

Expression profiling of 22 genes involved in the PI3K–AKT pathway identifies two subgroups of high-grade endometrial carcinomas with different molecular alterations

Lluís Catusus, Emanuela D'Angelo, Cristina Pons, Iñigo Espinosa and Jaime Prat

Department of Pathology, Hospital de la Santa Creu i Sant Pau, Autonomous University of Barcelona, Barcelona, Spain

Previously, we showed that *PIK3CA* and *p53* alterations in uterine endometrial carcinomas correlate with poor prognosis. However, the contribution of phosphatidylinositol 3-kinase (PI3K)–AKT deregulation to endometrial carcinogenesis is not completely understood. The purpose of this study was to analyze alterations of this pathway in endometrial carcinomas and correlate them with the most common genetic abnormalities. Expression profiling of 22 genes involved in PI3K–AKT signaling pathway was analyzed in 38 endometrial carcinomas using TaqMan low-density array (TLDA) analysis. The gene expression pattern was analyzed by hierarchical clustering analysis. Unsupervised clustering divided the high-grade endometrial carcinomas into two clusters. One cluster identified tumors with alterations in the PI3K–AKT signaling pathway (exon 20 *PIK3CA* mutations and/or *PTEN* mutations 9/15; 60%), and p16 protein overexpression (8/13; 62%). Almost all non-endometrioid adenocarcinomas (serous and clear cell adenocarcinomas) were segregated into this cluster. In contrast, the other cluster identified tumors with *p53* alterations (6/6; 100%), p16 protein overexpression (5/5; 100%), and exon 9 *PIK3CA* mutations (2/6; 33%). Exon 20 *PIK3CA* and *PTEN* mutations were not found in this subgroup. Low-grade endometrial carcinomas clustered in a third subgroup characterized by high frequency of *PTEN* mutations (10/17; 59%) and microsatellite instability (6/17; 35%). Our results show that gene expression profile differences in the PI3K–AKT signaling pathway identify two subgroups of high-grade endometrial carcinomas with different molecular alterations (PI3K–AKT pathway vs *p53* alterations) that may have distinct roles in endometrial carcinogenesis. Identification of these subgroups can provide insight into the biology of these tumors and may facilitate the development of future treatments.

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The phosphatidylinositol 3-kinase (PI3K)–AKT signaling pathway regulates the expression of several downstream target genes that inhibit apoptosis and promote cell proliferation.^{1,2} The effect of its deregulation on the clinical outcome of human tumors has recently become a subject of intense investigation.

Activation of this pathway has been associated with aggressive phenotype and poor prognosis in brain tumors (glioblastoma and neuroblastoma) as well as in various carcinomas, including breast, prostate, bladder, colon, and lung carcinomas.^{3–9}

Endometrial carcinoma is the most common malignant tumor of the female genital tract and the fourth most common female cancer in the Western World.¹⁰ It has been divided into two major types.^{11–13} Type I carcinomas are largely low-grade endometrioid carcinomas that occur in pre- and perimenopausal women. They are estrogen dependent, frequently preceded by endometrial hyperplasia,

Correspondence: Professor J Prat, MD, PhD, FRCPath, Department of Pathology, Hospital de la Santa Creu i Sant Pau, Avenida Sant Antoni Maria Claret 167, Barcelona 08025, Spain.

E-mail: jprat@santpau.cat

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and characterized by microsatellite instability, *PTEN*, *PIK3CA*, *CTNNB1*, and *K-RAS* mutations.^{14–19} Type II carcinomas are high-grade non-endometrioid carcinomas that develop in post-menopausal women and are commonly associated with *p53* alterations.^{20–22} Although surgery is still the main form of treatment, some patients with advanced-stage tumors have benefited from chemotherapy.^{23,24}

Recently, we reported that *PIK3CA* mutations are frequent events in endometrial carcinomas of any histological type. Whereas carcinomas with exon 20 *PIK3CA* mutations or *PIK3CA* mRNA overexpression are often high-grade carcinomas associated with myometrial invasion, tumors that carried exon 9 mutations are more likely low-grade endometrioid carcinomas.¹⁶ We also found that alterations in the PI3K-AKT signaling pathway (*PIK3CA* and/or *PTEN* mutations) alone did not influence overall survival; however, patients with deregulated PI3K-AKT pathway who also had *p53* alterations had shorter survival than patients with only *p53* alterations.²⁵

Nevertheless, the role of deregulations of the PI3K-AKT signaling pathway in the pathogenesis of endometrial carcinoma is only partially understood, as unrecognized alterations in less popular genes may be involved in tumor development. In an attempt to uncover new alterations of the PI3K-AKT pathway, we have examined the expression profiling of 22 involved genes in 38 endometrial carcinomas by Taqman low-density array (TLDA). Subsequently, we analyzed the gene expression pattern by a hierarchical clustering analysis and correlated the results with the clinicopathological features of the tumors and their molecular genetic alterations.

