Integrated genomic profiling of endometrial carcinoma associates aggressive tumors with indicators of PI3 kinase activation

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Although 75% of endometrial cancers are treated at an early stage, 15% to 20% of these recur. We performed an integrated analysis of genome-wide expression and copy-number data for primary endometrial carcinomas with extensive clinical and histopathological data to detect features predictive of recurrent disease. Unsupervised analysis of the expression data distinguished 2 major clusters with strikingly different phenotypes, including significant differences in disease-free survival. To identify possible mechanisms for these differences, we performed a global genomic survey of amplifications, deletions, and loss of heterozygosity, which identified 11 significantly amplified and 13 significantly deleted regions. Amplifications of 3q26.32 harboring the oncogene *PIK3CA* were associated with poor prognosis and segregated with the aggressive transcriptional cluster. Moreover, samples with *PIK3CA* amplification carried signatures associated with in vitro activation of PI3 kinase (PI3K), a signature that was shared by aggressive tumors without *PIK3CA* amplification. Tumors with loss of *PTEN* expression or *PIK3CA* overexpression that did not have *PIK3CA* amplification also shared the PI3K activation signature, high protein expression of the PI3K pathway member STMN1, and an aggressive phenotype in test and validation datasets. However, mutations of *PTEN* or *PIK3CA* were not associated with the same expression profile or aggressive phenotype. STMN1 expression had independent prognostic value. The results affirm the utility of systematic characterization of the cancer genome in clinically annotated specimens and suggest the particular importance of the PI3K pathway in patients who have aggressive endometrial cancer.

amplification | endometrial cancer | prognosis | comparative genomic hybridization | stathmin expression

With a 2% to 3% lifetime risk among women, endometrial cancer is the most common pelvic gynecologic malignancy in industrialized countries, and the incidence is increasing (1). Approximately 75% of cases are diagnosed with the tumor confined to the uterine corpus (1, 2), but after primary surgery 15% to 20% of these tumors recur and have limited response to systemic therapy. In light of these recurrences, patients who have localized endometrial cancer have 2 major needs: (1) adjuvant therapies that will reduce the recurrence rate, and (2) the ability to target these therapies to the patients in whom disease is most likely to recur. In addition, women who have metastatic disease require effective systemic therapy.

The needs for effective systemic therapies and for reliable prognostic markers have been addressed only partly. The most common basis for determining risk of recurrent disease has been the categorization of endometrial cancer into 2 subtypes. The majority are type I, associated with good prognosis, low stage and grade, and endometrioid histology. In contrast, type II cancers are characterized by high stage and grade, non-endometrioid histology, and poor prognosis. The prognostic value of this distinction is limited, however, because up to 20% of type I cancers recur, and half of type II cancers do not (2).

The molecular basis of the distinction between type I and II cancer is understood only partially. Type I cancer is associated with hyperestrogenic risk factors, is more often estrogen and progesterone receptor positive, diploid, microsatellite unstable, and *KRAS* or *PTEN* mutant. Type II cancer is more often aneuploid and harbors alterations in *CDKN2A*, *TP53*, and *ERBB2* (3). Such molecular alterations are of prognostic value (4) but have not provided a basis for improved therapy (3). Hormone receptor status influences the choice of treatment in metastatic disease, but most aggressive tumors are receptor negative (1).

We hypothesized that tumors with an aggressive phenotype are likely to be distinguished by underlying genetic alterations reflected in distinct transcriptional signatures, and we investigated whether tumors that recur share transcriptional signatures that suggest shared underlying genetic alterations.

