uicc
global cancer control

IJC
International Journal of Cancer

Molecular markers of endometrial carcinoma detected in uterine aspirates

Eva Colas[1,2*], Cristina Perez[3*], Silvia Cabrera[4], Nuria Pedrola[1], Marta Monge[1], Josep Castellvi[5], Fernando Eyzaguirre[4], Jesus Gregorio[4], Anna Ruiz[1], Marta Llaurado[1], Marina Rigau[1], Marta Garcia[1], Tugçe Ertekin[1], Melania Montes[1], Rafael Lopez-Lopez[6], Ramon Carreras[7], Jordi Xercavins[2,4], Alicia Ortega[8], Tamara Maes[3], Elisabet Rosell[3], Andreas Doll[1], Miguel Abal[1,6], Jaume Reventos[1,2] and Antonio Gil-Moreno[2,4]

[1] Biomedical Research Unit, Research Institute Vall d'Hebron University Hospital, Barcelona, Spain
[2] Universitat Autònoma de Barcelona Autonomous, University of Barcelona, Barcelona, Spain
[3] Oryzon Genomics, Parc Cientific de Barcelona, Barcelona, Spain
[4] Department of Gynecological Oncology, Vall d'Hebron University Hospital, Barcelona, Spain
[5] Department of Pathology, Vall d'Hebron University Hospital, Barcelona, Spain
[6] Department of Medical Oncology, Complexo Hospitalario Universitario SERGAS, Santiago de Compostela, Spain
[7] Department of Obstetrics and Gynecology, Hospital del Mar, Barcelona, Spain
[8] R+D Division, Biotechnology Development, Reig Jofre Group, Barcelona, Spain

Endometrial cancer (EC) is the most frequent of the invasive tumors of the female genital tract. Although usually detected in its initial stages, a 20% of the patients present with advanced disease. To date, no characterized molecular marker has been validated for the diagnosis of EC. In addition, new methods for prognosis and classification of EC are needed to combat this deadly disease. We thus aimed to identify new molecular markers of EC and to evaluate their validity on endometrial aspirates. Gene expression screening on 52 carcinoma samples and series of real-time quantitative PCR validation on 19 paired carcinomas and normal tissue samples and on 50 carcinoma and noncarcinoma uterine aspirates were performed to identify and validate potential biomarkers of EC. Candidate markers were further confirmed at the protein level by immunohistochemistry and Western blot. We identified ACAA1, AP1M2, CGN, DDR1, EPS8L2, FASTKD1, GMIP, IKBKE, P2RX4, P4HB, PHKG2, PPFIBP2, PPP1R16A, RASSF7, RNF183, SIRT6, TJP3, EFEMP2, SOCS2 and DCN as differentially expressed in ECs. Furthermore, the differential expression of these biomarkers in primary endometrial tumors is correlated to their expression level in corresponding uterine fluid samples. Finally, these biomarkers significantly identified EC with area under the receiver-operating-characteristic values ranging from 0.74 to 0.95 in uterine aspirates. Interestingly, analogous values were found among initial stages. We present the discovery of molecular biomarkers of EC and describe their utility in uterine aspirates. These findings represent the basis for the development of a highly sensitive and specific minimally invasive method for screening ECs.

Endometrial cancer (EC) is the most frequent of the invasive tumors of the female genital tract and the fourth most common in women in western countries.[1] The crude incidence of EC in the European Union range from 13 to 24 new cases per 100,000 women per year and a mortality rate of 4–5 cases per 100,000 per year. It is estimated that the lifetime risk of developing EC is ~1.7–2%.[2] In the United States, about 40,100 new cases of EC have been estimated in

Key words: endometrial cancer, molecular biomarkers, uterine aspirate, minimally invasive screening
Additional Supporting Information may be found in the online version of this article
E.C. and C.P. contributed equally to this work.
Miguel Abal, Jaume Reventos and Antonio Gil-Moreno are Principal investigators.
Conflict of interest: None
Grant sponsor: Spanish Ministry of Science and Innovation; **Grant numbers:** SAF 2005-06771, SAF 2008-03996; **Grant sponsor:** Spanish Ministry of Health; **Grant numbers:** RTICC RD06/0020/0058, CP08/00142, PI08/0797; **Grant sponsor:** Catalan Institute of Health; **Grant number:** DURSI 2005SGR00553; **Grant sponsor:** Fundacio La Marato de TV3; **Grant number:** 050431; **Grant sponsor:** ACCIO; **Grant number:** RDITSCON07-1-0001; **Grant sponsor:** National Programme of Biotechnology; **Grant number:** FIT-010000-2007-26; **Grant sponsor:** European Commission Program Fondo Europeo de Desarrollo Regional (FEDER)
DOI: 10.1002/ijc.25901
History: Received 10 Jun 2010; Accepted 3 Nov 2010; Online 4 Jan 2011
Correspondence to: Jaume Reventos, Biomedical Research Unit, Research Institute Vall d'Hebron University Hospital, Pg. Vall d'Hebron 119-129, 08035 Barcelona, Spain, E-mail: jreventos@ir.vhebron.net

