)</td>
<td>1.7–8.9</td>
<td>0.001</td>
</tr>
<tr>
<td>≥50%</td>
<td>116 (28)</td>
<td>3.9</td>
<td></td>
</tr>
<tr>
<td>pStathmin(S38)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>310 (75)</td>
<td>1.4–7.1</td>
<td>0.005</td>
</tr>
<tr>
<td>High</td>
<td>106 (25)</td>
<td>3.2</td>
<td></td>
</tr>
</tbody>
</table>

N, number of cases.

*Unadjusted HRs given for analyses of 399 cases with data available for all variables in the multivariate analysis.

Likelihood ratio test.
Correlation between levels of pStathmin(S38) and Stathmin in endometrial carcinomas. To further examine this hypothesis, we assessed the correlation between pStathmin(S38) and a panel of measures for cell proliferation, such as mitotic count, percentage of Ki67-positive tumor nuclei, and S-phase fraction by flow cytometry (validation series). Consistently, high pStathmin(S38) was significantly correlated with high proliferation assessed by all these methods (Fig. 2A–C). Also, high Stathmin protein expression was correlated with similar strength to high mitotic count and proportion of Ki67-positive tumor cells but not to S-phase fraction (Fig. 2D–F).

In sum, our data support that pStathmin(S38) is related to tumor cell proliferation and adds important and clinically relevant prognostic information in patients with endometrial cancer, also among presumed low-risk cases.

**Discussion**

Stathmin is shown to be a prognostic marker in various cancer types, such as breast and endometrial cancer (2, 32), and has recently been reported to predict lymph node metastases in a large multicenter study of endometrial cancer (10). In contrast, the prognostic impact and possible clinical use of phosphorylated Stathmin has not been much studied in human cancers (1, 3, 39), although the impact of phosphorylation at different Stathmin phospho-sites has been explored in some experimental models, mainly in relation to the effects on microtubule formation, proliferation, cell migration, and cancer invasion (3, 39, 40). In this study of endometrial cancer, pStathmin(S38) was strongly associated with different markers of tumor proliferation and showed a significant and independent association with patient survival above the information given by standard clinicopathologic features and by Stathmin expression. A prognostic impact of pStathmin(S38) has, to our knowledge, not been previously shown for any cancer type. Our findings have been validated in an independent patient cohort and indicate that pStathmin(S38) might be of practical use in the management of patients with endometrial carcinoma and also in regard to identifying patients with higher risk for recurrent disease among presumed low-risk cases.

FIGO stage reflects the results from lymph node investigation and is thus adjusted for in the Cox analysis taking FIGO stage into account. pStathmin(S38) maintains independent prognostic value when including tumors...
confined to the uterus (FIGO stage I/II) in the cohort collected between 1980 and 1990 with no patients routinely subjected to staging lymphadenectomy. pStathmin (S38) as Stathmin and histologic grade all lost their independent prognostic impact when analyzed in a Cox model where 78% of the patients had been subjected to staging lymphadenectomy. Still, pStathmin(S38) level showed independent prognostic impact in all other Cox models explored. Importantly, our study was not designed to assess the value of lymphadenectomy, but future studies should explore if pStathmin(S38) may be useful in a context of molecular staging instead of staging lymphadenectomy also associated with side effects.