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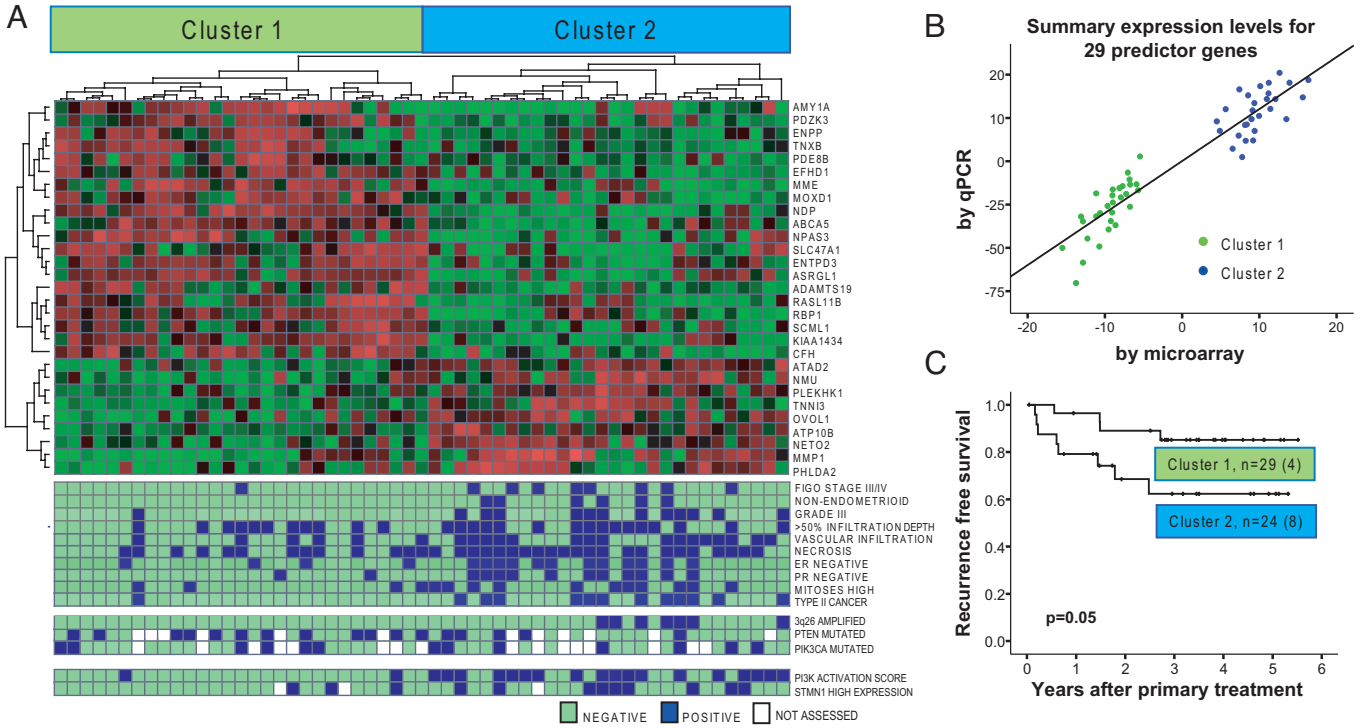

Fig. 1. Results of unsupervised clustering of expression data from 57 endometrial carcinomas. (*A*) Two major clusters are identified and recapitulated using the displayed predictor set of 29 genes. Features associated with each tumor are displayed in the bottom 3 panels. PI3K scores in the top tertile are called positive. (*B*) The ability of the 29 predictor genes to distinguish cluster 1 and 2 tumors was validated in these tumors by quantitative RT-PCR. (*C*) Recurrence-free survival was significantly lower for patients in cluster 2. Four tumors (all in cluster 2) were metastatic at presentation and omitted from this analysis.

Results and Discussion

Unsupervised Analysis of Expression Profiles Distinguishes Aggressive Tumors. We collected genome-wide expression and clinical and histopathologic data from a random sampling of 57 endometrial carcinomas in a population-based tissue bank of gynecologic cancer in Hordaland County, Norway. The characteristics of these patients were not significantly different from those of all patients diagnosed with endometrial carcinoma in a 10-year period from the same region (4) [supporting information (SI) Table S1].

An unsupervised analysis of these data distinguished 2 major groups of tumors (clusters 1 and 2, Fig. 1*A*). Significant analysis of microarrays (SAM) (5) between these clusters identified a 2-fold or higher change for 138 significant genes, of which 64 were up-regulated and 74 downregulated in cluster 2 (data not shown). A set of 29 genes, validated by quantitative RT-PCR, predicted the clusters with 100% accuracy (Fig. 1 *A* and *B*).

The 2 clusters had strikingly different clinical and histopathologic characteristics. Cluster 2 contained more aggressive tumors, with higher International Federation of Gynecology and Obstetrics (FIGO) stage, histologic grade, number of mitoses, presence of non-endometrioid histologic subtype, tumor necrosis, and vascular invasion, ($P < 0.001$ for the presence of any of these characteristics; Fig. 1*A*, Table S2). Cluster 2 contains almost all the type II tumors ($P < 0.001$) (Fig. 1*A*, Table S2) but it also contains almost 1/3 of the type I tumors, and these tumors have more vascular invasion, necrosis, and frequent mitoses than the type I tumors in cluster 1 ($P = 0.01$). The 29-gene summary set also was significantly correlated with aggressive cancer (Table S2).