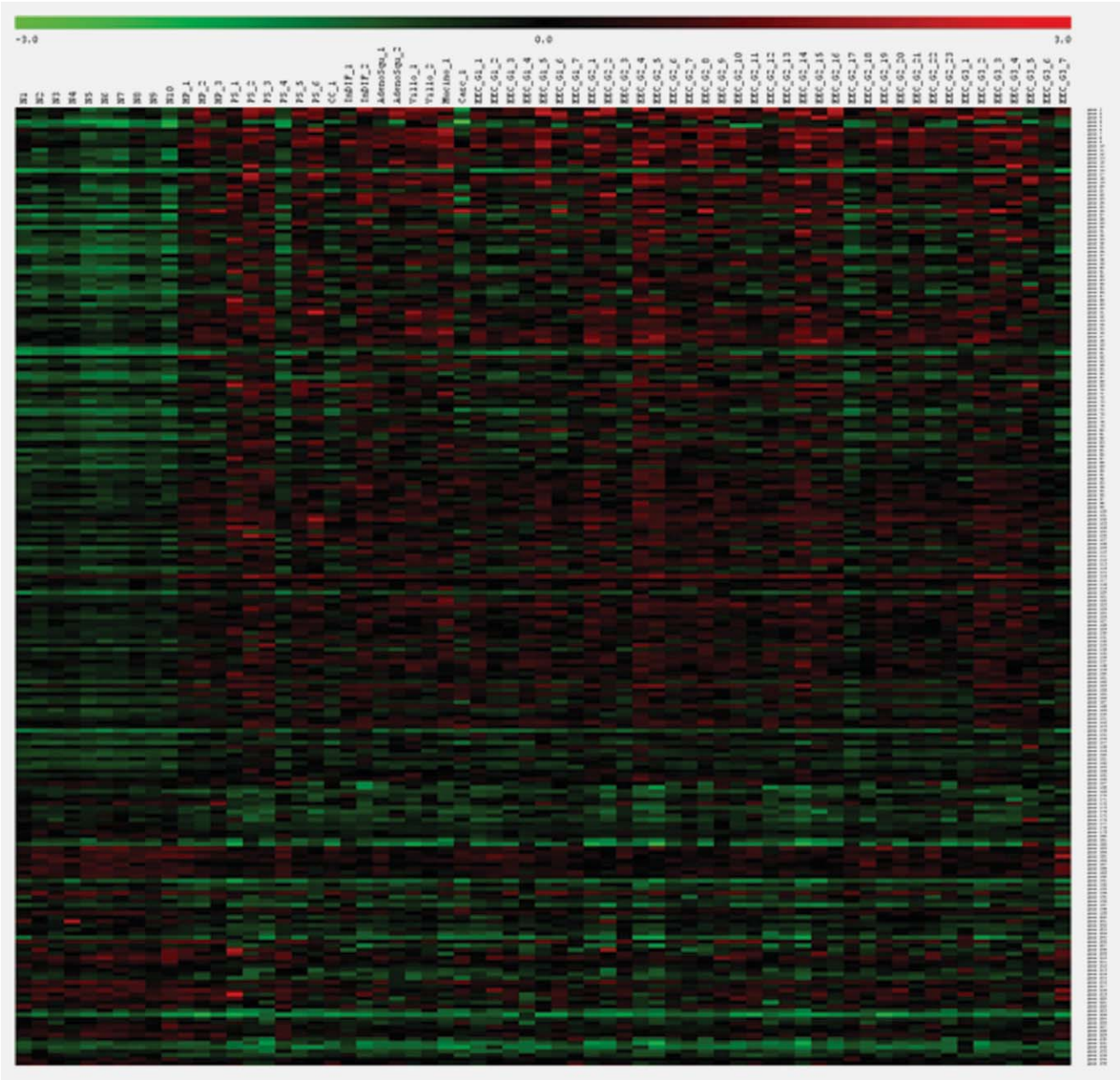

Figure 1. Hierarchical clustering of 236 (166 upregulated and 70 downregulated) differentially expressed genes between normal endometrial tissue and cancer tissue using as thresholds the *p* value and then fold change. Among the cancer tissues, there are 38 samples from endometrioid endometrial carcinomas (EECs) and 14 Type II carcinomas with different histotypes. Upregulation is indicated in red, downregulation in green and average regulation in black. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

2008 with 7,470 estimated deaths.[1] Age-standardized incidence rates continue to rise in most developed countries.

Uterine cancer usually occurs after menopause and is usually detected in its initial stages by presentation of disease-related symptoms, including unusual vaginal bleeding or discharge, trouble urinating, pelvic pain and pain during intercourse. Unfortunately, at the time of diagnosis, 20% of the patients present myometrial invasion and/or lymph node affectation, which are main indicators of an advanced disease, related to poor prognosis and decrease in survival rate. The standard treatment for EC varies depending on the stage of the disease. Primary treatment usually consists on staging surgery including hysterectomy, bilateral adnexectomy and pelvic and/or paraaortic lymphadenectomy, although there exist other options as hormone therapy and radiotherapy. Although EC is traditionally considered as an early diagnosis/good prognosis type of cancer, there is clearly room for improvement. An ideal scenario is to achieve diagnosis at Stage I where the 5-year survival rate ranges up to 95%. In addition, new

Table 1. Differential gene expression of endometrial cancer biomarkers in primary tumor when compared to control values

HUGO gene symbol	Entrez gene name	Fold change	*p* value	Location	Type(s)	Cancer	Plasma/Serum	Urine	Uterus
ACAA1	Acetyl-coenzyme A acyltransferase 1	1.26	0.11	Cytoplasm	Enzyme				x
AP1M2	Adaptor-related protein complex 1, mu 2 subunit	1.71	0.11	Cytoplasm	Transporter				
CGN	Cingulin	1.79	0.22	Plasma membrane	Other		x		
DCN	Decorin	−2.55	0.06	Extracellular space	Other				x
DDR1	Discoidin domain receptor tyrosine kinase 1	1.93	0.13	Plasma membrane	Kinase			x	
EFEMP2	EGF-containing fibulin-like extracellular matrix protein 2	−1.22	0.08	Extracellular space	Other	x	x	x	x
EPS8L2	EPS8-like 2	1.34	0.20	Unknown	Other		x	x	
FASTKD1	FAST kinase domains 1	1.71	0.06	Unknown	Other				
GMIP	GEM interacting protein	1.42	0.05	Unknown	Enzyme		x		
IKBKE	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase epsilon	1.37	0.17	Cytoplasm	Kinase	x	x		
P2RX4	Purinergic receptor P2X, ligand-gated ion channel, 4	1.70	0.12	Plasma membrane	Ion channel				x
P4HB	Prolyl 4-hydroxylase, beta polypeptide	1.90	0.13	Cytoplasm	Enzyme	x	x	x	x
PHKG2	Phosphorylase kinase, gamma 2 (testis)	1.34	0.09	Unknown	Kinase				
PPFIBP2	PTPRF interacting protein, binding protein 2 (liprin beta 2)	1.52	0.11	Nucleus	Phosphatase				x
PPP1R16A	Protein phosphatase 1, regulatory (inhibitor) subunit 16A	1.44	0.10	Plasma membrane	Other				
RASSF7	Ras association (RalGDS/AF-6) domain family (N-terminal) member 7	1.94	0.07	Unknown	Other				
RNF183	Ring finger protein 183	1.73	0.19	Unknown	Other				
SIRT6	Sirtuin (silent mating type information regulation 2 homolog) 6 (*S. cerevisiae*)	1.27	0.15	Nucleus	Enzyme				x
SOCS2	Suppressor of cytokine signaling 2	−1.69	0.06	Cytoplasm	Other	x			x
TJP3	Tight junction protein 3 (zona occludens 3)	1.57	0.17	Plasma membrane	Other	x			x

methods for prognosis and classification of EC are needed to combat this deadly disease.[3]