<table>
<thead>
<tr>
<th>Rank</th>
<th>Name of compound</th>
<th>Known target/action</th>
<th>N</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>Tanespimycin</td>
<td>HSP90 inhibitor</td>
<td>62</td>
<td>&lt;0.00001</td>
</tr>
<tr>
<td>2</td>
<td>Sirolimus</td>
<td>mTOR inhibitor</td>
<td>44</td>
<td>&lt;0.00001</td>
</tr>
<tr>
<td>3</td>
<td>LY-294002</td>
<td>PI3K inhibitor</td>
<td>61</td>
<td>&lt;0.00001</td>
</tr>
<tr>
<td>4</td>
<td>Quinostatin</td>
<td>PI3K inhibitor</td>
<td>2</td>
<td>0.0001</td>
</tr>
<tr>
<td>5</td>
<td>Thioridazine</td>
<td>Adrenerg and dopamine blocker</td>
<td>20</td>
<td>0.0001</td>
</tr>
<tr>
<td>6</td>
<td>Geldanamycin</td>
<td>HSP90 inhibitor</td>
<td>15</td>
<td>0.0007</td>
</tr>
<tr>
<td>7</td>
<td>Luteolin</td>
<td>Flavonoid; antiproliferative properties</td>
<td>4</td>
<td>0.0007</td>
</tr>
<tr>
<td>8</td>
<td>Apigenin</td>
<td>Flavonoid; antiproliferative properties</td>
<td>4</td>
<td>0.001</td>
</tr>
<tr>
<td>9</td>
<td>Vorinostat</td>
<td>HDAC inhibitor</td>
<td>12</td>
<td>0.001</td>
</tr>
<tr>
<td>10</td>
<td>Camptothecin</td>
<td>Topoisomerase I inhibitor</td>
<td>3</td>
<td>0.003</td>
</tr>
</tbody>
</table>

N, number of instances in which the compounds were tested in the Connectivity Map. The expression changes from the compounds tested were scored according to the pStathmin(S38) level signature. The P value for each compound represents the distribution of this score in the N instances, compared with the distribution of these scores among all compounds tested, using a permutation test [Lamb and colleagues (38)].

| Table 3. Top-ranked potential drugs and targets for therapy among patients with endometrial cancer with high pStathmin(S38), based on Connectivity Map |
|---|---|---|
| Rank | Name of compound | Known target/action |
| 1 | Tanespimycin | HSP90 inhibitor |
| 2 | Sirolimus | mTOR inhibitor |
| 3 | LY-294002 | PI3K inhibitor |
| 4 | Quinostatin | PI3K inhibitor |
| 5 | Thioridazine | Adrenerg and dopamine blocker |
| 6 | Geldanamycin | HSP90 inhibitor |
| 7 | Luteolin | Flavonoid; antiproliferative properties |
| 8 | Apigenin | Flavonoid; antiproliferative properties |
| 9 | Vorinostat | HDAC inhibitor |
| 10 | Camptothecin | Topoisomerase I inhibitor |

| Table 4. Correlations between pStathmin(S38) and PI3K alterations in the primary investigation series |
|---|---|---|---|
| pStathmin(S38) | Low n (%) | High n (%) | P<sup>a</sup> |
| 3q26 region (SNP array) | 49 (83) | 10 (17) | 0.047 |
| Amplified | 6 (55) | 5 (45) | 0.3 |
| PIK3CA copy number (FISH)<sup>b</sup> | 48 (86) | 8 (14) | 0.001 |
| ≥ 2 | 3 (30) | 7 (70) | 0.001 |
| PI3K activation score<sup>c</sup> | -4.1 | 10.1 | 0.02 |
| Mean score | 22 (65) | 12 (35) | n.s. |
| PIK3CA mutation<sup>d</sup> | 157 (74) | 54 (26) | 0.1 |
| No | 22 (65) | 12 (35) | n.s. |
| Yes | 73 (74) | 25 (26) | 0.2 |
| PTEN IHC<sup>e,f</sup> | 14 (61) | 9 (39) | 0.02 |
| High | 40 (75) | 13 (25) | 0.9 |
| Low | 152 (75) | 51 (25) | n.s. |
| PTEN mutations<sup>g</sup> | 40 (75) | 13 (25) | 0.9 |
| Yes | 152 (75) | 51 (25) | n.s. |

<sup>a</sup>χ<sup>2</sup>-square test except for PI3K activation score, where the Mann–Whitney U test was applied.

<sup>b</sup>Absolute copy number.

<sup>c</sup>Generated in mRNA microarray data from 122 primary tumors.

<sup>d</sup>Exon 9 and 20 mutations.

<sup>e</sup>Data from the retrospective validation series.