Most prominently, patients who had tumors in cluster 2 had significantly poorer recurrence-free survival ($P = 0.05$, Fig. 1*C*). Segregation into cluster 2 predicted recurrence better than FIGO stage, histologic subtype, or receptor status and slightly less well than grade (Table S3) but did not exhibit independent prognostic impact, probably because of the limited number of cases and events.

Regions of Significant Amplification, Deletion, and Loss of Heterozygosity. To identify the underlying somatic changes distinguishing aggressive tumors with the cluster 2 signature, we performed a genome-wide survey of copy-number changes and loss of heterozygosity (LOH) among 84 tumors. The majority of tumors exhibit a small number of amplifications (median of 4 in each tumor) and even fewer deletions (median of 1). Nevertheless, virtually every region of the genome is amplified or deleted in at least 1 tumor (Fig. 2*A*).

To distinguish copy-number changes associated with endometrial cancer from potentially random events, we applied the statistical method Genomic Identification of Significant Targets in Cancer (GISTIC) (6). GISTIC assigns each region of the genome 2 *G*-scores (Fig. 2*B*), each representing the combined frequency and amplitude of either local amplifications or deletions. It then compares these scores with similar scores generated from random permutations of the data to determine false discovery rate q-values (7), representing the likelihood of obtaining the observed *G*-scores from chance events alone. The *G*-scores tend to be larger for amplifications than deletions (Fig. 2*B*) because of the greater prevalence of amplifications (Fig. 2*A*). Conversely, deletions attain statistical significance (using a q-value threshold of 0.25, green lines in Fig. 2*B*) at lower prevalence because of their overall infrequency.

We found 11 significantly amplified and 13 significantly deleted regions of the genome (Table S4), including several that had been noted previously (8, 9). For each we selected the peak region (ie, the highest frequency and amplitude of events) as the region most likely to contain a cancer gene target. Known oncogenes are located within these peaks for 8 amplified regions,

