High Phospho-Stathmin(Serine38) expression identifies aggressive endometrial cancer and suggests an association with PI3Kinase inhibition

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pStathmin(S38) in endometrial carcinoma; survival and targeted therapy

Translational relevance

The oncoprotein Stathmin is recently shown to be a strong prognostic marker in endometrial cancer. In vitro studies have demonstrated 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) is 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 demonstrated. PI3K/mTOR/HSP90 are indicated as potential targets for therapy in pStathmin(S38) high cases. This suggests to further study pStathmin(S38)’s potential to predict therapy response in clinical trials of PI3K/mTOR/HSP90 inhibitors in endometrial carcinoma.

ABSTRACT/words

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Purpose: High Stathmin expression is recently associated with clinical progress of 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, clinico-pathologic phenotype and survival impact in endometrial cancer. A relationship with possible treatment targets were 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 IHC, FISH, flow cytometry, DNA oligonucleotide microarray, SNP array and Sanger sequencing, and related to clinico-pathologic 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. Based on transcriptional differences between high/low pStathmin(S38) tumors, 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, 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.
INTRODUCTION

Stathmin is a cytosolic phospho-protein known to be over-expressed 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). Further, it is considered important in signal transduction and involved in biological 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 post-translational 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 destabilizing effect on 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 de-stabilizing 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 (CDKs) 1/2 phosphorylate Ser25 and Ser38 and precede phosphorylation of Ser16 and Ser63 by other kinases, allowing the mitotic spindle to be properly organized (1, 7). Ser38 is suggested to be phosphorylated also by kinases belonging to the MAPK family as well as the PI3Kinase 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 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 two independent patient series, we identify and validate for the first time, that pStathmin(S38) adds independent prognostic information for cancer patients, as shown here for endometrial carcinomas. A link between strong pStathmin(S38) tissue staining and high tumor cell proliferation as well as the PI3Kinase pathway is supported by several measures, and transcriptional signatures associated with high pStathmin(S38) suggest drugs targeting PI3Kinase/mTOR signaling and HSP90 as particularly relevant to test in clinical trials of endometrial carcinomas.

MATERIAL 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, Norway, and related to clinical and histopathologic data in two 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 1980-1990 (16). All patients were treated at the Section for Gynecological Cancer, Haukeland University Hospital, 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 in the period 2001-2010. Clinico-pathologic 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 non-endometrioid tumors include clear cell, serous and undifferentiated carcinomas in both patient series. In the prospective investigation series, also the carcinosarcomas were included. Ninety-four tumors were classified as non-endometrioid. Of these, 18 (19%) clear cell carcinomas, 45 (48%) serous carcinomas, 10 (11%) undifferentiated carcinomas and 21 (22%) carcinosarcomas.

Median follow-up for survivors was 3.9 years (range 0.1-8). Patients were followed from the date of primary surgery until June 15th 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 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 non-endometrioid tumors or deeply infiltrating endometrioid grade 3 tumors. Adjuvant radiation and chemotherapy were given to 54 (11%), 57 (12%) patients.

**Validation series (n=286)**

The patients were diagnosed with primary endometrial cancer in the period 1981-90. Clinico-pathologic data, retrospectively obtained, included age at diagnosis, FIGO stage according to
pStathmin(S38) in endometrial carcinoma; survival and targeted therapy

the 1988 criteria, and histologic type and histologic grade, based on the results after histopathologic revision (I.M.S and L.A.A) (18). In the validation series, 31 tumors were classified as non-endometrioid; 15 (60%) clear cell carcinomas and 10 (40%) serous carcinomas. Median follow-up period for the survivors was 18.5 years (range 13.2-23.2). Last date of follow up was June 30th 2004. The treatment protocol for this period was abdominal hysterectomy with bilateral salpingo-oophorectomy as primary treatment. The pelvic and para-aortic lymph nodes were palpated and biopsied only if considered suspect, as previously reported (19). Radiation 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) (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 (TMA)

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

**Immunohistochemistry**

Details on the Stathmin staining are previously reported (10). For pStathmin(S38), staining procedures were performed using the Leica Microsystems Bond III Autostainer automated slide processing equipment. Heat Induced Epitope Retrieval was applied for ten minutes in Bond Epitope Retrieval Solution 1 (citrate buffer and surfactant, pH5.6-6.1, Leica Biosystems, IL, USA). Sections were blocked for peroxidase activity (Bond Refine Block, Leica Biosystems) and incubated during fifteen 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 ten minutes at room temperature for antibody detection. Finally, slides were briefly counterstained with Leica SurgiPath SelecTech Hematoxylin (Leica Biosystems) for five minutes. Samples of normal tonsils known to yield positive staining for pStatmin(S38) were used as positive controls and by substituting the primary antibody with diluent only, as negative control.

