Nestin: a biomarker of aggressive uterine cancers

Erica R. Hope^{1,2}, Paulette Mhaweche-Fauceglia³, Tanja Pejovic⁴, Christopher M. Zahn^{5,6},
Guisong Wang², Thomas P. Conrads^{2,6,7}, G. Larry Maxwell^{2,7,8}, Chad A. Hamilton^{1,2,6,7},
Kathleen M. Darcy^{2,6-7}, Viqar Syed^{6,7}

¹ Walter Reed National Military Medical Center, Department of Obstetrics and
Gynecology, 8901 Wisconsin Avenue, Bethesda, MD 20889

² Women's Health Integrated Research Center at Inova Health System, Department of
Defense Gynecologic Cancer Center of Excellence, 3289 Woodburn Road, Suites 370
and 375, Annandale, VA 22003

³ University of Southern California, Department of Pathology, 1100 N. State Street, Los
Angeles, CA 90033

⁴ Oregon Health & Science University, Department of Obstetrics and Gynecology, 3181
SW Sam Jackson Park Road, L466, Portland, OR, 97239

⁵ American College of Obstetricians and Gynecologists, 409 125th St SW, Washington,
DC 20024

⁶ Uniformed Services University, Department of Obstetrics & Gynecology, 4301 Jones
Bridge Road, Bethesda, MD 20814

⁷ John P. Murtha Cancer Center, 8901 Wisconsin Avenue, Bethesda, MD 20889

⁸ Inova Fairfax Hospital, Department of Obstetrics & Gynecology, 3300 Gallows Road,
Falls Church, VA 22042

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¥ Corresponding Authors:

Viqar Syed, PhD, Uniformed Services University, Department of Obstetrics & Gynecology, 4301 Jones Bridge Road, Bethesda, MD 20814, Phone: 301-295-3128, Fax: 301-295-6774, Email: viqar.syed@usuhs.edu

Kathleen M Darcy, PhD, DoD Gynecologic Cancer Center of Excellence, Women's Health Integrated Research Center at Inova Health System, 3289 Woodburn Road, Suite 370, Annandale VA, 22003, Phone: 571-205-2607, FAX: 703-560-0919, Email: DarcyK@WHIRC.org

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ABSTRACT

Objective. Evidence of potential prognostic and predictive value for nestin was investigated in well-annotated uterine cancers (UCs).

Methods. Nestin expression and previously-published biomarkers were evaluated by immunohistochemistry (IHC) in UC tissue microarrays. Biomarkers were categorized as low vs. high, and nestin was cut at 10% positive staining. Relationship between nestin and clinicopathologic factors, biomarkers and outcome were evaluated using exact/log-rank testing or logistic/Cox modeling.

Results. There were 323 eligible cases, 34% had advanced stage disease, 37% had type II disease, and 5% were carcinosarcomas. High nestin, observed in 19% of cases, was more common in advanced vs. early stage disease, type II cancers or uterine carcinosarcoma vs. type I cancers, grade 3 disease, positive lymphovascular space invasion (LVSI) and tumors >6 cm ($p<0.05$). Nestin was inversely correlated with ER, PR and TFF3, and correlated with p53 and IMP3. Women with high vs. low nestin had worse progression-free survival (PFS) and cancer-specific survival overall, and worse PFS in the subset who received no adjuvant therapy or radiation, or had early stage, type I disease or tumors with both low and high ER, PR, TFF3, PTEN, p53 or IMP3. The relationship between nestin and PFS was independent of stage, LVSI and risk categorization but not type of UC.

Conclusions. High nestin was more common in UCs with aggressive features and poor outcome. Nestin may represent a predictive biomarker for treatment selection for patients previously considered to be lower risk and a candidate for no or radiation-based adjuvant therapy, and compliment ER/PR testing.

INTRODUCTION

Uterine cancer is the fifth leading cause of cancer in women worldwide and the most common gynecologic cancer in the United States, with diagnosis in over 350,000 and 54,000, and deaths in 68,000 and 10,000 women each year in the world and U.S., respectively [1,2]. Since the 1970's, uterine cancer incidence has steadily increased while five-year overall survival has decreased [3]. The obesity epidemic and increasing average lifespans are likely associated with these trends.