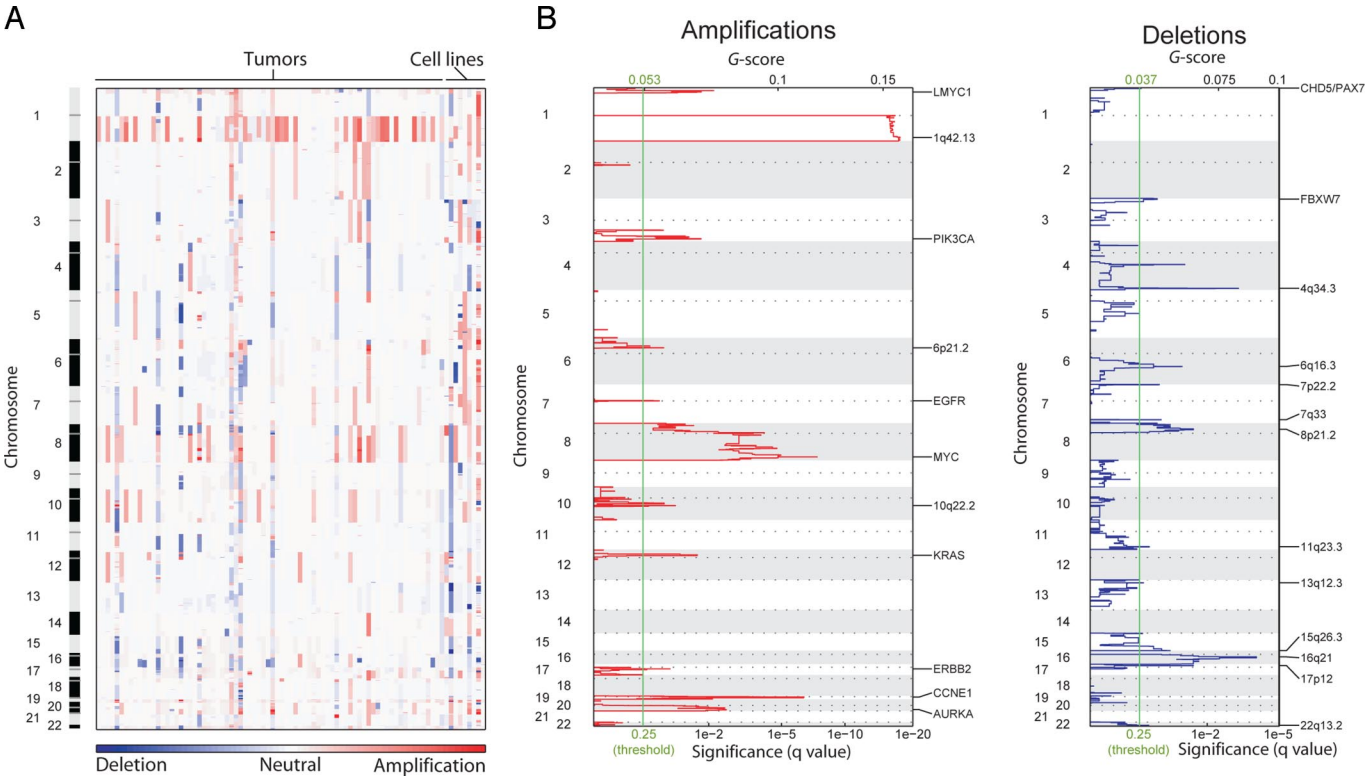

Fig. 2. Significant copy-number alterations in endometrial cancer. (*A*) Amplifications (*red*) and deletions (*blue*), determined by segmentation analysis of normalized signal intensities from 100K SNP arrays (see *Methods*), are displayed across the genome (chromosome positions, indicated along the *y* axis, are proportional to marker density) for 76 tumors and 9 cell lines. (*B*) GISTIC analysis of copy-number changes. The *G* score represents the frequency times average amplitude of the aberrations identified in (*A*). The false discovery rate q-values, representing the statistical significance associated with these scores (7), are displayed along the bottom. Regions with q-values < 0.25 (*green lines*) were considered significantly altered. The locations of the peak regions of maximal copy-number change and the known cancer-related genes within those peaks are indicated to the right of each panel.

and known tumor suppressors are located within deletion peaks on chromosomes 1 and 3 (Table S4), but functional data tying any of these genes to endometrial carcinogenesis are lacking. Also, 14 regions contain no known cancer genes. These peaks usually represent infrequent events ($< 17\%$ of tumors), with the exception of 1q amplification, in which the gene target is unclear because of the large size of the amplicon. The consistent breadth of this amplicon, in fact, may suggest more than 1 target (6). LOH generally reflects deletions, with the exception of prevalent copy-neutral LOH on 10q (Fig. S1) containing the known endometrial tumor suppressor *PTEN* (10).

Amplifications of *KRAS* and *PIK3CA* Associate with Poor Prognosis. Among the 11 significant amplifications, only 2 (3q26.32 and 12p12.1) are associated with recurrence-free survival (in both cases poor survival) after correction for multiple hypotheses (Table S5, Fig. 3*A*). We considered only amplifications because of the low prevalence of deletions. Amplification of 3q26.32 ($3q^{amp}$) also is associated with non-endometrioid (44% vs. 11% prevalence; $P = 0.02$) and high-grade ($P < 0.001$) histology. The association between 12p12.1 amplification and poor survival is surprising, because mutations of *KRAS*, which is within the peak region, are known to associate with better survival. However, we sequenced *KRAS* in 64 tumors and found mutant *KRAS* in none of the 12p12.1-amplified samples, although mutations were seen in 4 unamplified samples. Amplification of 12p12.1 is also associated with high grade ($P = 0.02$) and FIGO stage ($P = 0.04$). Although 3q26.32 and 12p12.1 tended to be amplified in the same tumors ($P = 0.03$), they usually did not coincide. We directed further analyses at $3q^{amp}$ because all the samples with this amplification segregated into expression cluster 2 ($P = 0.01$) (Fig. 1*A*), suggesting that the amplification could be associated with the cluster 2 transcriptional profile (*SI Text* Note 1).

