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High-throughput interrogation of PIK3CA, PTEN, KRAS, FBXW7 and TP53 mutations in primary endometrial carcinoma

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HIGHLIGHTS

- Mutations in *PIK3CA* as well as specific *PIK3CA* exon 20 mutations correlate with high-grade endometrial tumors in 1063 patients.
- We confirm that mutations in *TP53* and *PTEN* are more frequently observed in type II and type I tumors, respectively.
- The specific mutation in *PIK3CA* (H1047R) correlates with shorter survival and *FBXW7* mutations correlate with tumors showing lymph node invasion.

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ABSTRACT

Objective. Endometrial cancer patients may benefit from systemic adjuvant chemotherapy, alone or in combination with targeted therapies. Prognostic and predictive markers are needed, however, to identify patients amenable for these therapies.

Methods. Primary endometrial tumors were genotyped for >100 hot spot mutations in genes potentially acting as prognostic or predictive markers. Mutations were correlated with tumor characteristics in a discovery cohort, replicated in independent cohorts and finally, confirmed in the overall population ($n=1063$).

Results. PIK3CA, *PTEN* and *KRAS* mutations were most frequently detected, respectively in 172 (16.2%), 164 (15.4%) and 161 (15.1%) tumors. Binary logistic regression revealed that *PIK3CA* mutations were more common in high-grade tumors (OR = 2.03; $P=0.001$ for grade 2 and OR = 1.89; $P=0.012$ for grade 3 compared to grade 1), whereas a positive *TP53* status correlated with type II tumors (OR = 11.92; $P<0.001$) and PTEN mutations with type I tumors (OR = 19.58; $P=0.003$). Conversely, *FBXW7* mutations correlated with positive lymph nodes (OR = 3.38; $P=0.045$). When assessing the effects of individual hot spot mutations, the H1047R mutation in *PIK3CA* correlated with high tumor grade and reduced relapse-free survival (HR = 2.18; $P=0.028$).

Conclusions. Mutations in *PIK3CA*, *TP53*, *PTEN* and *FBXW7* correlate with high tumor grade, endometrial cancer type and lymph node status, whereas *PIK3CA* H1047R mutations serve as prognostic markers for relapse-free survival in endometrial cancer patients.

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Introduction

Endometrial cancer is the most frequent malignancy of the female genital tract and the fourth most common cancer in women in industrialized countries [1]. Although 75% of all endometrial cancers are diagnosed at an early stage with localized disease and beneficial disease outcome, 15–20% of all tumors recur. Treatment modalities for recurrent endometrial cancer are limited, however, leading to poor overall survival [2]. High-risk endometrial cancer patients might benefit from systemic adjuvant chemotherapy, although markers to allow efficient patient selection are currently lacking. Accordingly, clinical studies with adjuvant chemotherapy involved an insufficient number of high-risk patients and differed considerably with respect to the tumor type, lymph node resection and disease stage [3]. Identification of new molecular prognostic markers that improve selection of high-risk patients would therefore greatly facilitate the development of novel treatment modalities in endometrial cancer.

The increased availability of clinicopathologic data on endometrial carcinomas has led to a dualistic model of endometrial carcinogenesis, with endometrioid adenocarcinomas classified as type I and non-endometrioid (serous, clear cell carcinomas) tumors classified as type II [4]. Both cancer types might also be classified based on their underlying molecular mechanisms, whereby *PTEN*, *KRAS* and *PIK3CA* mutations and microsatellite instability are regularly observed in type I carcinomas [5,6], and type II cancers are more often associated with *TP53* mutations and HER2/neu overexpression [7–9]. Recently, mutations in *FBXW7*, a p53-dependent tumor suppressor gene known to be mutated in other cancer types [10], were also described in endometrial cancer, although correlation with tumor type, grade and clinical stage was not evaluated [10,11]. Moreover, most studies assessing these genetic pathways have used heterogeneous detection methods and considered only a small number of tumors with potential selection bias. Furthermore, since *KRAS*, *PIK3CA*, *TP53* and *FBXW7* are mutated in only ~5–25% of all endometrial tumors [12], the number of observed mutations in each study was limited and in-depth associations with clinical parameters were generally lacking. These findings therefore require independent validation experiments in much larger cohorts.