Uterine cancers are currently classified by histology. The majority of uterine cancers are considered type I disease, have a good prognosis, and are most closely associated with unopposed endogenous or exogenous estrogen [4]. Type II cancers include grade 3 endometrioid, serous, and clear cell [5], and the remaining cancers of the uterine corpus encompass carcinosarcomas and other sarcomas. These non-low-grade endometrioid cancers are typically more virulent and account for a disproportionate number of uterine cancer deaths [6,7]. The Cancer Genome Atlas (TCGA) Research Network and other groups are defining molecular distinctions and similarities between the different uterine cancer subtypes [8] and these biomarkers have been shown to have predictive [9] and biologic value [9-14].

Clinical management is based on the above classifications, which take into account histology and grade, in conjunction with known risk factors such as surgical stage, lymphovascular space invasion (LVSI), tumor size, and age at diagnosis [12,15], but no effective therapies exist for aggressive disease. Current risk stratification falls short of preventing over-treatment in many patients who would have never recurred and under-treatment in many who may ultimately die of recurrent or progressive disease.

Given the prevalence of low risk uterine cancer patients, identification of biomarkers that aid in risk stratification are needed.

Nestin, a class VI intermediate filament protein first described as a neural stem marker [16], regulates the TGF β pathway [17,18] and plays an important role in cancer cell migration, invasion, and metastasis by interacting with vimentin or desmin and modulating the expression of actin and cell adhesion molecules [17,19-21]. In vitro studies have shown that knockdown of nestin inhibits migration, invasion, and metastasis of cancer cells [17,22]. Nestin also plays a key role in angiogenesis [19]. Though unexplored in uterine cancer, nestin expression has been reported in ganglioglioma, ovarian, head and neck, prostate, bladder, and pancreatic cancers, and is a prognostic indicator of poor survival in many cancers [20,21]. In the current investigation, we examined the potential prognostic and predictive value of nestin expression levels in uterine cancer by studying associations with established prognostic clinical factors and biomarkers as well as measures of clinical outcome, including progression-free (PFS) and cancer-specific survival (CSS).

MATERIALS AND METHODS

Uterine Cancer Patients with Formalin-Fixed and Paraffin-Embedded Tumor

The University of Southern California archives and database were searched for uterine cancer patients treated from 1998 to 2010. All tissue specimens were collected and medical records reviewed under approved protocols from the Institutional Review Board (IRB). Endometrioid, clear cell, serous, or carcinosarcoma patients with de-identified clinical, outcome, and evaluable nestin data were eligible. Treatment decisions were made by physician and patients. For the purposes of this analysis, stage I & II disease were categorized as early stage uterine cancer, and stage III & IV disease were categorized as advanced stage uterine cancer. In addition, grade 1 and 2 (G1/G2) endometrioid carcinomas were categorized as type I uterine cancer. Serous adenocarcinomas, clear cell carcinomas, and grade 3 (G3) endometrioid carcinomas were categorized as type II uterine cancer. Carcinosarcomas including homologous, heterologous or not specified were evaluated collectively as a separate subtype.

Risk categorization represented a modification of the Keys GOG-99 and PORTEC classification [15,23] with myometrial invasion categorized at 50%. The low-risk group included patients with G1 or G2 endometrioid endometrial adenocarcinoma with stage IA disease (<50% myometrial invasion) and negative LVSI. The intermediate-risk patients with stage I or II disease were further subdivided into low- vs. high-intermediate risk groups based on age at diagnosis (<50, 50 to 69, 70+ years olds) and other clinical risk factors including positive LVSI, deep myometrial invasion ($\geq 50\%$), and G2/G3 disease. High-intermediate risk patients diagnosed at 70+ years of age needed one other clinical risk factor, at 50 to 69 years of age needed two other clinical risk

factors and at <50 years of age needed all three other clinical risk factors. Patients not meeting the high-intermediate risk criteria within the intermediate risk group were classified as low-intermediate risk. The high-risk group included patients with stage III/IV disease as well as those with stage I/II disease with a non-endometrioid histologic subtypes such as serous adenocarcinomas (with or without the papillary designation), clear cell carcinomas, and carcinosarcoma. For analysis purposes, patients with low and low-intermediate risk groups were aggregated into a lower risk category, and patients with high-intermediate and high risk were combined into a higher risk category.