Materials and methods

Selecting Tissue Samples

Samples from 38 endometrial carcinomas were retrieved from the Tumor Bank and the Surgical Pathology files of Hospital de la Santa Creu i Sant Pau, Barcelona, Spain. All cases were reviewed and classified using the 2003 World Health Organization (WHO) criteria. Tumors showing crowded glands resembling those of the normal endometrium were diagnosed as endometrioid adenocarcinomas. They were classified as high grade when the glandular differentiation decreased and the glands were replaced by solid nests. The term serous carcinoma was applied to tumors composed of cells with marked nuclear atypia, cellular budding, and psammoma bodies. The diagnosis of clear cell adenocarcinoma was made when the tumor consisted of clear or hobnail cells with large and pleomorphic nuclei and prominent nucleoli. Mixed adenocarcinomas were defined as neoplasms with endometrioid and non-endometrioid components with the minor component comprising at least 10% of the total volume of the tumor. We selected a similar number of low-grade and high-grade carcinomas. High-grade

tumors ($n = 21$) consisted of 8 grade 3 endometrioid adenocarcinomas, 8 non-endometrioid adenocarcinomas (4 serous, 3 clear cell, and 1 mixed serous-clear cell adenocarcinoma), and 5 mixed endometrioid and non-endometrioid adenocarcinomas. All low-grade tumors were grade 1 and 2 endometrioid adenocarcinomas ($n = 17$). Cases were anonymized and the study was approved by the institutional ethics committee. The cases had previously been subjected to determination of microsatellite instability status, and analysis of mutations in the *PTEN*, *PIK3CA*, and *p53* genes.^{16,25}

RNA Extraction and cDNA Synthesis

Total RNA from tumors and corresponding non-tumor tissues was extracted from fresh frozen biopsies using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and the RNeasy mini kit (Qiagen, Hilden, Germany) as specified by the manufacturer's instructions. RNA was eluted in 25 μ l of RNase-free water. RNA yield and quality were assessed by agarose electrophoresis and spectrophotometry, and then stored at -80°C . RNA was digested with DNase I (Invitrogen). Total RNA of 1 μ g was used for cDNA synthesis according to the protocol provided with the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). Recombinant RNasin ribonuclease inhibitor (Applied Biosystems) was added to prevent RNase-mediated degradation. The cDNA was stored at -20°C .

Gene Expression Analyses

Gene expression analyses were performed at mRNA level by TLDA. Pre-designed TaqMan probe and primer sets for target genes were chosen from an on-line catalog (Applied Biosystems). Once selected, the sets were factory loaded into the 384 wells of TLDA cards. Each TLDA card was configured into eight identical 24 gene sets in duplicate. In all, 22 genes were chosen based on literature reviews of the PI3K-AKT, apoptosis, and cell cycle molecular pathways and their involvement in carcinogenesis.^{1,2,26,27} Each set of genes also contained two housekeeping genes, *GAPDH* and *ABL-1* (Table 1). We mixed 5 μ l of single-stranded cDNA (equivalent to 100 ng of total RNA) with 45 μ l of nuclease-free water and 50 μ l of TaqMan Universal PCR Master Mix. After gentle mixing and centrifugation, 100 μ l of mixture was transferred into a loading port on a TLDA card. The card was centrifuged twice for 1 min at 1100 r.p.m. to distribute the samples from the loading port into each well. It was then sealed and placed in the micro fluidic card sample block of an Applied Biosystems 7900HT PCR system (Applied Biosystems). The thermal cycling conditions were 2 min at 50°C and 10 min at 95°C , followed by 40 cycles of 15 s at 95°C and 1 min at 60°C . Expression levels were measured in duplicate. Only

Table 1 Gene expression assays used for configuring the TLDA card

Gene symbol	Gene name	Ref. sequence	Assay ID	Amplicon length
<i>XIAP (BIRC4)</i>	X-linked inhibitor of apoptosis	NM_001167.2	Hs00236913_m1	116
<i>AKT1</i>	v-akt murine thymoma viral oncogene homolog 1	NM_005163	Hs00178289_m1	66
<i>TWIST1</i>	Twist homolog 1 (Drosophila)	NM_000474.3	Hs00361186_m1	115
<i>BAD</i>	BCL2-associated agonist of cell death	NM_004322.2	Hs00188930_m1	69
<i>CDKN1A (p21)</i>	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	NM_000389.2	Hs00355782_m1	66
<i>ABL1</i>	v-abl Abelson murine leukemia viral oncogene homolog 1	NM_005157.3	Hs00245443_m1	Endogenous control
<i>CDH1</i>	Cadherin 1, type 1, E-cadherin	NM_004360.3	Hs00170423_m1	117
<i>TP53</i>	Tumor protein p53	NM_000546	Hs00153340_m1	81
<i>CASP3</i>	Caspase 3, apoptosis-related cysteine peptidase	NM_004346.2	Hs00263337_m1	111
<i>PAK1</i>	p21/Cdc42/Rac1-activated kinase 1	NM_002576.4	Hs00176815_m1	93
<i>GAPDH</i>	Glyceraldehyde-3-phosphate dehydrogenase	NM_002046.3	Hs99999905_m1	Endogenous control
<i>PIK3CA</i>	Phosphoinositide-3-kinase, catalytic, α -polypeptide	NM_006218.2	Hs00180679_m1	104
<i>FAS</i>	TNF receptor superfamily, member 6	NM_000043.3	Hs00531110_m1	97
<i>AKT2</i>	v-akt murine thymoma viral oncogene homolog 2	NM_001626.3	Hs00609846_m1	129
<i>FRAP1 (mTOR)</i>	FK506 binding protein 12-rapamycin associated protein 1	NM_004958.3	Hs00234522_m1	71
<i>FOXO1A</i>	Forkhead box O1	NM_002015.3	Hs00231106_m1	103
<i>PTK2 (FAK)</i>	PTK2 protein tyrosine kinase 2	NM_005607.3	Hs00178587_m1	68
<i>CASP9</i>	Caspase 9, apoptosis-related cysteine peptidase	NM_001229.2	Hs00154260_m1	71
<i>PTEN</i>	Phosphatase and tensin homolog	NM_000314.4	Hs00829813_s1	154
<i>CCND1</i>	Cyclin D1	NM_053056.2	Hs00277039_m1	94
<i>NFKB1</i>	Nuclear factor κ -light polypeptide gene enhancer B-cells 1	NM_003998.2	Hs00765730_m1	66
<i>GSK3B</i>	Glycogen synthase kinase 3- β	NM_002093.2	Hs00275656_m1	73
<i>MDM2</i>	Mdm2 p53 binding protein homolog (mouse)	NM_002392.2	Hs01066938_m1	150
<i>CDKN1B (p27)</i>	Cyclin-dependent kinase inhibitor 1B (p27, Kip1)	NM_004064.3	Hs00153277_m1	71