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

**Proliferation markers**
Assessment of Ki67, mitotic count, and S-phase fraction in the validation series has previously been described (18, 24). Briefly, Ki67 IHC staining was assessed in 5µm full sections. After microwave epitope retrieval, the sections were incubated with a Ki67 polyclonal antibody (code no. A-047, Dako Cytomation Nordic Oslo, Norway). The percentage of staining positively stained nuclei was calculated from the area with most intense staining (“hot spot”), by counting ~1000 tumor cells at x1000 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 (x400).

Adjusted S-phase fraction, defined as the area between G1 and G2/M peaks, was calculated from DNA flow cytometric analyses from fresh, ethanol fixed tissue and estimated according to the method by Baisch et al,(25).

**Evaluation of staining**

Blinded for patient characteristics and outcome, the slides were evaluated by two of the authors (E.W. and H.S.), using a standard light microscope. A semi-quantitative 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 0-9, as described in several publications (26, 27). If heterogeneity was seen for the three cylinders of each case, the three cylinders were given one overall averaged score similar to the approach applied for investigations of full sections for comparison (For cut-off defining methods, see “Statistical analyses” below). A cut-off 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).
Fluorescence-in-situ-hybridization (FISH)

PIK3CA copy number alterations were assessed by fluorescent in situ hybridization (FISH) for 66 cases: The area of highest tumor grade was identified on H&E-stained slides. Tissue microarrays were prepared as reported above and TMA sections were treated at 56°C overnight before deparaffinization. Paraffin Pretreatment of TMA sections was performed according to the Reagent Kit protocol (Vysis) before hybridization. Dual color FISH was performed 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, IL, USA). Visualization of the gene probe was carried out by using fluorescent isothiocyanate (FITC)-conjugated sheep anti-digoxigenin (Roche Diagnostics, Rotkreuz, Switzerland) as described (28). Slides were counterstained with 125 ng/ml 4’,6-diamino-2-phenylindole in an antifade 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 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. SNP arrays interrogating 116 204 SNP loci were evaluated for 70 cases and Sanger sequencing of PIK3CA exon 9 and exon 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 44k (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 cut-off when identifying genes and pathways significantly differentially expressed between tumors with high versus low pStathmin(S38); using, respectively, Significance Analysis of Microarrays (SAM) (35) for single genes detection and Gene set enrichment analysis (GSEA) (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) (37), subtracting the sum of the expression values of genes down-regulated from genes up-regulated in the transfected cell lines. Expression values of each gene were normalized by a common mean and scaled to the same standard deviation.

**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 Connectivity Map.

**Statistical analyses**

Data were analyzed using SPSS (Statistical Package of Social Sciences), version 20.0 (SPSS, Inc., Chicago, IL). Probability of < 0.05 was considered statistically significant, except for the DNA microarray analyses. Mann-Whitney U test and the Spearman’s rank correlation was 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 performed 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 two sided log-rank (Mantel Cox) tests. Categories were compared using Pearson’s chi-square or Fisher’s exact test when appropriate. 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 cut off values, cut points were based on quartile limits, also considering 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 chi-square 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 versus 60% with pathological 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

dStathmin(S38) expression associates with clinico-pathologic phenotype and patient survival