Tissue Microarrays

Uterine cancer tissue microarrays (TMAs) were constructed using archival tissue from eligible patients as previously described [24]. Briefly, after carefully selecting the morphologically representative region from the hematoxylin & eosin (H&E)-stained section, 0.6 mm cores were punched from the individual donor formalin-fixed, paraffin-embedded blocks, and incorporated into the TMA paraffin receiver blocks. To account for tumor heterogeneity, core were sampled from three different areas of each tumor. One section from each TMA block was stained with H&E to confirm the presence of the tumor by light microscopy.

Nestin Immunohistochemistry (IHC) Assay and Scoring Criteria

Four μm thick sections prepared on positively charged slides from tumor blocks from individual patients or TMA blocks were deparaffinized and pretreated in citrate buffer pH 6.0 for 20 min. Sections were cooled 20 min and incubated 10 min at ambient temperature in 3% H_2O_2 to quench endogenous peroxidase activity. Blocking was performed using serum-free protein block, (Dakocytomation, Carpinteria, CA) for 30

min. Slides were loaded on a Dakocytomation autostainer and an anti-nestin antibody (1:50, LifeScience, Memphis, TN; monoclonal) was applied for 1 h. Diaminobenzidine tetrahydrochloride was added for development for 10 min, followed by counterstaining with hematoxylin. Negative control slides omitting the primary antibody were included in all assays.

Immunostaining was reviewed using conventional light microscopy and scored by a board-certified gynecologic pathologist (PMF), by intensity and percentage of positive tumor cells. Staining intensity was categorized as 0, 1+ (light brown), 2+ (moderate brown) or 3+ (dark brown), and by percentage of positive tumor cells categorized as 0 for none, 1 for $\leq 10\%$, 2 for 11-25%, 3 for 25-50%, 4 for 51-75% and 5 for $>75\%$. Cores were not evaluated if the core was lost, severely damaged, and/or did not have sufficient tumor cellularity. The reviewer was blinded to clinical data. Uterine cancer cases were excluded from statistical analysis if less than three cores were available for analysis secondary to inadequate cancer tissue or poor quality of the specimen. Tumor staining for nestin was very consistent across individual full tissue sections and across triplicate cores for individual patients. For purposes of primary analysis, nestin expression was categorized as low ($\leq 10\%$ of tumor cells with positive staining) versus (vs.) high ($>10\%$ of tumor cells with positive staining). This cut point was selected based on Western blot expression of nestin in immortalized and malignant endometrial cancer cell lines in vitro showing that low vs. high level expression rather than negative vs. positive expression.

Previously Published IHC Biomarkers of Uterine Cancer

IHC staining data in these uterine cancer TMAs were available for estrogen receptor (ER), progesterone receptor (PR), trefoil factor 3 (TFF3), phosphatase and tensin

homolog (PTEN), tumor protein p53 (p53), and insulin-like growth factor II mRNA-binding protein (IMP3) [9,10]. These biomarkers were initially scored based on overall percent positive tumor cells (0=none, 1=1-5%, 2=6-50%, 3=51-100%) and staining intensity (0 for negative; 1+ for weak; 2+ for moderate; and 3+ for strong/dark brown), and then categorized as low vs. high using the following threshold levels -- ER, PR, IMP3 and TFF3 were categorized as low when the additive percent and intensity scores were ≤ 3 vs. high when the additive score was > 3 . PTEN was categorized as low when percent tumor staining was $\leq 10\%$ positive tumor cells and intensity was ≤ 1 vs. high when $> 10\%$ of tumor cells were positive and intensity was 2+. The tumor suppressor p53 was classified as low when percent tumor staining was $\leq 10\%$ positive tumor cells and intensity was ≤ 2 vs. high when $> 10\%$ of tumor cells were positive and intensity was 3+.

Analysis of Public RNASeq Data for Nestin and Other Biomarkers.

Normalized level 3 RNASequencing (RNASeq)V2 RSEM expression for nestin, ER, PR, TFF3, p53 and IMP3 with relevant clinical annotations were downloaded from the TCGA Data Portal <https://tcga-data.nci.nih.gov/tcga/tcgaDownload.jsp> for patients with uterine corpus endometrial carcinomas.