Integrated Analyses Associate Markers of PI3 Kinase Activation with Aggressive Cancer. We wanted to investigate whether $3q^{amp}$ leads to an aggressive phenotype through activation of PIK3CA. Although *PIK3CA* has not been shown to be the $3q^{amp}$ target, we suspected it for 4 reasons: (*i*) *PIK3CA* is 1 of 36 genes within the peak region (indeed, PIK3CA amplifications were noted previously; see refs. 11, 12); (*ii*) tumors with $3q^{amp}$ overexpress *PIK3CA* compared with unamplified tumors ($P = 0.003$; Fig. 3*B*); (*iii*) similar amplifications in ovarian cancer act through *PIK3CA* (13); and (*iv*) the PI3 kinase (PI3K) pathway frequently is aberrant in endometrial cancer (11), including point mutations in *PIK3CA* (14).

We therefore looked for wider effects of PIK3CA activation in the transcriptome of tumors with $3q^{amp}$. We used published data (15) from cell lines transfected with mutationally activated *PIK3CA* to define a PI3K activation score (PI3K score), representing the expression levels of genes that correlate with activated PIK3CA (see *Methods*). Tumors with $3q^{amp}$ scored higher than unamplified samples ($P = 0.05$; Fig. 3*C*). However, the impact of this finding is limited by its borderline statistical significance and by the possibility that the PI3K score may reflect PI3K activation only in the model systems in which it was measured but not generally .

We therefore sought to corroborate this finding by seeing whether samples with $3q^{amp}$ have an expression profile opposite that induced by PI3K pathway inhibition. To that end, we queried the 50 most overexpressed and underexpressed genes in samples with $3q^{amp}$ relative to unamplified samples (Fig. S2*A*) using the