Therefore, we determined the incidence, clinical relevance and prognostic impact of somatic mutations in a much larger series of 1063 endometrial cancers. Since *KRAS*, *PIK3CA* and *TP53* have previously been proposed as molecular genetic markers for endometrial cancer and may serve as predictive markers for targeted therapies, the mutation status of these genes as well as some of the most closely-related genes (*EGFR*, *BRAF* and *NRAS* for *KRAS*, *PTEN* for *PIK3CA* and *FBXW7* for *TP53*) was determined.

Methods

Tumor collection

Study participants were drawn from five datasets: a discovery cohort from Leuven (Belgium; $n=407$) and four replication cohorts, respectively from Genk (Belgium; $n=77$), Bergen (Norway; $n=282$), Gothenburg (Sweden; $n=179$) and ANECS (Australia; $n=295$), comprising 1240 patients with primary endometrial cancer.

The following clinicopathological data were collected: age at diagnosis, type of endometrial cancer, histopathological differentiation and grade, FIGO stage (2009), lymph node status, recurrence and time to relapse. Histopathology was divided into endometrioid, clear cell carcinoma (CCC), serous and mixed subtypes. Endometrioid carcinomas were classified as type I cancers, whereas clear cell and serous carcinomas were classified as type II. Mixed subtypes were considered as a separate group. More detailed information about tumor histology can be found in Table 1.

Table 1
Sample histology overview: histopathological variables were missing for one sample.

Tumor histology and grading	Number of samples
Endometrial tumors	1117
Endometrioid (EEC or Type I)	970
Grade 1	393
Grade 2	378
Grade 3	198
Non-endometrioid (NEEC or Type II)	147
Serous	60
Clear cell	33
Mixed	54
Endo/serous	28
Endo/CCC	11
Endo/serous/CCC	5
Serous/CCC	1
Endo/CCC or serous, nd	9

Follow-up data included relapse-free survival (RFS), overall survival (OS) and current disease status (no evidence of disease (NED), alive with evidence of disease (AWED), dead of intercurrent disease (DID) and dead of disease (DOD)). Data on lymph node status were missing for the Bergen Endometrial Cancer Study, whereas data for recurrence were partially absent for the ANECS samples.

All studies obtained the relevant IRB approval in accordance with the principles of the Declaration of Helsinki. Informed consent was obtained according to these approvals. Complete patient cohort characteristics are available in the Supplementary Methods.

DNA extraction

Formalin-fixed paraffin-embedded tissue blocks from 1073 patients were obtained and 2–5 slides (5–20 μm) were used for DNA extraction. Additionally, 94 fresh-frozen non-overlapping tissue samples from LES were included. For each tumor block, an H&E slide was prepared and an experienced pathologist (A.C. and P.M.) delineated the tumor area. Tumor tissue was macro-dissected from the individual slides and DNA extraction was performed using an in-house optimized phenol/chloroform standard protocol. Generally, biopsies <3 mm in diameter, that failed to yield sufficient DNA after extraction, and biopsies with a low estimated tumor percentage on H&E could not be included in the study ($n=50$). As a result, after excluding patients that failed to fulfill inclusion criteria or failed to be successfully extracted, 1117 patients were used for genotyping.

Somatic oncogene profiling

The COSMIC (Catalogue Of Somatic Mutations In Cancer) database [13] was screened for mutations in *KRAS*, *BRAF*, *NRAS*, *PIK3CA*, *PTEN*, *EGFR*, *FBXW7* and *TP53* in solid tumors, and the most frequent mutations in each gene were selected (Supplementary Table S1). The iPLEX technology on a MassARRAY Compact Analyser (Sequenom Inc., San Diego, USA) was used for mutation analysis, as reported previously [14,15]. Detailed description is provided in the Supplementary Methods.

Statistical analysis

Data are summarized as frequencies and percentages for categorical variables. Data are summarized as frequencies and percentages for categorical variables. Age at diagnosis was analyzed as a binary variable ($<$ or $\geq$60 years). Binary logistic regression for the mutation status of every gene or for specific mutations was performed while adjusting for tumor type, FIGO, grading, and age. To show that observations did not depend on tissue type (DNA extracted from FFPE versus fresh frozen tissue) additional regression was performed while also correcting for

tissue type. Effects on lymph node status and recurrence were assessed in a separate analysis using binary logistic regression for the mutation status, tumor type, grading and age. Cox regression for RFS was used to assess the effect of the mutation status or of individual mutations, while correcting for tumor type, FIGO stage, grade and age. *P* values were two-tailed and $P<0.05$ was considered statistically significant. The analyses were not corrected for multiple variables, what could be considered a potential limitation of the study. Statistical analyses were performed using SPSS software v17.0 (SPSS Inc., Chicago, USA).