Statistical Analyses

Statistical analyses were performed using SPSS Version 22 (IBM Corp: Pittsburgh, PA). Relationships between immunohistochemical staining intensity of nestin and clinical characteristics or previously published biomarkers were evaluated with Fisher's exact test and/or logistic regression modeling. Survival analyses for time-to-event data were performed for biomarkers based on IHC staining or RNAseq data using Cox proportional hazard regression modeling with a Wald test, Kaplan-Meier method

with a log-rank test. Assessments of five-year PFS or CSS were evaluated using logistic regression modeling to calculate odds ratios (OR) and 95% confidence intervals (CI) and generating receiver operator curves (ROC) to calculate area under the curve (AUC) and 95% CI. PFS was calculated as the time in months from primary surgery to disease progression or uterine cancer death, or to the date of last contact for women who were alive with no evidence of disease progression or non-cancer death (censored). CSS was calculated as the interval from primary surgery to uterine cancer death or to the date of last contact for women who were alive or died of a cause other than uterine cancer (censored). OS was calculated as the time from primary surgery to death or to the date of last contact for those who were still alive (censored). Censoring of events was performed at 5 years after primary surgery. All reported p-values are two-sided with statistical significance set at $p < 0.05$. Significance levels with $p < 0.007$ passed a conservative Bonferroni correction for multiple testing for 7 biomarkers. Hazard ratio (HR) for Cox regression modeling and OR for logistic regression modeling are presented when 95% confidence intervals (CI) did not overlap 1.0.

RESULTS

The characteristics for the 323 eligible uterine cancer patients are presented in **Table 1** and with additional details in **Supplement S1**. Median age of the patients at the time of primary cancer surgery was 63 years. The distribution of cases by stage was 55.7% with stage I disease, 10.2% stage II, 22.6% stage III, and 11.5% stage IV. Fifty-seven percent of cases had type I disease (G1 or G2 endometrioid endometrial cancer), 37.5% had type II disease (serous or clear cell carcinomas or grade 3 endometrioid endometrial carcinoma), and 5.3% were carcinosarcomas of the uterine corpus. Thirty percent had tumors 6+ cm in size, and 33% exhibited LSVI. Median follow-up was 44 months with 64 (20.1%) cases with disease progression, 56 (17.3%) deaths attributable to uterine cancer, 22 (6.8%) non-cancer deaths, and 71.8% of patients received adjuvant therapy.

Tumor Nestin Expression in Uterine Cancer Patients

Uterine cancer patients were classified based on low [**Figure (Fig.) 1A**] or high (**Fig. 1B**) tumor expression of nestin. High IHC staining for nestin was observed in 19% of cases overall. High nestin expression was associated with advanced stage, type II cancers or carcinosarcomas, non-endometrioid cell type, high grade, large tumor size, presence of LVSI, but not age of diagnosis as either a continuous or categorical variable (**Table 1**). High expression of nestin was also more common in each of these subgroups (**Supplement S2**). The relationships between nestin and tumor stage, type of uterine cancer and risk categorization were also examined in greater detail. High nestin was observed in 10% of stage IA, 23% of stage IB, 24% of stage II, 30% of stage III and 27% of stage IV tumors ($p=0.002$) or in 6% of G1 endometrioid, 13% of G2 endometrioid,

22% of G3 endometrioid, 25% of clear cell and 40% of serous carcinomas as well as 61% of carcinosarcomas ($p<0.001$), respectively. Higher risk group patients were 5.6 times more likely to exhibit high tumor expression of nestin than the low and low-intermediate risk subgroups ($p<0.001$).

IHC Staining of Nestin and Other Biomarkers of Uterine Cancer

Type I endometrial tumors typically exhibit high ER, PR, TFF3, and loss of PTEN. Nestin expression was inversely associated with ER, PR, and TFF3, but not PTEN (**Table 2** and **Supplement S3**). Type II endometrial tumors are often characterized by high p53 or IMP3 expression. High nestin expression was more common in type II disease and tumors with high p53 or high IMP3 (**Table 2** and **Supplement S3**).

IHC Staining for Nestin a Biomarker of Poor Clinical Outcome

The relationship between nestin and clinical outcome was evaluated for potential prognostic, independent prognostic, or predictive value. High nestin expression was associated with worse PFS (**Fig. 2A**), CSS (**Supplement S4**), and overall survival (data not shown). Women with high vs. low tumor expression of nestin had an increased risk of disease progression (HR=2.787, 95% CI=1.798-4.320, $p<0.001$), cancer death (HR=2.325, 95% CI=0.323-4.086, $p=0.003$), and death due to any cause (HR=1.791, 95% CI=1.057-3.035, $p=0.03$).