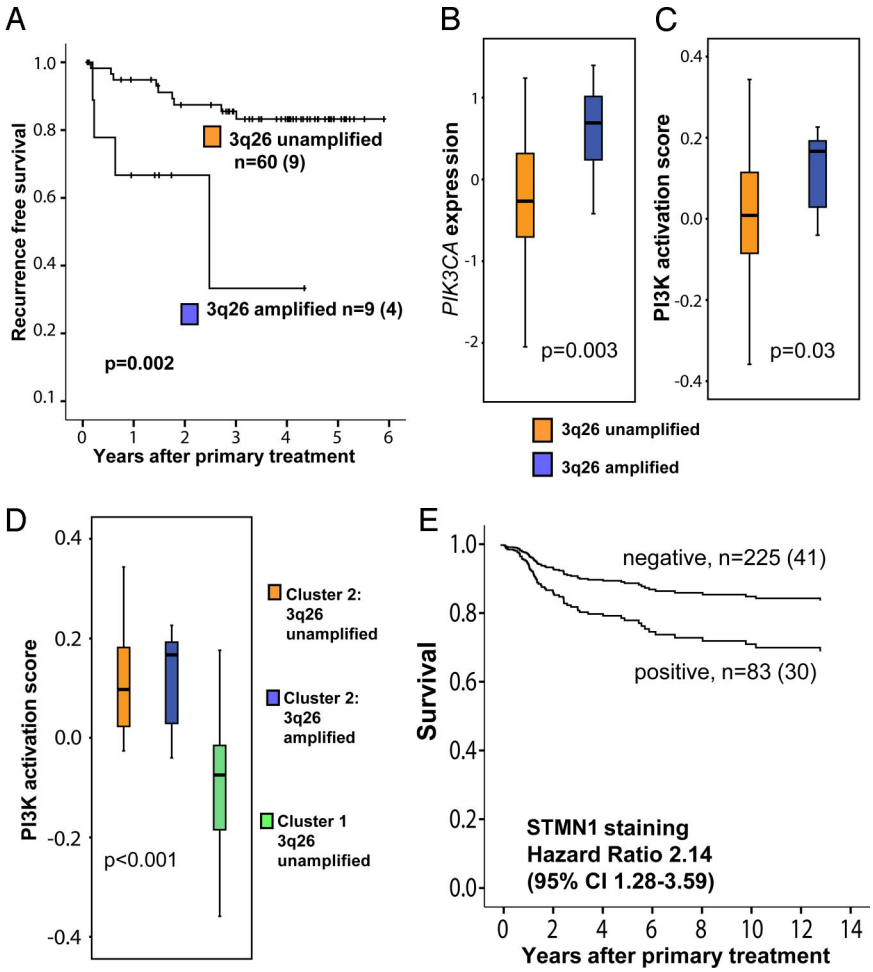

Fig. 3. Relations between *PIK3CA* amplification, PI3K activation, and survival. (*A*) Amplification of a region in 3q26 that includes *PIK3CA* is significantly associated with poor recurrence-free survival. These amplifications are associated with (*B*) overexpression of *PIK3CA* and (*C*) increased PI3K scores. (*D*) Among the broader set of poor-prognosis tumors in expression cluster 2, PI3K scores are equally high among tumors without 3q26 amplification and with 3q26 amplification, suggesting alternative methods of pathway activation. (*E*) Tumors with elevated protein expression of the PI3K pathway member STMN1 had significantly poorer survival, after controlling for age, FIGO stage, histologic subtype, and grade.

Connectivity Map (16). Among 164 small molecules represented in the Connectivity Map, the PI3K inhibitor LY-294002 (17) had an expression signature most significantly anticorrelated with the $3q^{amp}$ signature (Fig. S2 *B* and *C*, $P = 0.003$). LY-294002 is known to bind to additional kinases (18), raising the possibility that this anticorrelation results from nonspecific effects. The anticorrelation between the $3q^{amp}$ signature and inhibitors of adenylate cyclase and Hsp90 (Fig. S2*B*) also suggests potentially complex effects of the amplicon. Nevertheless, the findings that the $3q^{amp}$ signature correlates with a PI3K activation signature and anticorrelates with the signature of a PI3K inhibitor support the hypothesis that an effect of $3q^{amp}$ may be to increase PI3K activity.

We also validated the correlation between *PIK3CA* amplification and the PI3K score in an independent expression dataset. We first inferred amplification of 3q26–27 from local gene expression levels, as reflected in a "functional amplification" (FA) score (19). As expected, samples determined to have $3q^{amp}$ by SNP array analysis also had high 3q26–27 FA scores ($P < 10^{-5}$, Fig. S3*A*), confirming the score as a meaningful assessment of amplification status. We then inferred 3q26–27 amplification levels in a publicly available expression dataset of 134 endometrial tumors (http://expo.intgen.org/geo/home.do). The correlations between $3q^{amp}$ and both *PIK3CA* overexpression and the PI3K score validated ($P = 2 \times 10^{-10}$ and 7×10^{-5}, respectively; Fig. S3 *B* and *C*).

In addition, we validated the correlations between aggressive phenotype and both *PIK3CA* amplification and the cluster 2 signature in this independent dataset. Although survival data were unavailable, both available markers of poor survival, high-grade and non-endometrioid subtype (20) correlated with high 3q26–27 FA scores ($P = 0.001$ and 0.005, respectively; Fig. S3 *D* and *E*) and with high values of the 29-gene summary predictor for membership in cluster 2 ($P = 3 \times 10^{-4}$ and $P = 0.004$, respectively; Fig. S3 *F* and *G*).

The finding that both *PIK3CA* amplification and the cluster 2 expression profile indicate aggressive tumors, coupled with the association between *PIK3CA* amplification and the in vitro PI3K activation signature, suggested that the broader set of aggressive tumors in cluster 2 might share the in vitro PI3K activation signature. This conjecture seems to be true: tumors in cluster 2 without *PIK3CA* amplification have PI3K scores significantly higher than the scores of tumors in cluster 1 ($P < 0.001$) and equal to those in tumors with amplification of *PIK3CA* (Fig. *3D*). Moreover, the cluster 2 signature is highly anticorrelated with the signature of treatment with LY-294002 ($P = 0.02$; Fig. S4 *A* and *B*). Furthermore, tumors with high PI3K scores are associated with poor survival ($P = 0.03$, Fig. S4*C*) and other markers

of aggressive phenotype in both the test and validation datasets ($P = 0.01$ and $P = 0.001$, respectively).

A possible cause of overexpression of the PI3K activation signature among tumors without *PIK3CA* amplification is decreased expression of the downstream PI3K pathway member *PTEN*. Decreased *PTEN* expression was associated with increased PI3K scores in both our test and validation datasets ($P < 0.001$ and $P = 0.03$, respectively), regardless of *PIK3CA* amplification status. Decreased *PTEN* expression also was associated with markers of aggressive disease ($P = 0.02$), in agreement with prior studies (21).