Focusing on early detection, there is a necessity for screening methods with high sensitivity and specificity. To date, no characterized molecular marker has been validated for the diagnosis of EC. In addition, current methods of diagnosing EC often create discomfort to the patient and sometimes rely on subjective interpretation of visual images. Methods routinely used in the clinic for diagnosing EC include biopsy and/or transvaginal ultrasound. The final diagnosis of EC is usually done by pathology examination of an endometrial aspirate (20–30%) and by hysteroscopic-guided biopsy (70–80%). The rate of success diagnosis with hysteroscopy is over 90%, with false positives in the case of precursor lesions of the endometrial adenocarcinoma (hyperplasias with atypia); endometrial polyps that present a non-negligible degree of malignancy (0–4.8%) and must be removed although asymptomatic or benign appearance or in the case of diffuse forms of endometrial adenocarcinomas that are difficult to differentiate from an endometrial hyperplasia.

In our work, we aimed to identify new molecular markers of EC and to evaluate their validity on endometrial aspirates. For this, we first identified and validated new robust biomarkers of EC on hysterectomy tissue samples. We next evaluated whether gene expression profiles

on uterine aspirates efficiently mirror that of paired tissue samples. We finally confirmed the validity of these biomarkers on uterine aspirates to differentially classify carcinoma and control samples. The objective was to set up the basis for the development of a test that could be applied for the screening of risk groups of patients, to develop a reliable tool to ameliorate the sensitivity and specificity of the endometrial biopsy and to preclude unnecessary hysteroscopy.

Material and Methods

Sample description

Tumor samples were obtained from patients who underwent surgery for EC in the Department of Gynecological Oncology at the Vall d'Hebron University Hospital. Control tissue was obtained from nonaffected regions of endometrial tissue from the same patients. The protocol was previously approved by the Institutional Review Board, and informed consent was obtained from all of the patients involved in our study. During preparation of the specimens, care was taken to macroscopically dissect the carcinoma away from any adjacent myometrium. Samples were immediately frozen at -80° until processed for RNA extraction and gene expression analysis.

Endometrial aspirates were collected with the help of a Cornier pipelle, after complete informed consent was obtained from all patients. The aspirate (uterine fluid) was immediately transferred to an eppendorf tube containing 500 μl of a RNA preserving solution (RNA later, Ambion). The sample was centrifuged, and the pellet containing a representative population of cells from the uterine cavity was further processed for RNA extraction.

Microarray analysis

Total RNA was extracted with the RNeasy mini kit (Qiagen, Hilden, Germany), following the instructions provided by the manufacturer. Microarrays for gene expression were designed by the Tethys algorithm using the ENSEMBL database. Cy3- and Cy5-labeled aRNA was produced using the Message-Amplification kit by Ambion. Microarray hybridization was performed at 60°C and 17-hr hybridization time according to Agilent indications. Initial raw data were obtained using an Agilent DNA Microarray Scanner (G2505B) and Agilent acquisition software (Feature Extraction Software).

The mean fold change or *M* values can be ranked based on their probability of being different from 0, according to the absolute value of the regularized *t*-statistic,[4] which uses a Bayesian framework to derive a modified and improved *t*-Student statistics. To make fold change–based selection, the mean *M* distribution was used. This distribution is adjusted to a normal distribution, and an iterative process is used to define the mean *M* numbers that are outside the distribution. The cutoff is chosen as *n* times the standard deviations (σ) from the mean. This method generates a robust mean and standard deviation and allows to dynamically adjusting the cutoff value to the noise distribution of the data. Typically, values with mean FC $> 3\sigma$ or mean FC $< -3\sigma$ of the sample data distribution were selected.

An indirect analysis comparison, where the expression levels of particular biomarkers in tumor samples, was compared to a reference RNA pool obtained from a group of over 20 cell lines (melanoma, lung cancer, ovarian cancer, colon cancer and several noncancer cell lines). The expression level of particular genes in the normal samples (controls) was compared to the same reference pool, and final expression fold changes between tumor and normal endometrial tissue were generated *in silico* eliminating the reference pool.

Real-time quantitative PCR

Quality tests (Bioanalyzer) were performed before the analysis of gene expression by Taqman technology for the selected markers of EC. Real-time quantitative PCR (RT-qPCR) was performed following Applied Biosystem standard protocol for the 7900HT system.

RT-qPCR validation on uterine aspirates was performed with multiplex technology. Briefly, wells of the microfluidic card contain Applied Biosystems fluorogenic 5′ nuclease assays that detect the real-time amplification of the array selected targets. Relative levels of gene expression are determined from the fluorescence data generated during PCR using the ABI PRISM® 7900HT Sequence Detection System (7900HT SDS) Relative Quantification software.

Data analysis was made using the comparative ΔΔCt method of relative quantification. Differentially expressed genes were confirmed by thorough statistical analysis using a modified *T*-test.

Relative quantity (RQ) is the relative amount of RNA for a specific gene present on the tumor samples referred to the amount present on the control sample for the same gene. To calculate the RQ, the Ct values of each gene were normalized with respect to the Ct of the endogenous gene to get the delta Ct. The formula $2^{-(\mathrm{deltaCt})}$ was used to calculate the RQ.

A number of endogenous genes can be used as a control for normalization as well as other controls for normalization. We have tested four different housekeeping genes as possible endogenous genes for normalization purposes: 18S, B2M, PFN-1 and POLR2A. Finally, POLR2A was the most stable gene from all of them (data not shown), and all the calculations and statistics were done using it as endogenous. Its expression level was similar to the genes questioned in our test, different to 18S whose expression was quiet high compared to the genes selected for the test.