High tumor expression of nestin was not an independent predictor of PFS or CSS after adjusting for age, stage, and an aggregate of cell type and grade in the same model. Individual adjusted models (**Supplement S5**) demonstrated that the relationship between nestin and PFS was maintained after adjustment by treatment (adjusted HR=1.975), stage (adjusted HR=2.006), LVSI (adjusted HR=1.984) or a modified risk categorization based

on GOG-99 and PORTEC criteria (adjusted HR=1.878) each with 95% CIs that did not overlap 1, but not by the cell type and/or grade (95% CIs overlap 1.0).

Logistic regression modeling and receiver operator curve (ROC) analyses provided measures of predictive accuracy and demonstrated that high versus (vs) low nestin was associated with an increased risk of disease progression within 5-years (OR=3.450, 95% CI=1.942-6.129). The presence of high nestin predicted risk of 5-year PFS with an AUC of 0.608 (95% CI=0.535-0.681). Incorporation of nestin with stage and uterine cancer type improved the prediction of 5-year PFS to an AUC of 0.801 (95% CI=0.752-0.850). The accuracy for predicting 5-year PFS dropped to 0.624 with ER (95% CI=0.544-0.704), 0.566 with ER and PR (95% CI=0.482-0.650) or 0.568 with ER, PR and p53 (95% CI=0.484-0.652) added to the model with nestin, stage, uterine cancer type.

IHC Staining of Other Biomarkers and PFS

The relationship between the previously published biomarkers and PFS censored at 60 months was also assessed. Better PFS was observed in women with high vs. low ER, PR or TFF3 (**Fig. 2 B-D**, log-rank $p<0.001$), but not PTEN expression. Worse PFS was seen in women with high vs. low p53 or IMP3 (**Fig. 2E-F**, log-rank $p\leq 0.01$).

Relationship between IHC Staining of Nestin and Treatment on PFS

Subset analyses were performed to examine the relationship between nestin and PFS in greater detail. The first of these analyses examined the impact of nestin expression and treatment on PFS. Poor PFS was observed in women with high vs low nestin who received no or radiation-based adjuvant therapy (**Fig. 3A-B**, log rank $p<0.001$). Nestin

expression did not stratify PFS in the subset of patients who received chemotherapy or chemoradiation (**Fig. 3C**).

Relationship between Nestin and Prognostic Clinical Variables on PFS

The combination of nestin and prognostic clinical variables such as stage, LVSI, risk categorization (defined in methods), and type of uterine cancer (cell type by grade) on PFS was also assessed. Women with high nestin expression had worse PFS than those with low nestin expression in the subset with G1 or G2 endometrioid carcinoma, early stage disease, negative LVSI, lower risk, and higher risk patients (**Fig. 3D-H**). The level of nestin expression did not stratify PFS in the subset of women with G3 endometrioid carcinomas, non-endometrioid histologies, advanced stage or positive LVSI (**Supplement S6A-D**).

Relationship between Nestin and Other Biomarkers on PFS

The combined effect of nestin with type I biomarkers or type II biomarkers on PFS was also investigated. Categorized tumor expression of nestin consistently stratified PFS overall and when crossed with the other biomarkers. Women with high relative to low tumor expression of nestin consistently had worse PFS in the subset with low and with high ER (**Fig. 4A-B**), PR (**Fig. 4C-D**), p53 (**Fig. 4E-F**), PTEN (**Supplement S7A-B**), TFF3 (**Supplement S7C-D**) or IMP3 (**Supplement S7E-F**), respectively.

Transcript Expression of Nestin or Other Biomarkers and PFS

Data from the TCGA uterine cancer project was utilized to determine if transcript expression of nestin (or other biomarkers) had a similar relationship with PFS as observed using IHC-derived data or not (**Supplements S8-S10**). High vs. low tumor staining of either nestin or IMP3 was associated with worse PFS and an increased risk of

304 disease progression but neither of these biomarkers exhibited prognostic value at the
305 RNA-seq level. High vs. low tumor staining of p53 was associated with worse PFS
306 especially in type II cancers whereas higher transcript expression of *TP53* was associated
307 with better PFS and a reduced risk of disease progression in uterine cancers and the
308 subset of endometrioid but not serous cancers. Consistent with IHC-based results, *ERS1*,
309 *PGR* and *TFF3* were associated with better PFS and a reduced risk of disease progression
310 in uterine cancers.