Results

Population characteristics and mutation frequencies

Baseline characteristics of 1117 patients with primary endometrial cancer are given in Table 2. The median follow-up time of all patients was 35 months (range, 0–279 months). Overall, 970 (86.9%) and 93 (8.3%) patients had a type I or type II endometrial tumor, respectively, whereas 54 (4.8%) patients had a mixed tumor. Type II tumors were characterized by a higher FIGO stage, were more likely to be lymph node positive and had a higher risk of recurrence (Table 2).

Mutations were most frequently detected in *PIK3CA* (172 in 16.2% of the tumors), *PTEN* (164 in 15.4% of the tumors) and *KRAS* (161 in 15.1% of the tumors). Conversely, *FBXW7*, *TP53* and *NRAS* mutations were less frequent, with respectively 32 (3.0%), 30 (2.8%) and 19 (1.8%) mutations (Table 2). No mutations in *BRAF* and only a single mutation in *EGFR* were detected. *KRAS* and *NRAS* mutations and *TP53* and *PTEN* mutations were mutually exclusive, whereas all other mutations occurred independently from each other (Supplementary Table S2). These observations confirm recent findings [16,17].

Since we included 94 fresh frozen tumors in the Leuven cohort and since mutations can be more easily detected in fresh frozen than in FFPE tissue [18], we compared mutation frequencies between both tissue types. Mutation frequencies in *KRAS*, *PTEN*, *TP53*, *FBXW7* and *NRAS* did not differ (data not shown), but *PIK3CA* mutations were more frequent in fresh frozen than FFPE tumors (13.6% versus 30.0%, respectively; $P=0.001$).

Classification of grade 3 endometrioid and mixed (types I and II) tumors

Subsequently, we assessed whether the mutation status of individual genes correlated with any of the tumor characteristics. Since classification of grade 3 endometrioid tumors into either type I or type II is debatable due to their unfavorable prognosis [2,19,20], we first assessed how to classify these tumors. Survival analysis for endometrioid grade 1, 2, 3 and type II tumors revealed that poorly-differentiated endometrioid carcinomas behave similar to type II tumors, while showing a reduced RFS and OS (Fig. 1A–B) compared to well- and moderately-differentiated endometrioid tumors ($P<0.001$). Mutation profiling of grade 3 endometrioid tumors revealed, however, that these were more closely related to grade 1 and 2 tumors than to type II tumors (Supplementary Table S3). In particular, *PTEN* mutations were observed in respectively 16.8%, 15.9% and 18.7% of grade 1, 2 and 3 endometrioid tumors, and in only 1.1% of type II tumors. Likewise, *TP53* mutations were observed in 1.0%, 1.3% and 3.0% of grade 1, 2 and 3 endometrioid tumors compared to 16.1% of type II tumors (Table 3). We therefore considered grade 3 endometrioid tumors as type I. In contrast, mixed tumors could not be unambiguously classified as type I or II. They exhibited RFS rates intermediate to that of type I or II tumors (Fig. 1). Mutation profiling confirmed that mixed subtypes consist of mutations specifically occurring in both tumor types. In particular, *PTEN* mutations were observed in 16.8% of type I tumors, 1.1% of type II and 14.8% of the mixed tumors, whereas *TP53* mutations were observed in 16.1% of type II, 1.5% and 7.4% of type I and mixed tumors, respectively (Table 3). Since there were too few mixed tumors to be analyzed as a separate subgroup, they were removed from further analysis. The only exception is for mixed endometrioid/mucinous tumors ($n=15$). Although these tumors have recently been correlated with lymph node metastasis [21], we also classified them as endometrioid (type I) tumors. There were no significant differences between mixed endometrioid/mucinous

Table 2
Proportions of EEC, NEEC and mixed tumors by patient demographic and clinicopathologic characteristics: Clinical data are stratified for type I, type II and mixed tumor types. Percentages are given as column %. *P* values assessing the difference between type I, type II and mixed tumors were assessed using a Pearson's Chi Square test. Abbreviations: NED, no evidence of disease; AWED, alive with evidence of disease; DOD, dead of intercurrent disease; DOD, dead of disease. Data are expressed as number (%) of patients. Clinical variables were missing for age ($n=2$), histopathological grade ($n=1$), FIGO ($n=11$), lymph node involvement ($n=657$), recurrence ($n=108$) and follow-up ($n=287$).