M+M ROC

The area under the curve (AUC) values was calculated based on a receiver-operating-characteristic (ROC) analysis to evaluate the performance of each marker.

Western blot and Immunohistochemistry

For immunohistochemistry validation, tissue microarrays (TMAs) were constructed at the Pathology Department of the Vall d'Hebron University Hospital. Representative areas from 70 paraffin-embedded carcinomas (56 endometrioid, six serous papillary, one mucinous, four clear cell carcinomas and three carcinosarcomas) and 11 non-neoplastic endometria (four atrophic, three proliferative, one secretory endometrial and three hyperplasias) were carefully selected and marked on individual paraffin blocks. Two tissue cores of 1 mm in diameter were obtained from each paraffin block and were precisely arrayed in a new paraffin block. Sections of 5 μm were obtained from all TMA paraffin blocks. The protocol was approved by the Institutional Review Board, and informed consent was obtained from all of the patients. P4HB, PPP1R16A and EPS8L2 were detected by the indirect immunoperoxidase assay with citrate buffer, pH 7.3, for antigen retrieval. Sections were incubated with a primary antibodies against P4HB (LS-C38385; Lifespan Biosciences, Seattle, WA) and PPP1R16A (MaxPab polyclonal antibody; Cat# H00084988-B01, Abnova, Taipei, Taiwan) for 1 hr at room temperature using a dilution 1:500 and 1:100, respectively, and EPS8L2 (MaxPab; Cat# H00064787-B01, Abnova) overnight at 1:100 dilution. Thereafter, sections were incubated with peroxidase-conjugated goat antimouse immunoglobulin (EnVision Dual System, DAKO, Glostrup, Denmark). Endogenous peroxidase activity was quenched with 3% H_2O_2. Sections were washed, and reactions were developed with diaminobenzidine, followed by counterstaining with hematoxylin. Semiquantitative evaluation of PH4B was performed by three independent investigators, scoring the intensity of the stained and the percentage of positive cells.

P4HB protein levels were analyzed by Western blot in four different paired samples of EC and their respective adjacent normal endometrial, as described.[5] P4HB levels were examined with the antibody LS-C38385 (1:250), and actin was used as loading control.

Results

Identification of new robust molecular markers of EC

A total of 52 human ECs and three preneoplastic atypical hyperplasias were processed for the analysis of gene expression using Agilent Technology. The gene expression levels for each of these samples, detected with more than 25,000 probes, were compared to a control mean expression value obtained from ten different normal endometria. Human samples included all histological types and tumor grades in a representative manner to identify a diagnostic tool applicable to a complete panel of ECs (73% endometrioid, 10% serous and 17% other Type II ECs; Supporting Information Table 1). Supervised hierarchical clustering of 236 genes (166 upregulated and 70 downregulated) clearly differentiated between tumor and nontumor samples (Fig. 1).

A first round of candidate gene selection, based on statistical data mining, rendered a list of 100 potential biomarkers for further confirmation by RT-qPCR. The differential candidate gene expression levels between normal endometrial tissue and ECs were assessed in an independent series of samples including paired samples of primary Type I and Type II ECs and the adjacent normal endometrium from 17 patients. We also validated the biomarkers onto two samples of normal endometrium and two samples of endometrioid

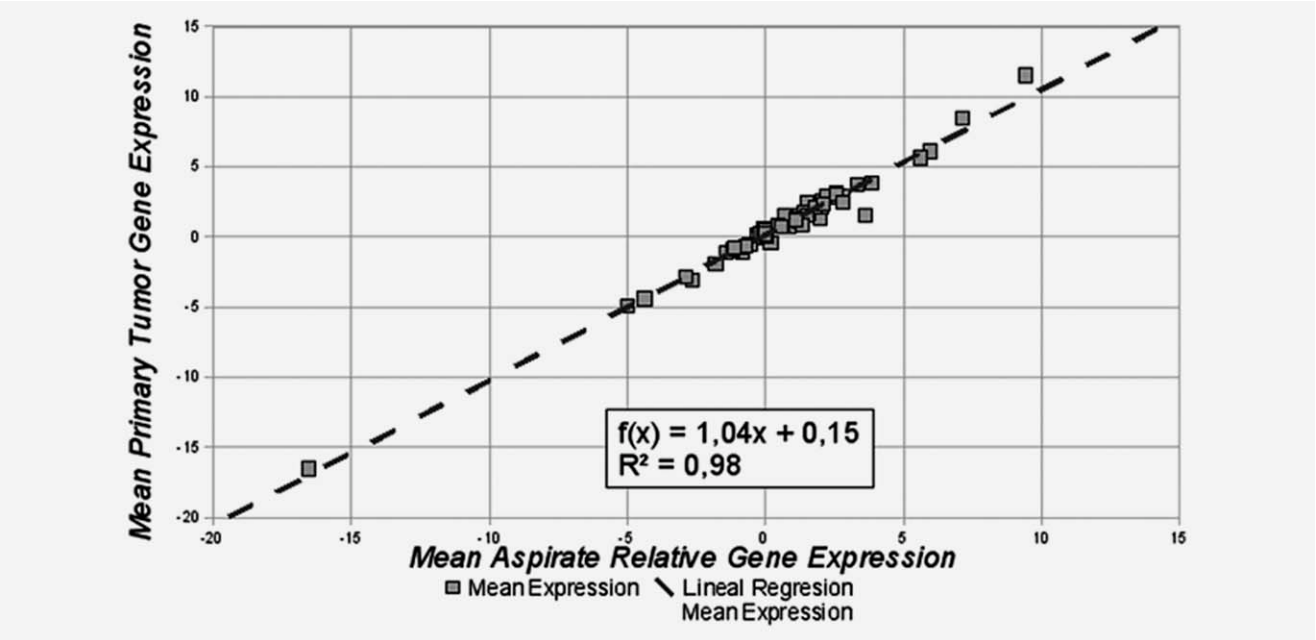

Figure 2. Analysis of gene expression correlation of the identified endometrial carcinoma markers in primary tumors and uterine fluids. Lineal regression and coefficient of determination R^2 when comparing the RT-PCR data obtained from primary tumor carcinomas and aspirate samples from the same patients for the selected genes.