TLDA, TaqMan low-density array.

the genes with reproducible amplification curves of both duplicates were analyzed and presented. TLDA cards were analyzed with RQ Manager Software for automated data analysis. Gene expression values RQ were calculated based on the $\Delta\Delta C_t$ method. Delta cycle threshold (C_t) values, defined as the point at which the fluorescence rises above the background fluorescence, were calculated with SDS 2.3 software (Applied Biosystems). Normal endometrium pool tissue was used as a calibrator and *ABL-1* house-keeping gene was the reference for normalization. Gene upregulated was arbitrarily designated as $\times 2$ or higher, and gene downregulated as ≤ 0.5 when compared with the expression of the reference sample.

Hierarchical Clustering Analysis

Hierarchical clustering analysis of TLDA data was performed using the *Cluster* program and the results were visualized using the *TreeView* program from Stanford as previously reported.²⁸ Both programs can be downloaded at: <http://rana.lbl.gov/eisen/>. Clustered data were shown with tumors on the horizontal axis and genes on the vertical axis.

Immunohistochemical Analysis

Protein p16(INK4a) expression was evaluated by immunohistochemistry on tissue arrays constructed with a tissue arrayer device (Beecher Instruments, Sun Prairie, WI, USA). Immunohistochemistry was

performed on paraffin-embedded tissue sections using the EnVision system (Dako, Glostrup, Denmark) and diaminobenzidine as the chromogen. Sections were stained with monoclonal anti-p16 antibody (MTM, Heidelberg, Germany) using optimized protocols. The reactions were carried out in an autostainer immunostainer. The staining intensity and area extent of the immunohistochemistry was evaluated using the semiquantitative scoring system. The stained sections were semiquantitatively scored by two pathologists. The score (H-score) was calculated by multiplying the staining intensity (0 = no staining; 1 = weak; 2 = moderate; and 3 = strong) by the percentage of immunoreactive tumor cells (0–100). P16 immunostaining was considered positive when the H-score was ≥ 150 . This cutoff was chosen because it was close to the median protein expression in our cases.

Statistical Analysis

Statistical analysis was performed using the statistical package SPSS/win 17.0 (SPSS, Chicago, IL, USA). The following clinicopathological parameters were evaluated: age, tumor size, histological type and grade, depth of myometrial invasion, lymphovascular invasion, clinicopathological stage, hormone receptor status, MI status, p16 protein expression, *PIK3CA*, *PTEN*, and *p53* mutations, and patient outcome. A value of $P \leq 0.05$ was considered statistically significant.

Results

Clinical and Pathological Findings

The clinicopathological features of the 38 endometrial carcinomas are summarized in Table 2. In brief, age of the patients ranged from 35 to 82 years (mean 68 years). Tumor size varied from 1.2 to 11 cm (mean 5 cm). Of the 38 cases, 25 (66%) were endometrioid, 8 (21%) non-endometrioid (4 serous, 3 clear cell, and 1 mixed clear cell-serous carcinoma), and 5 (13%) mixed endometrioid and non-endometrioid adenocarcinomas (3 endometrioid-clear cell and 2 endometrioid-serous carcinomas). Over half (55%) of the tumors were grade 3. Most tumors were FIGO stage I ($n=28$; 74%). Myometrial invasion was found in 34 cases ($<1/2$ of the myometrial thickness in 17 cases and $>1/2$ in 17). Vascular invasion was observed in one third of cases.

TLDA and Clustering Analysis

Because the expression values of *GAPDH* showed distinct variations among the samples, *ABL-1* was exclusively used as the housekeeping gene. In addition, three of 22 genes implicated in the PI3K-AKT pathway were excluded because their signal intensities were too weak.

According to the clustering index, samples clustered into three major groups (clusters 1, 2, and 3;

Figure 1). Cluster 1 consisted of 15 tumors, which were mostly high-grade endometrial carcinomas (14/15; 93%). They included 1 low-grade endometrioid carcinoma, 5 high-grade endometrioid carcinomas, 7 non-endometrioid carcinomas (4 serous and 3 clear cell carcinomas), and 2 mixed carcinomas (1 endometrioid-clear cell and 1 endometrioid-serous carcinomas). Except *Foxo1A* and *p27*, most genes were highly expressed, particularly *CCND1* and *E-CADH*, which were consistently upregulated by more than eightfold compared with normal endometrium pool. Cluster 2 contained 17 tumors, which were mostly low-grade endometrioid carcinomas (15/17; 88%). They included 15 low-grade endometrioid carcinomas, 1 high-grade endometrioid carcinoma, and 1 mixed endometrioid-clear cell carcinoma. These tumors showed upregulation of *p21*, *FAK*, *PAK1*, and *CCND1* and downregulation of *CASP3*, *Foxo1A*, *PIK3CA*, *p27*, and *NFKB*. Last, cluster 3 contained six tumors, which were mostly high-grade endometrial carcinomas (5/6; 83%). They included 1 low-grade endometrioid carcinoma, 2 high-grade endometrioid carcinomas, 1 non-endometrioid carcinoma (mixed serous-clear cell carcinoma), and 2 mixed carcinomas (1 endometrioid-clear cell and 1 endometrioid-serous carcinoma). These tumors showed overexpression of *PTEN* and underexpression of *XIAP*. Median RQ values of analyzed genes according to the three clusters are shown in Table 3. Plot box graphs of RQ values expression for *FOXO1A*, *FAK*, *GSK3-β*, and *PTEN* in the three clusters are shown in Figure 2.