Total cases analyzed $n=1117$	EEC (Type I) $n=970$ (86.9%)	NEEC (Type II) $n=93$ (8.3%)	Mixed types $n=54$ (4.8%)	*P* value Type I versus type II	*P* value Type I versus mixed	*P* value Type II versus mixed
Age (66 ± 10)						
>=60 ($n=760$, 68.0%)	655 (67.6%)	69 (75.0%)	36 (66.7%)	0.145	0.887	0.279
<60 ($n=355$, 31.8%)	314 (32.4%)	23 (25.0%)	18 (33.3%)			
Histopathological grade						
Grade 1 ($n=393$, 33.8%)	393 (40.6%)	–	–	–	–	–
Grade 2 ($n=378$, 33.6%)	378 (39.0%)	–	–			
Grade 3 ($n=198$, 32.1%)	198 (20.4%)	–	–			
FIGO 2009						
I ($n=828$, 74.1%)	750 (77.9%)	48 (52.7%)	30 (57.7%)	<0.001	<0.001	0.231
II ($n=79$, 7.1%)	70 (7.3%)	8 (8.8%)	1 (1.9%)			
III ($n=159$, 14.2%)	125 (13.0%)	19 (20.9%)	15 (28.9%)			
IV ($n=40$, 3.6%)	18 (1.9%)	16 (17.6%)	6 (11.5%)			
Lymph node involvement						
Positive nodes ($n=80$, 7.2%)	54 (14.7%)	12 (26.1%)	14 (29.8%)	0.047	0.009	0.691
Negative nodes ($n=380$, 34.0%)	313 (85.3%)	34 (73.9%)	33 (70.2%)			
Recurrence						
Yes ($n=160$, 14.3%)	122 (13.9%)	29 (34.5%)	9 (19.6%)	<0.001	0.281	0.073
No ($n=849$, 76.0%)	757 (86.1%)	55 (65.5%)	37 (80.4%)			
Follow up data						
NED ($n=579$, 51.8%)	525 (71.7%)	31(44.3%)	23 (82.1%)	<0.001	0.484	0.008
AWED ($n=24$, 2.1%)	19 (2.6%)	4 (5.7%)	1 (3.6%)			
DID ($n=103$, 9.2%)	94 (12.8%)	8 (11.4%)	1 (3.6%)			
DOD ($n=124$, 11.1%)	94 (12.8%)	27 (38.6%)	3 (10.7%)			