Table 2. Differential expression of biomarkers (mean RQ) in aspirate samples from patients having endometrial cancer compared to aspirates from control patients not having endometrial cancer

				Global tumor *vs.* normal			Initial-stage tumor *vs.* normal		
Gene	Mean RQ	SEM	*p* value	AUROC	Sensitivity (%)	Specificity (%)	AUROC	Sensitivity (%)	Specificity v
ACAA1	1.47	0.48	<0.0001	0.78	88.46	66.67	0.82	91.67	66.67
AP1M2	1.69	0.42	<0.0001	0.83	76.92	75.00	0.80	100.00	54.17
CGN	2.35	1.31	<0.0001	0.85	76.92	87.50	0.78	66.67	87.50
DDR1	0.25	0.20	0.002	0.74	92.31	70.83	0.73	91.67	66.67
EPS8L2	1.52	0.53	0.0167	0.84	80.77	79.17	0.83	100.00	62.50
RASSF7	0.41	0.29	<0.0001	0.81	76.92	87.50	0.80	75.00	87.50
TJP3	1.65	0.56	0.0016	0.81	80.77	79.17	0.77	75.00	83.33
PPFIBP2	1.69	0.66	<0.0001	0.80	92.31	62.50	0.82	91.67	70.83
PPP1R16A	1.34	0.49	<0.0001	0.82	73.08	83.33	0.76	75.00	75.00
IKBKE	2.88	1.62	<0.0001	0.90	92.31	75.00	0.91	100.00	75.00
FASTKD1	1.54	0.50	0.0002	0.83	69.23	87.50	0.84	100.00	58.33
GMIP	2.00	0.65	<0.0001	0.85	84.62	79.17	0.83	91.67	66.67
P2RX4	1.56	0.38	<0.0001	0.80	100.00	58.33	0.77	100.00	58.33
PHKG2	1.54	0.73	0.0094	0.86	92.31	70.83	0.78	83.33	70.83
SIRT6	1.92	0.79	<0.0001	0.84	73.08	95.83	0.75	58.33	95.83
RNF183	1.85	0.77	0.0001	0.88	76.92	95.83	0.92	83.33	95.83
P4HB	3.65	2.37	<0.0001	0.95	100.00	87.50	0.94	100.00	87.50
SOCS2	1.61	0.55	<0.0001	0.93	96.15	75.00	0.93	100.00	75.00
EFEMP2	0.27	0.18	<0.0001	0.88	80.77	87.50	0.94	91.67	87.50
DCN	2.09	0.93	<0.0001	0.81	92.31	70.83	0.80	91.67	62.50

AUROC values for the biomarkers determined from aspirate samples in affected (global: all types and grades of endometrial cancer) and nonaffected individuals, and in affected (only initial stages) and nonaffected individuals.

endometrial tumor tissue obtained by biopsy-guided hysteroscopy (Supporting Information Table 2). This additional validation on biopsies aimed to prove whether the biomarkers were suitable to discriminate ECs from normal tissue on samples obtained through the standard procedure used on a day-to-day basis. Both with primary carcinomas and biopsies, we found a good correlation between cDNA microarray data and RT-qPCR expression levels when comparing tumor tissue and normal endometrium, with a validation of 80% of genes with a p value less than 0.01 (data not shown).

Among the validated genes, a second round of candidate gene selection refined the list to 20 potential biomarkers for EC based on fold expression and p value, but also based on public database information as subcellular localization, type of protein, association with cancer or biological factors that theoretically would improve their utility as a biomarker such as protein presence on biological fluids or specificity of expression in endometrial tissue. The list of the selected candidate genes included ACAA1, AP1M2, CGN, DDR1, EPS8L2, FASTKD1, GMIP, IKBKE, P2RX4, P4HB, PHKG2, PPFIBP2, PPP1R16A, RASSF7, RNF183, SIRT6, TJP3, EFEMP2, SOCS2 and DCN (Table 1).

Uterine aspirates reliably mirror the molecular alterations found in primary tumors

The next step in setting up the basis for a minimally invasive screening test based on molecular biomarkers to be applied on uterine aspirates was to verify whether the endometrial aspirate is a reliable surrogate of its primary tumor. Endometrial aspirates contain fragmented samples of the uterine cavity content, embedded in blood or mucus. Samples obtained from hyperplastic or tumoral endometria are usually very abundant, but those from atrophic endometria show scant epithelial strips or no endometrial tissue to be evaluated histologically. We thus compared the expression levels of the selected candidate genes on a series of nine paired samples of uterine aspirates and primary tumors from the same patients (Supporting Information Table 3). As can be seen in Figure 2, the expression levels of all the candidate genes demonstrated a high degree of correlation between the uterine fluids and their corresponding primary tumors. This data demonstrated that uterine fluids are representative of the molecular alterations characterizing the primary tumors and so could be used to assess biomarkers for EC.