dStathmin(S38) IHC staining was mainly cytoplasmic (Figure 1A-B). High level of dStathmin(S38) was significantly associated with features of aggressive tumors, such as non-endometrioid histology, high histologic grade and high FIGO stage, as well as with recurrent disease (Table 1). Also, high dStathmin(S38) predicted lymph node metastasis (OR=3.3, P<0.001). In the primary investigation series, a trend towards high dStathmin(S38) in serous and undifferentiated carcinomas and carcinosarcomas compared to clear cell carcinomas (P=0.1) was seen. However, this finding was not present in the smaller validation series. High dStathmin(S38) was also associated with shorter disease specific survival in both patient series studied (Figure 1C-D). In the subsets of presumed low risk endometrioid cases: grade 1/2 endometrioid tumors, and FIGO stages I/II grade 1/2 endometrioid tumors, high dStathmin(S38) was still associated with significantly worse outcome compared to 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 Figure 1 A-F). Among the FIGO I/II endometrioid, histologic grade 1 or 2 cases, 26 of 309 patients had recurrences. Fourteen of these were vaginal recurrences, with no
pStathmin(S38) in endometrial carcinoma; survival and targeted therapy

survival impact if cured, but with a likely quality of life impact.

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% CI: 0.96-4.9, p=0.07) when adjusted for the same histopathologic variables.

pStathmin(S38) IHC staining and scoring in TMAs were compared to 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 are 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) performed as a prognosticator compared to 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), while Stathmin did not (Supplementary Table 1). 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 2). When age was included in the model along with FIGO stage instead of myometrial infiltration,
pStathmin(S38) in endometrial carcinoma; survival and targeted therapy

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) loses 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 three clusters of which two were enriched for cases with an aggressive phenotype. High pStathmin(S38) was more frequent (P=0.03) in these two clusters also showing worse survival compared to the third cluster (Supplementary figure 2). Still, many of the cases segregating into the worse outcome clusters did not show 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 furthermore 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(S38) by IHC (Supplementary Table 3). These results, together with known functions of Stathmin and phosphorylation of the protein in relation to mitosis, support that pStathmin(S38) might be important for tumor cell proliferation in endometrial carcinomas. To further examine this hypothesis, we assessed the correlation between pStathmin(S38) and a panel of measures for
pStathmin(S38) in endometrial carcinoma; survival and targeted therapy

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 (Figure 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 (Figure 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 endometrial cancer patients, also among presumed low risk cases.

**PI3K/mTOR are suggested as potential targets in tumors with high pStathmin(S38)**

Connectivity Map version 02 (38) was queried for compounds negatively correlated to high pStathmin(S38) expression in endometrial carcinomas. The gene list acquired from class comparison analysis based on pStathmin(S38) IHC expression status was used. Amongst 1309 small molecules represented in Connectivity Map, inhibitors of PI3K/mTOR and HSP90 were the top ranked therapeutics identified as potential drugs to patients with high tumor pStathmin(S38), as listed in Table 3.

We then explored the correlation between PI3Kinease pathway alterations and pStathmin(S38) expression in the tumors. High pStathmin(S38) associated significantly with amplification of the 3q26 region harboring PIK3CA as estimated by SNP array, increased absolute copy number of PIK3CA, estimated by FISH, and a high PI3K activation score (37), although not with PTEN immunostaining, PTEN mutations or PIK3CA mutations (Table 4), neither with any significant association when investigating the correlation with PIK3CA exon 9 and exon 20 separately (p=0.7 and p=0.2, respectively). Taken together, high pStathmin(S38) expression associates with several potential measures of PI3K signaling activity which further
underlines the potential for drugs inhibiting the PI3K signaling pathway in pStathmin(S38)-high endometrial carcinoma.

**DISCUSSION**

Stathmin is shown to be a prognostic marker in various cancer types, such as breast and endometrial cancer (2, 32), and is recently reported to predict lymph node metastases in a large multicenter study of endometrial cancer (10). In contrast, the prognostic impact and possible clinical utility 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 clinico-pathologic 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 endometrial carcinoma patients, also in regard to identifying patients with higher risk for recurrent disease amongst 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
pStathmin(S38) in endometrial carcinoma; survival and targeted therapy

prognostic impact when analyzed in a Cox model including from FIGO stage 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.

Both pStathmin(S38) and Stathmin IHC 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 is not previously 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 is demonstrated, our findings strongly advocate the inclusion of pStathmin(S38) as a biomarker in relevant clinical trials of advanced endometrial cancers, studying 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 1-20 and PIK3R1 are described for endometrial carcinomas (43-45). Several of the mutations in exon 9 and exon 20 are previously 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 phosphorylation of Stathmin being linked to the PI3K pathway (9), supported by functional studies demonstrating phosphorylation of Stathmin by PAK1 downstream of Rac1 (46). Based on drug signatures (38), we found several compounds relevant for targeting the PI3Kinase 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.
CONCLUSION:

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 predictive marker for response to PI3K/mTOR/HSP90 inhibitors.