DISCUSSION

In our study, high nestin expression was more common in advanced stage, non-endometrioid subtypes, positive LVSI, tumor size 6+ cm, and women with disease progression or cancer death. Women with high vs. low nestin had worse progression-free survival (PFS) and cancer-specific survival (CSS) overall, and worse PFS in the subset who received no or radiation-based adjuvant therapy or had early stage, type I disease, and biologically appropriate relationship with conventional biomarkers. Nestin was an independent prognostic factor for PFS or CSS after controlling for stage, LVSI, or risk categorization, but not after correction for type of uterine cancer (cell type and grade). The accuracy of predicting disease progression at five years was 60% for nestin alone and 80% when nestin was combined with stage and uterine cancer type.

Nestin is a regulator of TGF- β [17,18] and in vitro experiments have demonstrated that it plays a role in invasion, metastasis, and angiogenesis [17,22]. The association of nestin expression with prognosis has been demonstrated in ganglioglioma, ovarian, head and neck, prostate, bladder, and pancreatic cancer [20,22,25,26], and from the present work, in uterine cancer. Nestin may have utility as a theragnostic triage companion test and opportunity for further risk stratification for clinical management. This may play a particularly important role in patients who may not receive adjuvant therapy but may actually be at a higher risk of recurrence than previously thought due to high nestin expression. The decrease in PFS was also seen in patients treated with adjuvant radiation, but not among patients treated with chemotherapy alone or chemoradiation as these patients are among the highest risk for recurrence. High nestin was more common in tumors with aggressive histologic features, but portended worse

PFS in the lower risk cancers. We speculate that nestin was not able to stratify PFS in women had advanced stage disease or G3 endometrioid endometrial cancer or non-endometrioid cancers as we lack effective therapies for these subsets. This hypothesis is supported by the observation that similar PFS was observed in women who received adjuvant chemotherapy or chemoradiation.

One hallmark of invasive and metastatic cancer is the induction of epithelial-mesenchymal transition (EMT) [27-29]. Tumor EMT is a phenotypic switch that promotes the acquisition of a fibroblastoid-like morphology by epithelial tumor cells, resulting in enhanced tumor cell motility and invasiveness, increased metastatic propensity, and resistance to chemotherapy [27,29]. Tumor cells undergoing EMT secrete specific factors, including cytokines, chemokines, and growth factors, such as interleukin (IL)-1, tumor necrosis factor alpha (TNF- α), and TGF- β , that promote tumor progression [28,29].

Progesterone has a fundamental role in reproductive biology, uterine cancer treatment, and has been implicated as a chemopreventive agent, for a number of cancers including endometrial cancer [30-32]. A potential key mechanism of the therapeutic and chemopreventive activity involves the progesterone-dependent downregulation of the transforming growth factor-beta (TGF- β) pathway, a driver of cancer cell viability and invasion. Progestin is a therapeutic option for endometrial cancer patients who are poor surgical candidates or wish to maintain fertility [12,33], has a protective effect on the endometrium, and is also being explored for chemopreventive activity alone or in combination with vitamin D [17]. Progestin induces cellular differentiation, the mechanism by which includes suppression of TGF- β signaling and inhibition of EMT

[9,10]. Studies are underway to determine the relationship between progesterin signaling and nestin. Future uses of nestin may include evaluation as a surrogate biomarker and in the development of chemopreventive combinations, such as with progesterin, especially in patients with multiple risk factors for uterine cancer development (e.g. Lynch syndrome, obesity, chronic anovulation).

High nestin expression did not retain independent prognostic relevance after correction for cell type and grade, but did stand up to adjustments for clinical features not always readily apparent at the time of diagnosis. The observation that nestin expression stratified both PFS and CSS overall and in all subsets of lower risk patients illustrates the potential utility of nestin in triaging the apparent low risk patients for more aggressive treatment. The predictive value of nestin was optimal when combined with stage and uterine cancer cell type with accuracy levels achieving 80%. Predictive assessments in the subsets of patients receiving no or radiation therapy has the potential to benefit a significant proportion of uterine cancer patients in the US and globally each year.