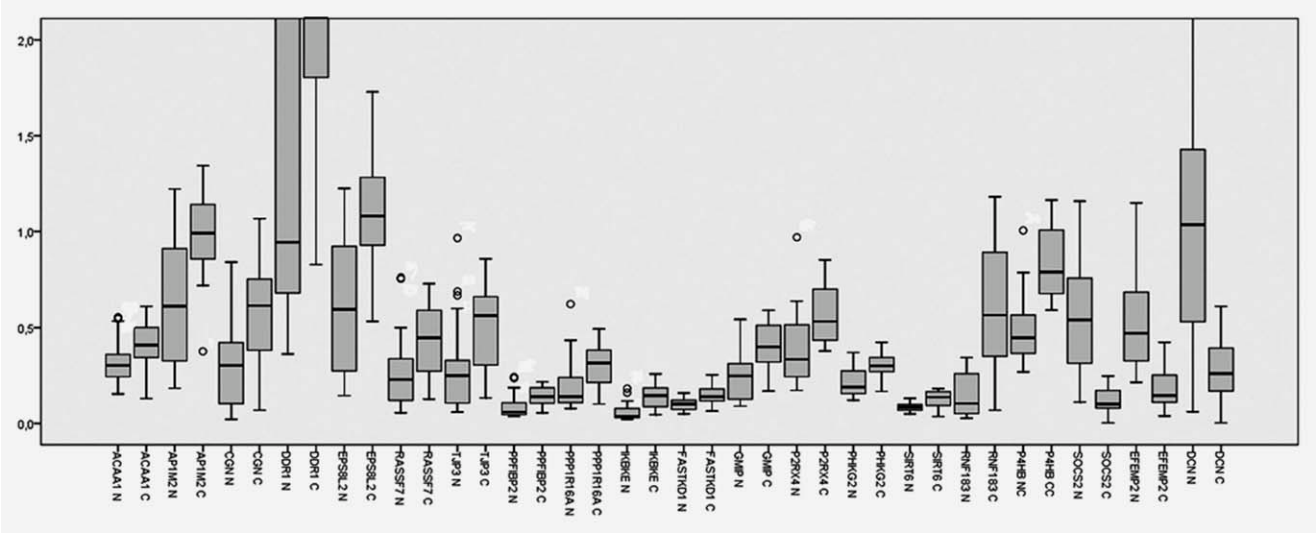

Figure 3. Box–Whisker plot for the selected candidates in uterine aspirates from patients diagnosed with endometrial carcinoma (C) and nonaffected women (N). The horizontal white lines within the boxes denote the medians, and the boxes span the 25th and 75th percentiles of the distributions. The vertical lines above and below each box indicate the range of the distribution. Outliers defined as 3× SD are indicated as black circles.

Validation of the new EC biomarkers in uterine aspirates

As the results suggested, the group of the 20 selected candidate genes could be used to define an increased likelihood of EC based on their expression levels in samples obtained from uterine fluid. We thus aimed to address in a further step of validation whether the selected candidate genes were differentially expressed in endometrial aspirates from EC patients ($n = 26$; 21 endometrioid adenocarcinomas and five tumor samples from different Type II carcinomas), compared to healthy donors ($n = 24$; atrophic endometrium, normal endometrium with polyps from postmenopausal women and samples from premenopausal women both in secretory phase and in proliferative phase of the cycle). Again, the series included representative tumors from all histological type and grade (Supporting Information Table 4). Hence, RT-qPCR data were collected for the set of 20 selected candidate genes and quantified relative to POLR2A levels as a housekeeping gene. The results clearly confirmed the differential expression of biomarkers in aspirate samples from patients having EC compared to aspirates from patients not having EC (Table 2). The RQ values for the aspirates corresponding to the 26 tumor samples and the 24 samples that were not EC (normal) are illustrated in a box plot (Fig. 3).

The sensitivity and specificity for each individual gene represented by the area under the ROC (AUROC) curve for each gene when comparing the RQ values from the 26 tumor samples and the 24 control samples were also calculated (Table 2). As can be observed, the markers identified in these studies have excellent sensitivity and/or specificity for defining an increased likelihood of EC in minimally invasive uterine aspirates. More interestingly, the suitability of these genes as markers for early detection was confirmed when we focused on initial stages among tumor aspirates (Table 2). No differences were found when the comparison was performed between histology types, although the limited number of samples precluded conclusive evaluation. In conclusion, the AUROC values for these biomarkers indicate that these markers could have a utility in the diagnosis of EC.

Validation of the new biomarkers at the protein level

We finally sought to analyze and validate at the protein level the expression of three candidate genes, P4HB, PPP1R16A and EPS8L2, by immunohistochemistry on ECs. For this, we constructed TMAs to cover the complete range from normal tissue to different types and grades of ECs (see Material and Methods). As shown in Figure 4, TMA immunohistochemistry confirmed the differential expression of the three proteins at the tumoral glands (T) when compared to the normal endometrial glands (n). P4HB, PPP1R16A and EPS8L2 presented a specific cytoplasmatic expression within the tumoral cells in all carcinoma histological types and grades and an absence or faint cytoplasmatic stain within the normal epithelial glands (Fig. 4; upper panels).

Likewise, Western blotting further confirmed the specific differential expression of P4HB as the most specific and sensitive EC marker among candidate genes, in tumor samples compared to paired normal endometrial tissue from four different patients (Fig. 4, lower panel). All these results further demonstrate the suitability of these candidate genes as EC markers.

Discussion

Current methods for detecting EC include pathology assessment on uterine aspirates, hysteroscopy-guided biopsies and curettage method, which is considered the gold standard but can cause significant discomfort. In addition, this diagnosis has only a moderate ability to predict final pathology in EC