FIGURE LEGENDS:

Figure 1. pStathmin(S38) IHC staining; high (A) and low (B) pStathmin(S38) levels. High level of pStathmin(S38) is significantly associated with poor disease specific survival in both the investigation (C) and validation cohorts (D). Survival curves are estimated by the Kaplan-Meier method. For each category the number of cases is given followed by the number of endometrial carcinoma deaths.

Figure 2. Correlation between levels of pStathmin(S38) and Stathmin in relation to markers for proliferative activity in primary tumors: mitotic counts per 10 high power field (x400), proportion of Ki67 positive tumor cells and assessment of S-phase fraction (validation series). $r_s = \text{Spearman’s correlation coefficient.}$

Acknowledgements: We thank Britt Edvardsen, Bendik Nordanger, Gerd Lillian Hallseth, Tormund Njølstad and Hua My Hoang for technical assistance.
pStathmin(S38) in endometrial carcinoma; survival and targeted therapy

References:

pStathmin(S38) in endometrial carcinoma; survival and targeted therapy

pStathmin(S38) in endometrial carcinoma; survival and targeted therapy

Low pStathmin(S38), n=145 (29)  
High pStathmin(S38), n=362 (33)  
P<0.001

Low pStathmin(S38), n=188 (39)  
High pStathmin(S38), n=63 (25)  
P=0.002

Figure 1.
Figure 2

**pStathmin(S38)**

- **A**: Mitotic count with box plots showing low and high categories. 
  - $r_s = 0.24$, $P < 0.001$

- **B**: % Ki67 positive nuclei with box plots showing low and high categories. 
  - $r_s = 0.37$, $P < 0.001$

**Stathmin**

- **D**: Mitotic count with box plots showing low and high categories. 
  - $r_s = 0.35$, $P = 0.002$

- **E**: % Ki67 positive nuclei with box plots showing low and high categories. 
  - $r_s = 0.24$, $P = 0.001$

- **F**: % of cells in S-phase with box plots showing low and high categories. 
  - $r_s = -0.004$, $P = 0.98$
Table 1. Correlation between pStathmin(S38), clinico-pathologic phenotype and Stathmin expression in endometrial carcinomas.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Categories</th>
<th>Primary investigation series</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Low n (%)</td>
<td>High n (%)</td>
<td>P</td>
<td></td>
</tr>
<tr>
<td>Patient age</td>
<td>&lt; 65</td>
<td>187 (74)</td>
<td>65 (26)</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td></td>
<td>≥ 65</td>
<td>182 (68)</td>
<td>84 (32)</td>
<td></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>
<td></td>
</tr>
<tr>
<td></td>
<td>Non-endometrioid</td>
<td>53 (54)</td>
<td>45 (46)</td>
<td></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>
<td></td>
</tr>
<tr>
<td></td>
<td>Grade 3</td>
<td>94 (57)</td>
<td>71 (43)</td>
<td></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>
<td></td>
</tr>
<tr>
<td></td>
<td>III / IV</td>
<td>47 (56)</td>
<td>39 (44)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Recurrence</td>
<td>No</td>
<td>305 (75)</td>
<td>101 (25)</td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>46 (58)</td>
<td>33 (42)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stathmin</td>
<td>Low</td>
<td>304 (81)</td>
<td>71 (19)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>26 (33)</td>
<td>53 (67)</td>
<td></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.
Table 2. Cox’s 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 HR&lt;sup&gt;a&lt;/sup&gt;</th>
<th>95% CI</th>
<th>p&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Adjusted HR</th>
<th>95% CI</th>
<th>p&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histologic subtype</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endometrioid</td>
<td>355 (85)</td>
<td>1</td>
<td>4.1-20.8</td>
<td>&lt;0.001</td>
<td>1</td>
<td>1.01-12.1</td>
<td>0.048</td>
</tr>
<tr>
<td>Non-endometrioid</td>
<td>61 (15)</td>
<td>9.3</td>
<td>1.7-8.9</td>
<td>0.001</td>
<td>3.5</td>
<td>1.6-8.2</td>
<td>0.003</td>
</tr>
<tr>
<td>Histologic grade</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade 1/2</td>
<td>308 (74)</td>
<td>1</td>
<td>2.9-16.1</td>
<td>&lt;0.001</td>
<td>1</td>
<td>0.7-8.7</td>
<td>0.2</td>
</tr>
<tr>
<td>Grade 3</td>
<td>108 (26)</td>
<td>6.9</td>
<td>1.7-8.9</td>
<td>0.001</td>
<td>1</td>
<td>1.6-8.2</td>
<td>0.003</td>
</tr>
<tr>
<td>Myometrial infiltration</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 50%</td>
<td>300 (72)</td>
<td>1</td>
<td>1.4-7.1</td>
<td>0.005</td>
<td>1</td>
<td>1.1-5.7</td>
<td>0.035</td>
</tr>
<tr>
<td>≥ 50%</td>
<td>116 (28)</td>
<td>3.9</td>
<td>1.4-7.1</td>
<td>0.005</td>
<td>3.6</td>
<td>1.1-5.7</td>
<td>0.035</td>
</tr>
<tr>
<td>pStathmin(S38)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>310 (75)</td>
<td>1</td>
<td>1.4-7.1</td>
<td>0.005</td>
<td>1</td>
<td>1.1-5.7</td>
<td>0.035</td>
</tr>
<tr>
<td>High</td>
<td>106 (25)</td>
<td>3.2</td>
<td>1.4-7.1</td>
<td>0.005</td>
<td>2.5</td>
<td>1.1-5.7</td>
<td>0.035</td>
</tr>
</tbody>
</table>