Biomarkers assist in clinical management decision making of uterine cancer patients, distinguishing those cancers with more or less aggressive features [9-14,33]. Markers of type I disease (ER, PR, and TFF3) were inversely associated with nestin expression and, conversely, type II biomarkers (p53 and IMP3) were more common with high nestin expression. Nestin stratified PFS in tumors either with low or with high ER, PR, TFF3, PTEN, p53 and IMP3. This profile of PFS stratification, particularly in patients with type I disease that receive no or adjuvant radiation therapy, offers an opportunity for nestin to act as a clinical biomarker along with ER and PR to more effectively stratify risk in these individuals. Given that type I disease accounts for the

majority of uterine cancers [1-3], the potential impact on women in the US and the world is high. Independent validation will be required to document the true clinical value of nestin in triaging the lowest risk uterine cancer patients with the worst PFS. Development and deployment of robust companion biomarker assays with prognostic and predictive value is needed to enhance the personalized clinical management of uterine cancer patients beyond that provided by estrogen receptor or progesterone receptor (ER/PR).

Some biomarkers are coordinately regulated at the transcript and protein levels whereas others are not. There are numerous post-transcriptional, translational and post-translational mechanisms [34,35] that may explain, at least in part, any disparity in the relationship between IHC staining and PFS vs. transcript expression and PFS. In addition, the confounding effect of tumor purity on transcript expression and disparities with IHC staining demonstrated in an assessment of >10,000 samples across 21 cancer types from TCGA [36] provide a potential alternate or complimentary explanation for the observed inconsistencies between IHC- vs. RNASeq-based results.

High nestin expression is more common in aggressive uterine cancers, but appears to be more predictive of poor outcome in patients with lower risk, including those with early stage disease and G1/G2 endometrioid histologies with and without LVSI. These findings are consistent with the mechanistic studies of nestin and clinical outcome assessments of nestin expression in other cancers [20,22,25,26]. It is a testable and biologically relevant protein that may have utility as a triage tool to identify lower risk patients who may require more aggressive treatment. Additional studies will be required

403 to more thoroughly assess the prognostic, predictive, and theragnostic potential of nestin
404 and to optimize its deployment in therapeutic and chemopreventive settings.

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539

Table 1. Clinical characteristics in eligible uterine cancer patients and relationship with tumor expression of nestin.

	Nestin Tumor Expression ^Φ					Fisher's exact p-value
	Total	Low		High		
		Cases	%	Cases	%	
Eligible patients	323	260	80.5	63	19.5	
Age at diagnosis						NS
<50	40	34	13.1	6	9.5	
50-69	175	144	55.4	31	49.2	
70+	108	82	31.5	26	41.3	
Tumor stage [¥]						0.003
Early	213	182	70.0	31	49.2	
Advanced	110	78	30.0	32	50.8	
Uterine cancer type						<0.001
Type I	185	169	65.0	16	24.4	
Type II	121	85	32.7	36	57.1	
Carcinosarcoma	17	6	2.3	11	17.5	
Cell type						<0.001
Endometrioid	236	223	80.2	28	42.4	
Non-endometrioid	87	55	19.8	38	57.6	
Grade						<0.001
1 or 2	185	181	65.1	17	25.8	
3	138	97	34.9	49	74.2	
Tumor size						0.046
<6 cm	225	188	72.3	37	58.7	
6+ cm	98	72	27.7	26	41.3	
LVSI						0.001
Negative	215	185	71.2	30	47.6	
Positive	108	75	28.8	33	52.4	
Risk Category						<0.001
Low	32	29	11.5	3	4.8	
Low-intermediate	83	79	30.4	4	6.3	
High-intermediate	53	49	18.8	4	6.3	
High	155	103	39.6	52	82.5	

Abbreviations: NS = not significant (p>0.05), LVSI = lymphovascular space invasion

^φ Nestin expression was categorized as low (≤10% positive tumor cells) or high (>10%

positive tumor cells).

[¥] Early represents stage I and II disease. Advanced includes stage III and IV disease. See

methods for the definitions for the risk categorization.