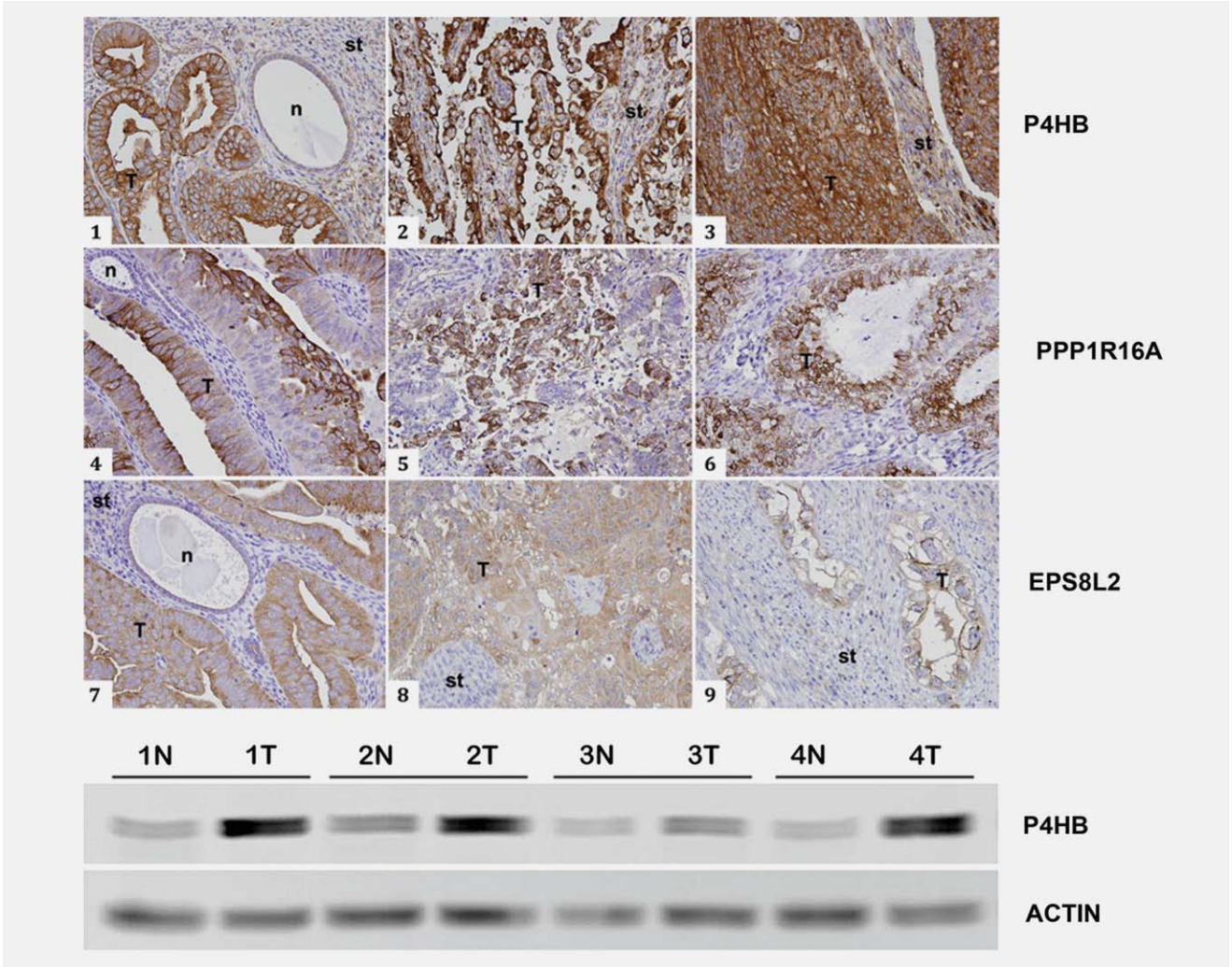

Figure 4. Specific endometrial carcinoma expression of candidate biomarkers at the protein level. Immunohistochemistry of P4HB, PPP1R16A and EPS8L2 in representative examples of endometrial carcinoma (upper panels). Specific staining at tumor glands (T) in contrast to faint labeling in normal glands (n) and stroma (st) in different histological types including endometrioid (1, 3, 4, 5, 6, 7, 8), serous (2) and clear cell (9) carcinomas. Western blot analysis of P4HB in paired samples including tumor tissue (T) and adjacent normal endometrium (N) from four different patients. Actin was used as a loading control. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

and may require a trained pathologist for interpretation; therefore, it is not suitable as a general screening tool. Additional factors should be considered in selecting patients for a surgical staging procedure.[6]

Transvaginal ultrasound measuring the thickness of the endometrium also represents a standard minimally invasive method for diagnosing EC. In a study of patients having postmenopausal bleeding, using a cutoff of 4 mm, it was found that transvaginal ultrasound had 100% sensitivity and 60% specificity.[7] In women without vaginal bleeding, the sensitivity of the endometrial thickness measurement was 17% for a threshold of 6 mm and 33% for a threshold of 5 mm.[8]

To date, imaging techniques have a role on presurgery stratification rather than on diagnosis. Magnetic resonance imaging (MRI) has become an indispensable tool in the assessment of malignant diseases. In gynecological malignancies, this modality has to assume greater responsibility, particularly in the evaluation of cervical and ECs. In addition to conventional imaging, innovative techniques such as dynamic contrast-enhanced MRI and diffusion-weighted MRI show promise in offering early assessment of tumor response.[9] Concerning PET/PET-computed tomography (CT) in the management of gynecological malignancies, the promise of this technique is becoming increasingly evident. 2-Fluoro-2-deoxy-D-glucose-PET appears to have a potential role in assessing response to treatment and forecasting prognosis. With regard to its role in EC, its benefit is particularly emphasized in the setting of post-therapy surveillance of the disease, although, in a limited series, it also appears to give additional information in the pretreatment states. PET may be of value in detecting the extrauterine lesions that are not visualized with CT/MRI.[10]

The precise molecular events that occur during the development, progression/invasion and formation of metastasis in EC are largely uncharacterized and are still poorly understood.[3] Type I cancers are typically known to have alterations in PTEN, KRAS2, DNA mismatch repair defects, CTNNB1, and have near diploid karyotype. Type II cancers typically have TP53 mutations and ErBB2 overexpression and are mostly nondiploid.[11]

A number of studies have examined gene expression profiles for classifying uterine cancers. Sugiyama *et al.* reported 45 genes highly expressed in Type I cancers and 24 highly expressed in Type II cancers.[12] Risinger *et al.* reported distinct gene expression profiles among different histologic subtypes of ECs by microarray analysis, with 191 genes exhibiting greater than two-fold difference in expression between endometrioid and nonendometrioid ECs.[13] In our group, we have identified a number of genes associated with endometrial carcinogenesis.[14] Serum markers for the detection of uterine cancer have also been reported in the literature. Yurkovetsky *et al.* identified that prolactin is a serum biomarker with sensitivity and specificity for EC.[15] They found that serum CA 125, CA 15-3 and CEA are higher in patients with Stage III disease when compared to Stage I, and a five-biomarker panel of prolactin, GH, eotaxin, E-selectin and TSH discriminated EC from ovarian and breast cancer. Nevertheless, none of these potential biomarkers has been validated nor reached the clinical practice.

All these evidences generate a scenario where (*i*) there is a need for less invasive methods of screening for EC, which are less subjective in interpretation, and (*ii*) there is a need for new markers that are useful for the early detection of EC. Clearly, there is room for improvement in the tools currently available for screening for EC. In our work, we present two main findings: first, the identification and validation of new potent molecular biomarkers for the detection of EC. Second, the successful application of these biomarkers to a minimally invasive uterine fluid representative of the primary tumor. The result is a minimally invasive and a highly sensitive and specific method for the identification of EC. This could be of help for the pathologists as a molecular precise tool for diagnosing ECs and for the gynecologists for reducing unnecessary biopsies.