Table 2 Clinicopathological features of 38 endometrial carcinomas

Age (mean)	68 years (35–82 years)
Tumor size (mean)	5 cm (1.2–11 cm)
<i>Histological type</i>	
EEC low grade (grades 1–2)	17 (45%)
EEC high grade (grade 3)	8 (21%)
NEEC	8 (21%)
Mixed EEC-NEEC	5 (13%)
<i>Grade</i>	
1	8 (21%)
2	9 (24%)
3	21 (55%)
<i>Stage</i>	
I	28 (74%)
II	2 (5%)
III	5 (13%)
IV	3 (8%)
<i>Myometrial invasion</i>	
No	4 (10%)
< 50%	17 (45%)
> 50%	17 (45%)
<i>Vascular invasion</i>	
No	25 (66%)
Yes	13 (34%)

EEC, endometrioid endometrial carcinoma; NEEC, non-endometrioid endometrial carcinoma.

Association of Clusters Subgroups with the Clinicopathological Parameters and Molecular Alterations

A relationship between the three clustering subgroups, the clinicopathological prognostic parameters, and the most common molecular genetic alterations was analyzed (Figure 1 and Table 4). Histological type and grade varied significantly according to the tumor distribution into the three clusters ($P=0.000$ for both). Most low-grade endometrioid carcinomas (15/17; 88%) were in cluster 2, whereas the majority of non-endometrioid carcinomas (7/8; 88%) were in cluster 1. The non-endometrioid carcinomas segregated into this cluster represented a heterogeneous group of tumors composed of four serous, and three clear cell carcinomas. In addition, high-grade endometrioid carcinomas were mainly distributed in cluster 1 (5/8; 63%), whereas mixed tumors were allocated into the three clusters. All grade 1 tumors (8/8; 100%), and almost all grade 2 tumors (7/9; 78%) were segregated into cluster 2, whereas grade 3 carcinomas were mostly found in clusters 1 and 3 (19/21; 90%). Vascular invasion was more commonly encountered in cluster 3 tumors (3/6; 50%) than in clusters 1 (5/15; 33%) and 2 (5/17; 29%).

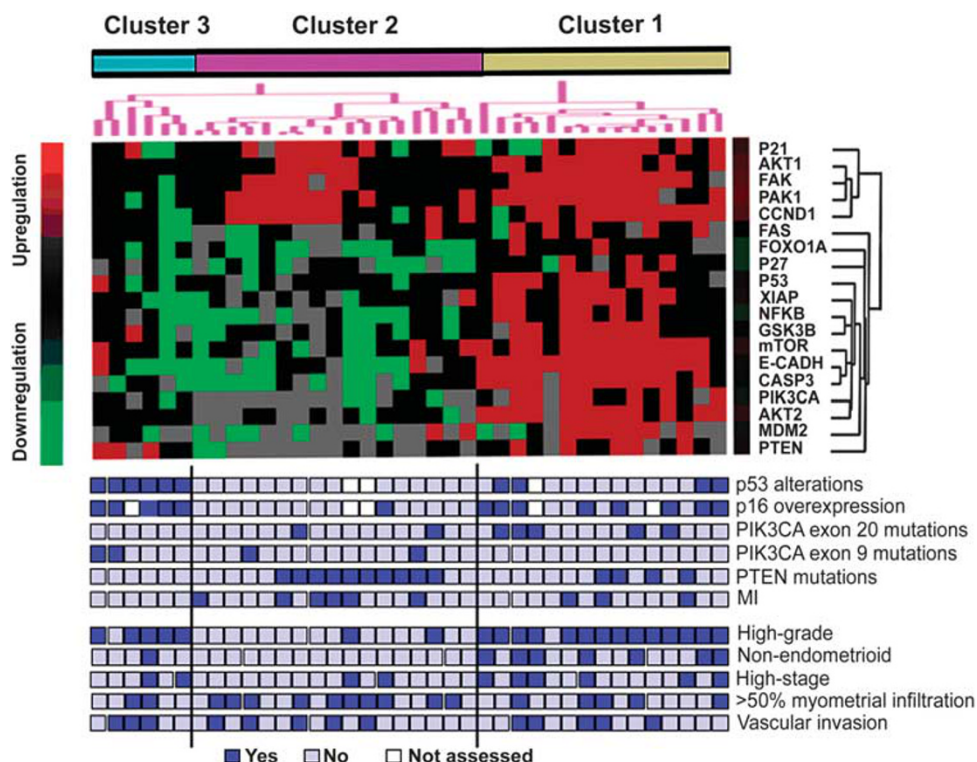


Figure 1 Hierarchical clustering analysis of mRNA expression of 19 genes in 38 endometrial carcinomas. Upregulated and downregulated expression is indicated as red and green cubes, respectively. Genes that did not vary in their expression level are shown in black and genes with unsatisfactory results are labeled in gray. Enclosed in the clustering image, results of *p53*, *p16*, *PIK3CA*, *PTEN*, and MI analysis as well as clinicopathological parameters, such as high-grade (grade 3), non-endometrioid, high-stage (stage 2 or higher), myometrial invasion (>50%), and vascular invasion, are graphically represented for each case.