545

Table 2. Relationship between nestin and other biomarkers in the uterine cancers.					
	Nestin Tumor Expression ^φ				Fisher's exact p-value
	Low		High		
	Cases	%	Cases	%	
Type I Biomarkers [¥]					
ER					0.001
Low	81	33.9	35	59.3	
High	158	66.1	24	40.7	
PR					<0.001
Low	81	33.5	39	67.2	
High	161	66.5	19	32.8	
TFF3					<0.001
Low	78	35.1	37	63.8	
High	144	64.9	21	36.2	
PTEN					NS
Low	152	63.3	33	55.0	
High	88	36.7	27	45.0	
Type II Biomarkers [¥]					
p53					0.009
Low	144	59.3	24	40.0	
High	99	40.7	36	60.0	
IMP3					0.003
Low	181	76.1	32	55.2	
High	57	23.9	26	44.8	

546 Abbreviations: NS = not significant (p>0.05); ER (estrogen receptor); PR (progesterone
 547 receptor); TFF3 (trefoil factor 3); PTEN (phosphatase and tensin homolog); p53 (tumor
 548 protein p53); IMP3 (insulin-like growth factor II mRNA-binding protein 3).

549 ^φ Nestin expression was categorized as low (≤10% positive tumor cells) or high (>10%
 550 positive tumor cells).

551 [¥] See methods for details regarding the scoring and categorization of each biomarker.

552

FIGURE LEGENDS

Figure 1. Tumor expression of nestin and relationship with clinical outcome.

Immunohistochemical expression of nestin was categorized as low ($\leq 10\%$ positive tumor cells) or high ($>10\%$ positive tumor cells) and representative photomicrographs provided for low (**A**) and high (**B**) staining in endometrioid endometrial carcinomas. Lower power images provided in the upper right corner of panels **A-B**.

Figure 2. The relationship between other biomarkers and progression-free survival.

Progression-free survival (**A**) plot censored at 60 months were generated using Kaplan-Meier method, and survival distributions were compared using log-rank test for women with low (**blue line**) vs. high (**green line**) tumor expression of nestin. Hazard ratio (HR) and 95% confidence interval (CI) were generated from univariate Cox regression modeling for progression-free survival (**A**). Progression-free survival plots censored at 60 months were generated using Kaplan-Meier method and survival distributions were compared using log-rank test for women with low (**blue line**) vs. high (**green line**) tumor expression of ER (**B**, $p < 0.001$), PR (**C**, $p < 0.001$), TFF3 (**D**, < 0.001), p53 (**E**, $p = 0.01$), IMP3 (**F**, $p < 0.001$). Abbreviations: ER (estrogen receptor); PR (progesterone receptor); TFF3 (trefoil factor 3); p53 (tumor protein p53); IMP3 (insulin-like growth factor II mRNA-binding protein 3). See methods for details regarding the scoring and categorization of each biomarker.

Figure 3. Combination of nestin and prognostic clinical variables on progression-

free survival. Progression-free survival plots censored at 60 months were generated using Kaplan-Meier method and survival distributions were compared using log-rank test for the subset of women with low (**blue line**) vs. high (**green line**) tumor expression of

nestin who received no adjuvant treatment (**A**, $p < 0.001$), radiation (**B**, $p < 0.001$) or chemotherapy/chemoradiation (**C**, $p > 0.05$), or had G1 or G2 endometrioid endometrial cancer (**D**, $p < 0.001$), early stage disease (**E**, $p < 0.001$), negative LVSI (**F**, $p = 0.001$), lower risk disease (**G**, $p < 0.001$) or higher risk disease (**H**, $p = 0.03$). Early stage includes stage I and II disease. LVSI refers to lymphovascular space invasion. See methods for definitions for the risk categorizations.

Figure 4. Combination of nestin and other biomarkers on progression-free survival.

Progression-free survival plots censored at 60 months were generated using Kaplan-Meier (KM) method and survival distributions were compared using pairwise log-rank test for the subset of women categorized by low (blue line) or high (green line) expression of nestin and by either low ER (**A**), high ER (**B**), low PR (**C**), high PR (**D**), low p53 (**E**) or high p53 (**F**). Abbreviations: ER (estrogen receptor); PR (progesterone receptor); p53 (tumor protein p53). See methods for details regarding the scoring and categorization of each biomarker.

591 **Figure 1.**

592

593 **Figure 2.**

595 **Figure 3.**

597 **Figure 4.**