Moreover, the American Cancer Society (ACS) concluded that there was insufficient evidence to recommend screening for EC in women at average risk or those who were at an increased risk because of a history of unopposed estrogen therapy, tamoxifen therapy, late menopause, nulliparity, infertility or failure to ovulate, obesity, diabetes or hypertension. The ACS recommends that women at average and increased risk should be informed about the risks and symptoms (in particular, unexpected bleeding and spotting) of EC at the onset of menopause and should be strongly encouraged to immediately report these symptoms to their physician. Women at very high risk for EC because of (*i*) known HNPCC genetic mutation carrier status; (*ii*) a substantial likelihood of being a mutation carrier (*i.e.*, a mutation is known to be present in the family) or (*iii*) the absence of genetic testing results in families with a suspected autosomal dominant predisposition to colon cancer should consider beginning annual testing for the detection of early EC at age 35 years. The evaluation of endometrial histology with the endometrial biopsy is still the standard for determining the status of the endometrium. Women at high risk should be informed that the recommendation for screening is based on expert opinion, and they also should be informed about potential benefits, risks and limitations of testing for the detection of early EC.[16]

Among the clinical applications of the molecular biomarkers that we identified, a test based on our findings could be appropriate to screening programs in high-risk feminine populations, as a highly sensitive and specific minimally invasive method for the diagnosis of EC on uterine aspirates. The high sensitivity and specificity demonstrated by the new identified biomarkers among uterine aspirates corresponding to initial stages of ECs further strengthen their potential on early detection. A clinical study in a large cohort of patients within several clinical institutions is actually ongoing to evaluate the validity and clinical applications of this new EC diagnostic test.

References

1. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Murray T, Thun MJ. Cancer statistics, 2008. *CA Cancer J Clin* 2008;58:71–96.
2. Baekelandt MM,Castiglione M; ESMO Guidelines Working Group. Endometrial carcinoma: ESMO clinical recommendations for diagnosis, treatment and follow-up. *Ann Oncol* 2009;20:29–31.
3. Abal M, Llaurado M, Doll A, Monge M, Colas E, Gonzalez M, Rigau M, Alazzouzi H, Demajo S, Castellvi J, Garcia A, Ramon y Cajal S, et al. Molecular determinants of invasion in endometrial cancer. *Clin Transl Oncol* 2007;9:272–7.
4. Baldi P, Long AD. A Bayesian framework for the analysis of microarray expression data: regularized t -test and statistical inferences of gene changes. *Bioinformatics* 2001;17:509–19.
5. Monge M, Doll A, Colas E, Gil-Moreno A, Castellvi J, Garcia A, Colome N, Perez-Benavente A, Pedrola N, Lopez-Lopez R, Dolcet X, Ramon y Cajal S, et al. Subtractive proteomic approach to the endometrial carcinoma invasion front. *J Proteome Res* 2009;8:4676–84.
6. Francis JA, Weir MM, Ettler HC, Qiu F, Kwon JS. Should preoperative pathology be used to select patients for surgical staging in endometrial cancer? *Int J Gynecol Cancer* 2009;19:380–4.
7. Gull B, Karlsson B, Milsom I, Granberg S. Can ultrasound replace dilation and curettage? A longitudinal evaluation of postmenopausal bleeding and transvaginal sonographic measurement of the endometrium as predictors of endometrial cancer. *Am J Obstet Gynecol* 2003;188: 401–8.
8. Fleischer AC, Wheeler JE, Lindsay I, Hendrix SL, Grabill S, Kravitz B, MacDonald B. An assessment of the value of ultrasonographic screening for endometrial disease in postmenopausal

women without symptoms. *Am J Obstet Gynecol* 2001;184:70–5.
9. Harry VN, Deans H, Ramage E, Parkin DE, Gilbert FJ. Magnetic resonance imaging in gynecological oncology. *Int J Gynecol Cancer* 2009;19:186–93.
10. Basu S, Li G, Alavi A. PET and PET-CT imaging of gynecological malignancies: present role and future promise. *Expert Rev Anticancer Ther* 2009;9:75–96.
11. Doll A, Abal M, Rigau M, Monge M, Gonzalez M, Demajo S, Colas E, Llaurado M, Alazzouzi H, Planaguma J, Lohmann MA, Garcia J, et al. Novel molecular profiles of endometrial cancer-new light through old windows. *J Steroid Biochem Mol Biol* 2008;108:221–9.
12. Sugiyama Y, Dan S, Yoshida Y, Akiyama F, Sugiyama K, Hirai Y, Matsuura M, Miyata S, Ushijima M, Hasumi K, Yamori T. A large-scale gene expression comparison of microdissected, small-sized endometrial cancers with or without hyperplasia matched to same-patient normal tissue. *Clin Cancer Res* 2003;9: 5589–600.
13. Risinger JI, Maxwell GL, Chandramouli GV, Jazaeri A, Aprelikova O, Patterson T, Berchuck A, Barrett JC. Microarray analysis reveals distinct gene expression profiles among different histologic types of endometrial cancer. *Cancer Res* 2003;63:6–11.
14. Planaguma J, Diaz-Fuertes M, Gil-Moreno A, Abal M, Monge M, Garcia A, Baro T, Thomson TM, Xercavins J, Alameda F, Reventos J. A differential gene expression profile reveals overexpression of RUNX1/ AML1 in invasive endometrioid carcinoma. *Cancer Res* 2004;64:8846–53.
15. Yurkovetsky Z, Ta'asan S, Skates S, Rand A, Lomakin A, Linkov F, Marrangoni A, Velikokhatnaya L, Winans M, Gorelik E, Maxwell GL, Lu K, et al. Development of multimarker panel for early detection of endometrial cancer. High diagnostic power of prolactin. *Gynecol Oncol* 2007;107:58–65.
16. Smith RA, Cokkinides V, Brawley OW. Cancer screening in the United States, 2009: a review of current American Cancer Society guidelines and issues in cancer screening. *CA Cancer J Clin* 2009;59:27–41.