Table 3 Median RQ values of investigated genes (range of values in parentheses) according to the three clusters

	Cluster 1	Cluster 2	Cluster 3	P-value ^a
<i>CASP3</i>	6.58 (1.18–27.15)	0.49 (0.02–1.33)	0.52 (0.33–0.66)	0.000
<i>CCND1</i>	9.69 (0.42–65.98)	2.78 (0.42–7.45)	0.95 (0.30–1.43)	0.006
<i>AKT2</i>	7.74 (1.48–34.51)	0.84 (0.13–1.44)	1.22 (0.75–1.62)	0.000
<i>XIAP</i>	2.96 (0.99–6.42)	0.90 (0.14–2.0)	0.45 (0.19–0.78)	0.000
<i>FAS</i>	3.75 (0.28–17.78)	1.58 (0.08–9.61)	0.73 (0.22–1.28)	0.032
<i>AKT1</i>	3.69 (1.43–7.66)	1.97 (0.54–5.65)	0.79 (0.51–1.31)	0.000
<i>MTOR</i>	5.19 (1.43–16.30)	0.91 (0.25–3.17)	1.25 (0.34–2.74)	0.000
<i>MDM2</i>	3.73 (0.35–8.12)	1.28 (0.22–4.63)	0.92 (0.0–1.48)	0.054
<i>GSK3-β</i>	3.67 (1.34–12.70)	0.64 (0.10–1.64)	1.00 (0.09–2.12)	0.000
<i>PAK1</i>	2.93 (1.10–7.48)	3.18 (0.65–10.64)	0.59 (0.23–1.08)	0.001
<i>FOXO1A</i>	1.71 (0.52–7.01)	0.43 (0.06–1.50)	0.75 (0.41–1.33)	0.000
<i>PTEN</i>	2.76 (0.65–10.70)	0.82 (0.11–1.76)	2.01 (0.73–2.66)	0.064
<i>FAK</i>	4.24 (1.05–8.08)	2.30 (1.25–7.46)	0.90 (0.37–1.66)	0.000
<i>PIK3CA</i>	3.15 (0.50–7.84)	0.46 (0.19–0.63)	0.87 (0.37–1.71)	0.003
<i>E-CADH</i>	15.10 (0.50–91.54)	0.69 (0.10–2.42)	0.73 (0.01–2.56)	0.000
<i>P27</i>	1.39 (0.38–4.61)	0.50 (0.17–1.08)	0.63 (0.47–1.02)	0.005
<i>P21</i>	3.87 (0.30–13.35)	3.89 (0.44–16.51)	0.94 (0.27–2.08)	0.119
<i>P53</i>	3.72 (0.72–14.39)	0.85 (0.36–1.89)	1.33 (0.10–5.42)	0.000
<i>NFKB</i>	2.12 (0.91–6.80)	0.39 (0.10–0.98)	0.57 (0.31–1.09)	0.000

^aP-values were calculated using Kruskal–Wallis test.

Upregulated genes (median RQ ≥ 2) are shown in red, downregulated genes (median RQ ≤ 0.5) in green, and genes that did not vary in their expression level (median RQ interval 0.5–2) are shown in black.

Moreover, myometrial invasion (>50%) was more frequent in cluster 2 tumors (10/17; 59%) than in clusters 1 (5/15; 33%) and 3 (2/6; 33%). In contrast,

cluster 2 tumors were more frequently low stage (stage 1, 15/17; 88% vs stage ≥ 2, 2/17; 12%) than clusters 1 and 3 (stage 1, 13/21; 62% vs stage ≥ 2, 8/21; 38%).

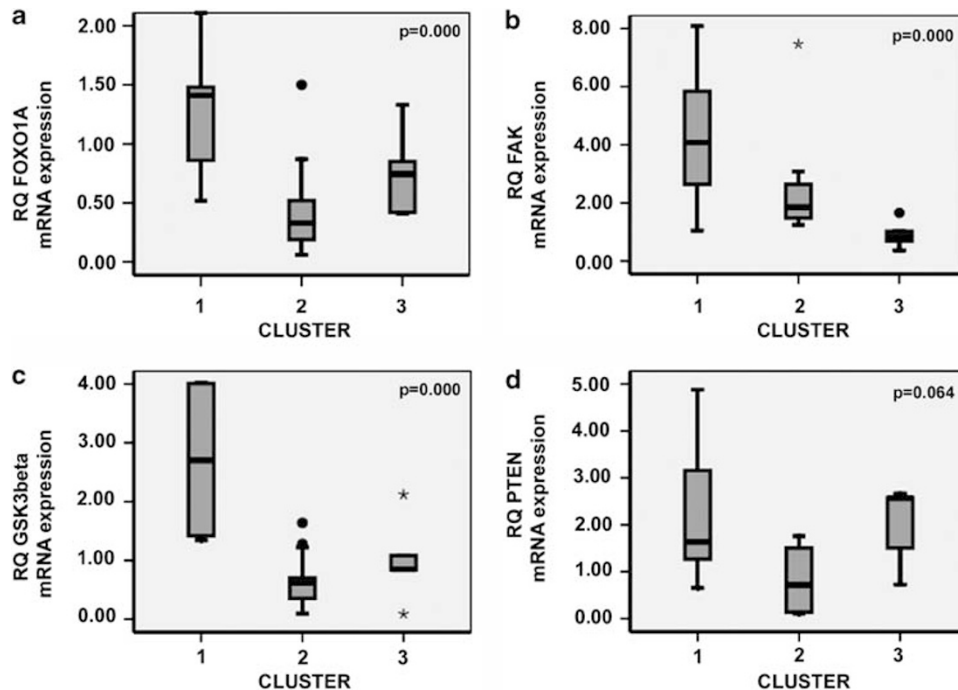


Figure 2 Plot box graphs representing RQ values expression for *FOXO1A* (a), *FAK* (b), *GSK3-β* (c), and *PTEN* (d) in the three clusters.