Conversely, among the 45 tumors with expression data that we sequenced for *PTEN*, mutations did not associate with high PI3K scores ($P = 0.6$; Fig. S4*D*). On the contrary, we observed more mutations in the nonaggressive cluster 1 than in cluster 2 ($P = 0.04$; Fig. 1*A*), in line with prior studies associating *PTEN* mutations with indolent disease (22, 23).

Overexpression and mutation of *PIK3CA* also seem to have different implications. We found significantly higher *PIK3CA* expression in tumors with aggressive features, including those without *PIK3CA* amplification ($P = 0.05$ and $P = 0.0009$ among test and validation data, respectively). However, among the 41 tumors with expression data that we sequenced for *PIK3CA*, mutations did not associate with high PI3K scores ($P = 0.8$; Fig. S4*E*) or features of aggressive disease ($P = 0.5$). We also cannot confirm the finding that exon 20 mutations correspond to aggressive tumors (24) (data not shown). These results were surprising in light of evidence that overexpression of mutated, but not wild-type, *PIK3CA* leads to transformation (25, 26) and suggest either that *PIK3CA* suffers from prevalent cryptic mutations or that transformation assays did not capture the effects of wild-type *PIK3CA* overexpression in human tumors. Although *PIK3CA* mutations previously have been noted primarily in endometrioid cancers (11, 12), we also find no correlation with histologic subtype ($P = 1$).

Expression of the PI3 Kinase Pathway Member STMN1 is an Independent Prognostic Indicator. The suggestion that PI3K activation associates with poor prognosis suggested that measuring PI3K activity might improve prognostication of localized endometrial cancer. Expression of STMN1 previously has been shown to correlate with PI3K activity in breast cancer (27) and can be measured by immunohistochemistry in paraffin-embedded tissue. We therefore measured STMN1 expression by immunohistochemistry (*SI Methods* and Fig. S4 *F* and *G*) in 72 tumors, including 66 with SNP array and 53 with expression data (Fig. 1*A*). Although *STMN1* is not a member of our PI3K activation signature, STMN1 expression correlated with PI3K scores ($P = 0.05$). High STMN1 expression also correlated with *PIK3CA* amplification ($P = 0.04$) and overexpression ($P = 0.04$) and with segregation in cluster 2 ($P = 0.03$), supporting our prior associations between these features and PI3K pathway activation.

High STMN1 expression also was associated with poor recurrence-free survival in our original tumor set ($P = 0.006$) and with poor recurrence-free ($P = 0.01$) and overall ($P = 0.01$) survival in a validation set of 241 tumors from a population-based series of all endometrial carcinomas in Hordaland County from 1981 to 1990 (4, 21). In both tumor sets, STMN1 expression correlated with grade, mitotic rate, presence of necrosis or vascular invasion, and type II status (Table S6). Nevertheless, across all 313 cases (except for 5 cases with missing clinical data), high STMN1 expression was a prognostic indicator independent of FIGO stage, histologic subtype, grade, and age ($P = 0.004$; Fig. 3G, Table S7). In particular, high STMN1 expression was associated with poor prognosis in the otherwise low-risk endometrioid subgroup ($P = 0.007$, data not shown).

Ultimately, the goals of integrated genomic analyses of localized tumors are to enable development of clinical assays to distinguish aggressive tumors requiring therapy beyond resection and of effective therapeutics for such tumors. We have shown that both transcriptional and copy-number profiles of endometrial tumors contain prognostic information that is partly reflected in expression levels of *PIK3CA*, in vitro *PI3K* activation signatures, *PTEN*, and STMN1. We also find that *PTEN* and *PIK3CA* mutations seem to have different transcriptional and phenotypic correlates than changes in expression of these genes. These results suggest that further investigation of the specific consequences of mutation and altered expression is warranted. They also emphasize the potential utility of clinical assays for PI3K pathway activation to identify patients who have aggressive disease and the particular relevance of therapeutics that inhibit this pathway.