N: number of cases; HR: Hazard ratio; CI: confidence interval; FIGO: International Federation of Gynecology and Obstetrics

<sup>a</sup>Unadjusted HRs given for analyses of 399 cases with data available for all variables in the multivariate analysis; <sup>b</sup>Likelihood ratio test
Table 3. Top ranked potential drugs and targets for therapy among endometrial cancer with high pStathmin(S38), based on Connectivity Map.

<table>
<thead>
<tr>
<th>Rank</th>
<th>Name of compound</th>
<th>Known target/action</th>
<th>N</th>
<th>P</th>
</tr>
</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; anti-proliferative properties</td>
<td>4</td>
<td>0.0007</td>
</tr>
<tr>
<td>8</td>
<td>apigenin</td>
<td>Flavonoid; anti-proliferative 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 amongst all compounds tested, using a permutation test (Lamb, Science 2006).
Table 4. Correlations between pStathmin(S38) and PI3K alterations in the primary investigation series.

<table>
<thead>
<tr>
<th></th>
<th>pStathmin(S38)</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low</td>
<td>High</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3q26 region (SNP array)</td>
<td>Unamplified</td>
<td>49 (83)</td>
<td>10 (17)</td>
<td>0.047</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Amplified</td>
<td>6 (55)</td>
<td>5 (45)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PIK3CA copy number (FISH)</td>
<td>= 2</td>
<td>48 (86)</td>
<td>8 (14)</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>≥ 3</td>
<td>3 (30)</td>
<td>7 (70)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PI3K activation score</td>
<td>mean score</td>
<td>-4.1</td>
<td>10.1</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>PIK3CA mutation</td>
<td>No</td>
<td>157 (74)</td>
<td>54 (26)</td>
<td>n.s.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>22 (65)</td>
<td>12 (35)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PTEN IHC</td>
<td>High</td>
<td>73 (74)</td>
<td>25 (26)</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>14 (61)</td>
<td>9 (39)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PTEN mutations</td>
<td>No</td>
<td>40 (75)</td>
<td>13 (25)</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>152 (75)</td>
<td>51 (25)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

^a^Chi square test except for PI3K activation score where the Mann-Whitney U test was applied

^b^Absolute copy number. ^c^Generated in mRNA microarray data from 122 primary tumors.

^d^Exon 9 and exon 20 mutations. ^e^Data from the retrospective validation series. ^f^The antibody 6H2.1 was tested; there was no significant association between pStathmin(S38) and a second antibody tested (A2B1, P=0.3). ^g^Any PTEN mutations versus wild-type samples.
High Phospho-Stathmin(Serine38) expression identifies aggressive endometrial cancer and suggests an association with PI3Kinase inhibition

Elisabeth Wik, Even Birkeland, Jone Trovik, et al.

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