The cases have been previously subjected to determination of the most frequent molecular genetic alterations of endometrial carcinomas, including microsatellite instability status, and mutational analysis of *PTEN*, *PIK3CA*, and *p53* genes.^{16,25} Only *PTEN* mutations and *p53* alterations varied significantly according to the tumor distribution into the three clusters ($P=0.017$ and $P=0.000$, respectively). *PTEN* mutations were more frequent in cluster 2 tumors (10/17; 59%) than in cluster 1 carcinomas (4/15; 27%). Moreover, no *PTEN* mutation was found in cluster 3. *P53* alterations were present in all cluster 3 tumors (6/6; 100%), in some cluster 1 tumors (4/14; 29%), and in none of cluster 2 tumors. Carcinomas with exon 20 *PIK3CA* mutations (seven cases) were mostly high-grade carcinomas, and were almost all segregated into cluster 1 (five cases). None was found in cluster 3. In contrast, tumors that carried exon 9 *PIK3CA* mutations (four cases) were mostly low-grade endometrioid carcinomas (three cases), and were segregated into clusters 2 and 3. None was encountered in cluster 1. MI was found more commonly in cluster 2 (6/17; 35%) than in cluster 1 (3/15; 20%) or cluster 3 carcinomas (0/6; 0%), but this association was not statistically significant. P16 protein was strongly expressed (score > 150) in 14 of 33 (42%) endometrioid tumors, more frequently in mixed endometrioid and non-endometrioid carcinomas (4/4; 100%), and non-endometrioid carcinomas (6/7; 86%) than in high-grade endometrioid carcinomas (2/6; 33%) or in low-grade endometrioid carcinomas (2/16; 13%; $P=0.000$). Expression of p16 protein varied

significantly according to the tumor distribution into the three clusters ($P=0.000$); that is, it was strongly positive in all cluster 3 tumors (5/5), 62% (8/13) cluster 1 tumors, and only 7% (1/15) of cluster 2 tumors.

Discussion

The PI3K–AKT pathway is frequently deregulated in various human epithelial tumors, including endometrial carcinomas. Alterations include mutation or amplification of *PIK3CA*, loss of *PTEN* function, mutation or amplification of *AKT*, and activation of growth factor receptors. Deregulated PI3K–AKT signaling pathway has been associated with aggressive tumor phenotype and poor prognostic parameters, including high grade, large size, nodal involvement, vascular invasion, and high proliferative fraction in breast cancer.^{3–9}

Microarrays and real-time reverse transcription-PCR (qRT-PCR) analyses for identification of differences in gene expression patterns have increased our understanding of relevant genetic events, and have contributed to further characterize the molecular genetic model for carcinogenesis. Gene expression profiling coupled with statistical method by clustering analysis is an important tool to evaluate genetic heterogeneity in carcinomas and is useful to develop expression-based classifications for many types of cancer, as well as markers of disease outcome.^{29,30}

Table 4 Clinical and molecular features of 38 endometrial carcinomas distributed in the three cluster subgroups

	Total n (%)	Cluster 1 15 (39%)	Cluster 2 17 (45%)	Cluster 3 6 (16%)	P- value ^a
<i>Histological subtype</i>					
EEC low grade	17	1	15	1	0.000
EEC high grade	8	5	1	2	
NEEC	8	7	0	1	
EEC-NEEC	5	2	1	2	
<i>Grade</i>					
1	8	0	8	0	0.000
2	9	1	7	1	
3	21	14	2	5	
<i>Vascular invasion</i>					
No	25	10	12	3	NS
Yes	13	5	5	3	
<i>Stage</i>					
1	28	9	15	4	0.094
2	2	2	0	0	
3	5	3	2	0	
4	3	1	0	2	
<i>Myometrial invasion</i>					
<50%	21	10	7	4	NS
>50%	17	5	10	2	
<i>PIK3CA mutations</i>					
No	27	10	13	4	NS
Yes	11	5	4	2	
<i>PTEN mutations</i>					
No	24	11	7	6	0.017
Yes	14	4	10	0	
<i>Microsatellite status</i>					
MS	29	12	11	6	NS
MI	9	3	6	0	
<i>P53 alterations</i>					
No	25	10	15	0	0.000
Yes	10	4	0	6	
<i>P16 immunoexpression</i>					
No (score ≤150)	19	5	14	0	0.000
Yes (score >150)	14	8	1	5	

EEC, endometrioid endometrial carcinoma; NEEC, non-endometrioid endometrial carcinoma; NS, nonsignificant.

^aP-values were calculated using Fisher's test.

In this study, we have analyzed the gene expression profile differences of the PI3K-AKT signaling pathway in 38 endometrial carcinomas, including endometrioid, non-endometrioid, and mixed endometrioid and non-endometrioid carcinomas. Gene expression analyses were performed at mRNA level by TLDA,³¹⁻³³ and the gene expression pattern was analyzed by a hierarchical clustering analysis.²⁸

Two groups of high-grade endometrial carcinomas with different molecular alterations were identified. One group (cluster 1) had exon 20 *PIK3CA* and/or *PTEN* mutations and the other (cluster 3) had *p53* alterations and exon 9 *PIK3CA* mutations. Exons 9 and 20 of *PIK3CA* encode different domains and it is

reasonable to speculate that they may have different oncogenic potential.³⁴⁻³⁶ These findings are compatible with our recent study in which mutations of exons 9 and 20 had prognostic implications.^{16,25} Although both groups of tumors have similar clinicopathological features, our results support the existence of a dualistic model for high-grade endometrial carcinomas.