Aside from the PI3K pathway, our general survey of chromosomal changes in endometrial carcinoma also identified approximately 20 other regions of significant copy-number change. Most of these copy-number changes involve tens to hundreds of genes, so even when known oncogenes or tumor suppressors are within the regions most affected by these copy-number changes, the genomic data are ambiguous as to the actual target. In many cases, including amplification of 3q26.32, the size of these events suggests the possibility of multiple targets. Moreover, functional data tying even known oncogenes and tumor suppressors to carcinogenesis in model systems of endometrial cancer are lacking, for the most part. The limited number of significant regions of copy-number change suggests that comprehensive, systematic experiments to identify these oncogenes and tumor suppressors in endometrial cancer are feasible. Such experiments are likely to point to therapeutic targets for women with all stages of endometrial carcinoma.

Materials and Methods

Patient Series. For the primary investigation series, primary endometrial carcinomas were frozen immediately during hysterectomies conducted from 2001 to 2003. All samples were reviewed by a pathologist according to published criteria (28). Treatment included bilateral salpingo-oophorectomy and pelvic lymphadenectomy. Adjuvant therapy was recommended for patients who had disease of FIGO surgical stage IIB or higher or non-endometrioid histology. Patients were followed from primary surgery until June, 2007 or death, with a median follow-up for survivors of 3.6 years (range, 0.8–5.5). Deaths not attributable to endometrial cancer were censored. No patient was lost to follow-up.

RNA Analysis. RNA was extracted from biopsies with at least 50% (usually > 80%) tumor content using the RNeasy kit (Qiagen). Quality and yield were assessed by agarose electrophoresis, the Agilent Bioanalyser 2100, and spectrophotometry. RNA was prepared in 2 batches and hybridized to Agilent 21K and 22K arrays, respectively, according to manufacturer's instructions (www.agilent.com). Arrays were scanned using the Agilent Microarray Scanner Bundle.

Signal intensities were determined using J-Express (www.molmine.com) and filtered to remove genes with signal intensities below 2 SD over background in either channel (Cy5, Cy3) in more than 30% of samples. Batch adjustment was performed as described previously (29). Genes were mean-centered across the tumor set.

Hierarchical clustering was performed using the 3500 genes with highest variance using weighted average linkage and Pearson correlation as similarity measures. Clustering with more or fewer genes gave stable results (data not shown). A SAM analysis using these clusters as class labels identified 138 significantly changed genes, 29 of which were selected for their combined discriminatory power as described in *SI Methods*. Messenger RNA levels for these 29 genes and *PTEN* were validated by quantitative PCR using the TaqMan Low Density Array (Applied Biosystems) according to manufacturer's instructions (29).

For the external dataset (Affymetrix U133 + 2 arrays), individual probes were sequence-matched against Aceview (NCBI35) (19) to construct transcript-level probe sets. Summary expression levels then were derived by batch normalization across samples via RMA (30).

The PI3K score was obtained by comparing previously published expression data of 9 replicate transfections of activated PIK3CA with 5 GFP controls (15)

and includes the 495 genes surpassing a Bonferroni-corrected 2-sided *t* test p-value of 0.05. To evaluate this signature, expression data for each gene were normalized to a common mean and scaled to the same SD. For each sample, the activation score is the sum of genes significantly up-regulated in the cells with activated PIK3CA (relative to the cells with GFP control) minus genes significantly down-regulated in those cells.

DNA Analysis. Genomic DNA was extracted from surgically dissected, fresh-frozen primary tumors and from 9 cell lines: Ishicawa, Hec1A, KLE, AN3-CA, EFE184, MFE-280, MFE-296, MFE-319, and RL-95–2. Tumors were needle dissected to ensure 80% purity.

PIK3CA, *KRAS*, and *PTEN* were sequenced as previously described (31). Genomic DNA was analyzed by SNP arrays interrogating 116,204 SNP loci (Affymetrix) and the GISTIC algorithm, as previously described (6). SNP, gene, and cytogenetic band locations are based on the hg16 (July 2003) genome build (genome.ucsc.edu).

Statistics. For relations of molecular data to clinical phenotype, Pearson's χ^2-(χ^2), Fisher's exact-, Mann-Whitney-, or Kruskal-Wallis tests were used as appropriate. P-values represent 2-sided tests except when testing the 1-sided hypothesis that $3q^{amp}$ correlates with measures of PI3K activation. Univariate survival analyses were performed by the Kaplan-Meier method. The log-rank (Mantel-Cox) test with Bonferroni correction was used to compare survival curves for different categories. Variables with significant impact on survival ($P \leq 0.05$) were examined further by log-minus-log plot before incorporation in the Cox proportional hazards regression model.

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