<sup>f</sup>The antibody 6H2.1 was tested; there was no significant association between pStathmin(S38) and a second antibody tested (A2B1; P = 0.3).

<sup>g</sup>Any PTEN mutations vs. wild-type samples.
Both pStathmin(S38) and Stathmin immunohistochemical expression were strongly associated with the different proliferation markers investigated, possibly reflecting that both phosphorylated and unphosphorylated Stathmin might contribute to mitotic progression by microtubular stabilization and destabilization during the various phases of cell division (1). In line with this, we found gene sets related to cell-cycle progression and proliferation to be particularly enriched among pStathmin(S38)-high tumors. A link between pStathmin(S38) and tumor cell proliferation has not previously been reported in human cancer, although one study indicated that strong Stathmin expression was associated with higher Ki67 levels in regenerating liver tissue (41).

Moreover, transcriptional profiling indicated a relationship between pStathmin(S38) levels and inhibitors of PI3K and mTOR signaling, pointing to possible treatment effects of such drugs in pStathmin(S38)-high cases in particular. Thus, although only indirect measures for an association between pStathmin(S38) and drug response are shown, our findings strongly advocate the inclusion of pStathmin(S38) as a biomarker in relevant clinical trials of advanced endometrial cancers examining the potential predictive value of this marker.

Stathmin has previously been associated with several potential measures for PI3K activation, such as PTEN loss (2), high levels of a transcriptional PI3K signature, and amplification of the 3q26 region, harboring PIK3CA (20, 32), although not with PIK3CA mutations (32). Here, we found a similar pattern for pStathmin(S38), and in addition an association between high pStathmin(S38) and increased absolute PIK3CA copy number by FISH. Various molecular alterations may potentially contribute to PI3K signaling activation (42), including PIK3CA amplifications. In line with this, we find higher PIK3CA copy number in pStathmin(S38)-high cases, potentially contributing to PI3K signaling activation in these tumors. Mutations in PIK3CA exons 1 to 20 and PIK3R1 are described for endometrial carcinomas (43–45). Several of the mutations in exon 9 and 20 have previously been associated with aggressive histopathologic features and suggested to be PI3K signaling activating mutations (44). Other PIK3CA and PIK3R1 mutations not included in the present study are also suggested to activate the PI3K signaling pathway, and further testing for any potential link to pStathmin(S38) level is needed. This is also the case for other PI3K-related molecular alterations not assessed in this study.

Any potential mechanistic link between Stathmin expression and PI3K signaling is poorly understood. However, one study suggests a link between phosphorylation of Stathmin and the PI3K pathway (9), supported by functional studies showing phosphorylation of Stathmin by PAK1 downstream of Rac1 (46). On the basis of drug signatures (38), we found several compounds relevant for targeting the PI3K pathway related to high pStathmin(S38) expression. The top-ranked HSP90 inhibitors are shown to be crucial for functional folding of various proteins in multiple pathways, including AKT in the PI3K pathway (47–50). Also, HSP90 and PI3K inhibitors in combination are more effective than single drugs in cell line studies of various cancer types (51, 52), and a clinical trial in advanced gastric cancer with combined HSP90/PI3K inhibition has been initiated (www.clinicaltrials.gov August 2012: NCT01613950). Whether pStathmin(S38) may predict the response to combined HSP90/PI3K inhibition should be further studied in such settings.

Conclusions

Our study supports that pStathmin(S38) is an independent prognostic marker in endometrial carcinomas and significantly associated with tumor cell proliferation. To our knowledge, pStathmin(S38) is a prognostic biomarker not previously reported for human cancers. The present data also suggest a potential for drugs inhibiting the PI3K signaling pathway to pStathmin(S38)-high cases in particular, and we provide a rationale for further studies testing pStathmin(S38) as a predictive marker for response to PI3K/mTOR/HSP90 inhibitors.

Disclosure of Potential Conflicts of Interest

W. Ricketts is employed as CSO and has ownership interest (including patents) in OvaGene Oncology. No potential conflicts of interest were disclosed by the other authors.

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