A recent study has reconfirmed that the *PIK3CA* mutations occur almost exclusively in invasive tumors, whereas upstream mutations of the PI3K-AKT pathway (*PTEN* and *K-RAS* mutations) occur with equal frequency in early- and advanced-stage tumors. This finding suggests that the *PIK3CA* mutations cooperate with these alterations in the malignant transformation.³⁷ In addition, an integrated analysis of genome-wide expression and copy-number data disclosed two major clusters of primary endometrial carcinomas with different phenotype and prognosis. Amplifications of 3q26.32, harboring the oncogene *PIK3CA*, were associated with poor prognosis and segregated with the aggressive transcriptional cluster. This finding emphasizes the involvement of the PI3K-AKT pathway in aggressive endometrial carcinomas.³⁸

P16 protein overexpression has been recently proposed as a marker of high-grade endometrial carcinoma. In our series, we confirmed this finding as we found intense p16 overexpression in both clusters of high-grade endometrial carcinomas. Our results are similar to those obtained by a recent comparative analysis of 117 endometrial carcinomas; that is, strong immunoexpression of p16 was identified in 16% of endometrioid carcinomas (7% of low-grade endometrioid carcinomas, and 25% of high-grade endometrioid carcinomas), and 77% of non-endometrioid carcinomas (92% of serous carcinomas, and 45% of clear cell carcinomas). These findings suggest that p16 protein expression correlates with tumor type and degree of differentiation, and it is useful for the differential diagnosis of endometrial carcinomas.³⁹ It has been confirmed that overexpression of p16 in serous endometrial carcinomas is unrelated to human papilloma virus infection.⁴⁰

Advances in the understanding of molecular genetic events in endometrial carcinomas has led to the development of novel and individualized therapeutic strategies. Although surgery is still the main treatment option,^{23,24} identification of two groups of high-grade endometrial carcinomas with different molecular alterations in the PI3K-AKT pathway can facilitate additional therapeutic strategies in these patients. Thus, further studies on the response to chemotherapy on patients with tumors in the two subgroups are justified. In fact, there are actually inhibitors of PI3K and mTOR that may be used as potential anticancer agents.⁴¹

In summary, hierarchical clustering analysis of genes involved in the PI3K-AKT pathway revealed two subgroups of high-grade endometrial carcinomas

that correlated with the presence or absence of p53 alterations and the activation of the PI3K-AKT pathway (*PTEN* and/or exon 20 *PIK3CA* mutations). PI3K-AKT pathway alterations and p53 abnormalities may have distinct roles in high-grade endometrial tumorigenesis. Identification of these subgroups can provide insight into the biology of these tumors and may facilitate the development of future treatments.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

References

- Vivanco I, Sawyers CL. The phosphatidylinositol 3-kinase AKT pathway in human cancer. *Nat Rev Cancer* 2002;2:489-501.
- Cully M, You H, Levine AJ, *et al*. Beyond PTEN mutations: the PI3K pathway as an integrator of multiple inputs during tumorigenesis. *Nat Rev Cancer* 2006;6:184-192.
- Chakravarti A, Zhai G, Suzuki Y, *et al*. The prognostic significance of phosphatidylinositol 3-kinase pathway activation in human gliomas. *J Clin Oncol* 2004;22:1926-1933.
- Tang JM, He QY, Guo RX, *et al*. Phosphorylated Akt overexpression and loss of PTEN expression in non-small cell lung cancer confers poor prognosis. *Lung Cancer* 2006;51:181-191.
- Saal LH, Johansson P, Holm K, *et al*. Poor prognosis in carcinoma is associated with a gene expression signature of aberrant PTEN tumor suppressor pathway activity. *Proc Natl Acad Sci USA* 2007;104:7564-7569.
- Opel D, Poremba C, Simon T, *et al*. Activation of Akt predicts poor outcome in neuroblastoma. *Cancer Res* 2007;67:735-745.
- Kato S, Iida S, Higuchi T, *et al*. PIK3CA mutation is predictive of poor survival in patients with colorectal cancer. *Int J Cancer* 2007;121:1771-1778.
- Ogino S, Noshio K, Kirkner GJ, *et al*. PIK3CA mutation is associated with poor prognosis among patients with curatively resected colon cancer. *J Clin Oncol* 2009;27:1477-1484.
- Aleskandarany MA, Rakha EA, Ahmed MA, *et al*. PIK3CA expression in invasive breast cancer: a biomarker of poor prognosis. *Breast Cancer Res Treat*; published online 22 August 2009; e-pub ahead of print.
- American Cancer Society. *Cancer Facts and Figures* 2009. American Cancer Society: Atlanta, GA, 2009.
- Bokhman JV. Two pathogenetic types of endometrial carcinoma. *Gynecol Oncol* 1983;15:10-17.
- Prat J, Gallardo A, Cuatrecasas M, *et al*. Endometrial carcinoma: pathology and genetics. *Pathology* 2007;39:72-87.
- Lax SF. Molecular genetic pathways in various types of endometrial carcinoma: from a phenotypical to a molecular-based classification. *Virchows Arch* 2004;444:213-223.
- Catusus L, Machin P, Matias-Guiu X, *et al*. Microsatellite instability in endometrial carcinomas clinicopathologic correlations in a series of 42 cases. *Hum Pathol* 1998;29:1160-1164.
- Bussaglia E, del Rio E, Matias-Guiu X, *et al*. PTEN mutations in endometrial carcinomas: a molecular and clinicopathologic analysis of 38 cases. *Hum Pathol* 2000;31:312-317.
- Catusus L, Gallardo A, Cuatrecasas M, *et al*. PIK3CA mutations in the kinase domain (exon 20) of uterine endometrial adenocarcinomas are associated with adverse prognostic parameters. *Mod Pathol* 2008;21:131-139.
- Machin P, Catusus L, Pons C, *et al*. CTNNB1 mutations and beta-catenin expression in endometrial carcinomas. *Hum Pathol* 2002;33:206-212.
- Moreno-Bueno G, Hardisson D, Sánchez C, *et al*. Abnormalities of the APC/beta-catenin pathway in endometrial cancer. *Oncogene* 2002;21:7981-7990.
- Lagarda H, Catusus L, Arguelles R, *et al*. K-ras mutations in endometrial carcinomas with microsatellite instability. *J Pathol* 2001;193:193-199.
- Strang P, Nordstöm B, Nilsson S, *et al*. Mutant p53 protein as a predictor of survival in endometrial carcinoma. *Eur J Cancer* 1996;32A:598-602.
- Lax SF, Kendall B, Tashiro H, *et al*. The frequency of p53, K-Ras mutations, and microsatellite instability differs in uterine endometrioid and serous carcinoma: evidence of distinct molecular genetic pathways. *Cancer* 2000;88:814-824.
- Sherman ME, Bur ME, Kurman RJ. P53 in endometrial cancer and its putative precursors: evidence for diverse pathways of tumorigenesis. *Hum Pathol* 1995;26:1268-1274.
- Markman M. Unresolved issues in the chemotherapeutic management of gynecologic malignancies. *Semin Oncol* 2006;33:S33-S38.
- Boruta II DM, Gehrig PA, Fader AN, *et al*. Management of women with uterine papillary serous cancer: a Society of Gynecologic Oncology (SGO) review. *Gynecol Oncol* 2009;115:142-153.
- Catusus L, Gallardo A, Cuatrecasas M, *et al*. Concomitant PI3K-AKT and p53 alterations in endometrial carcinomas are associated with poor prognosis. *Mod Pathol* 2009;22:522-529.
- Bader AG, Kang S, Zhao L, *et al*. Oncogenic PI3K deregulates transcription and translation. *Nat Rev Cancer* 2005;5:921-929.
- Samuels Y, Ericson K. Oncogenic PI3K and its role in cancer. *Curr Opin Oncol* 2006;18:77-82.
- Eisen MB, Spellman PT, Brown PO, *et al*. Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci USA* 1998;95:14863-14868.
- Ge X, Yamamoto S, Tsutsumi S, *et al*. Interpreting expression profiles of cancers by genome-wide survey of breadth of expression in normal tissues. *Genomics* 2005;86:127-141.

- 30 van't Veer LJ, Dai H, van de Vijver MJ, *et al*. Gene expression profiling predicts clinical outcome of breast cancer. *Nature* 2002;415:530–536.
- 31 Bustin S. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. *J Mol Endocrinol* 2002;29:23–39.
- 32 Akilesh S, Shaffer DJ, Roopenian D. Customized molecular phenotyping by quantitative gene expression and pattern recognition analysis. *Genome Res* 2003;13:1719–1727.
- 33 Abruzzo L, Lee K, Fuller A, *et al*. Validation of oligonucleotide microarray data using microfluidic low-density arrays: a new statistical method to normalize real-time RT-PCR data. *BioTechniques* 2005;38:785–792.
- 34 Gymnopoulos M, Elsliger M-A, Vogt PK. Rare cancer specific mutations in PIK3CA show gain of function. *Proc Natl Acad Sci USA* 2007;104:5569–5574.
- 35 Huang CH, Mandelker D, Schmidt-Kittler O, *et al*. The structure of a human p110alpha/p85alpha complex elucidates the effects of oncogenic PI3Kalpha mutations. *Science* 2007;318:1744–1748.
- 36 Zhao L, Vogt PK. Helical domain and kinase domain mutations in p110alpha of phosphatidylinositol 3-kinase induce gain of function by different mechanisms. *Proc Natl Acad Sci USA* 2008;105:2652–2657.
- 37 Oda K, Okada J, Timmerman L, *et al*. PIK3CA cooperates with other phosphatidylinositol 3'-kinase pathway mutations to effect oncogenic transformation. *Cancer Res* 2008;68:8127–8136.
- 38 Salvesen HB, Carter SL, Mannelqvist M, *et al*. Integrated genomic profiling of endometrial carcinoma associates aggressive tumors with indicators of PI3 kinase activation. *Proc Natl Acad Sci USA* 2009;106:4834–4839.
- 39 Reid-Nicholson M, Iyengar P, Hummer AJ, *et al*. Immunophenotypic diversity of endometrial adenocarcinomas: implications for differential diagnosis. *Mod Pathol* 2006;19:1091–1100.
- 40 Chiesa-Vottero AG, Malpica A, Deavers MT, *et al*. Immunohistochemical overexpression of p16 and p53 in uterine serous carcinoma and ovarian high-grade serous carcinoma. *Int J Gynecol Pathol* 2007;26:328–333.
- 41 Chon HS, Hu W, Kavanagh JJ. Targeted therapies in gynecologic cancers. *Curr Cancer Drug Targets* 2006;6:333–363.