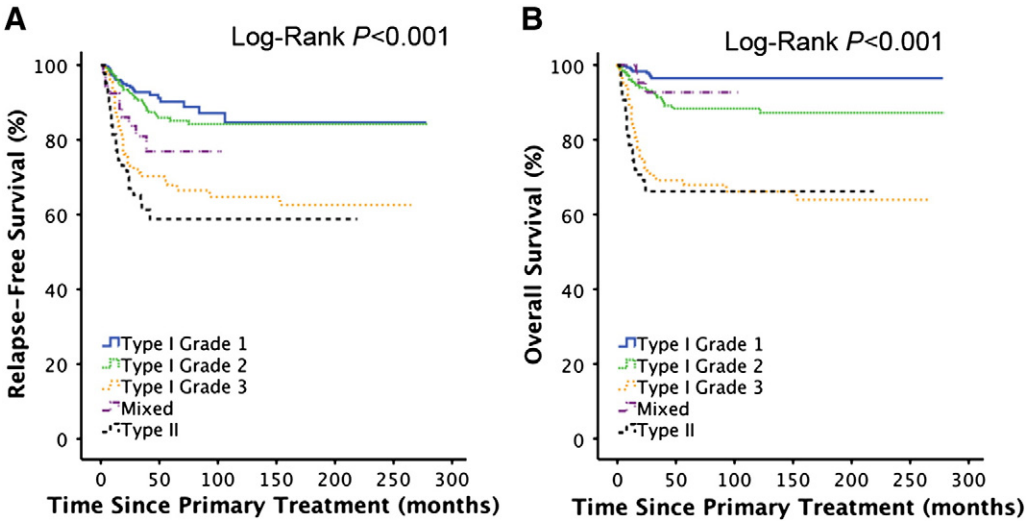

Fig. 1. Effects of type I (grade 1, 2 or 3), type II and mixed tumors on disease outcome in endometrial cancer. Kaplan–Meier survival curves for relapse-free survival (RFS; A) and overall survival (OS; B) in patients with endometrial cancer according to different histopathological subtypes. Type I tumors refer to endometrioid adenocarcinomas, whereas type II cancers refer to serous and clear cell carcinomas. Grades 1, 2 and 3 refer to well-, moderately- and poorly-differentiated endometrioid tumors, respectively.

tumors and all other endometrioid tumors (data not shown), possibly due to the small number of endometrioid/mucinous tumors.

Mutation status of TP53, PTEN and PIK3CA correlates with type and grade

When correlating the mutation status of every gene with tumor characteristics in our discovery cohort (Leuven; $n=318$), *TP53* mutations were more frequent in type II than type I tumors (OR = 22.99; CI = 3.90–135.44; $P=0.001$ using binary logistic regression). In a similar analysis, a positive *PTEN* status was predictive for type I tumors (OR = 7.69; CI = 1.00–58.99; $P=0.050$), whereas *PIK3CA*-mutations were significantly more common in high-grade tumors ($P=0.011$) with *PIK3CA*-mutant tumors being 2.97-fold and 2.14-fold more likely to be grade 2 or 3 relative to grade 1 (CI = 1.42–6.21; $P=0.004$ and CI = 0.95–4.81; $P=0.066$, respectively). No significant effects were detected for other genes (Table 3). When additionally correcting for tissue type, associations for each of these genes remained significant (e.g., *PIK3CA*-mutations were significantly more common in high-grade tumors with *PIK3CA*-mutant tumors; $P=0.033$).

In an effort to replicate these findings, similar tests were performed in cohorts from Genk ($n=68$), Norway ($n=264$), Sweden ($n=160$) and Australia ($n=253$). Although associations in the individual populations were mostly not significant, pooling of the replication cohorts revealed that associations for *TP53* and *PTEN* could be independently replicated (OR = 10.54; $P<0.001$ for *TP53* and OR = 11.32; $P=0.017$ for *PTEN*). Likewise, *PIK3CA*-mutations correlated with tumor grade ($P=0.033$), with *PIK3CA*-mutant tumors being 1.96-fold and 1.85-fold more likely to be grade 2 or 3 relative to grade 1 (CI = 1.17–3.29; $P=0.011$ and CI = 0.98–3.49; $P=0.059$, respectively).

Finally, we also performed analyses in the overall population ($n=1063$). As expected, *TP53* mutations significantly correlated with type II tumors (OR = 11.92; CI = 5.26–26.99; $P<0.001$), whereas *PTEN* mutations correlated with type I tumors (OR = 19.58; CI = 2.68–142.89; $P=0.003$). *PIK3CA* mutations were associated with grade 2 and 3 tumors (OR = 2.03; CI = 1.35–3.05; $P=0.001$ for grade 2 versus grade 1 and OR = 1.89; CI = 1.15–3.09; $P=0.012$ for grade 3 versus grade 1). Interestingly, when eliminating all fresh frozen tumors ($n=90$), the outcome of the meta-analysis of all remaining FFPE tumors ($n=973$) did not change for any of the genes. For instance, association of *PIK3CA* mutation status with grade was still significant ($P=0.004$) thus indicating that *PIK3CA* mutations correlated with high grade in tumors that were only derived from FFPE material.

FBXW7 mutation status correlates with lymph node involvement

Since clinical data for lymph node involvement were only available for 413 patients (38.8%) we could not run independent analyses in separate cohorts. Therefore, we assessed whether lymph node status correlated with mutations occurring in any of the genes using binary logistic regression in the overall population. This correlation was significant independently of age, grade and tumor type (OR = 3.38; CI = 1.03–11.16; $P=0.045$).

H1047R in PIK3CA correlates with relapse-free survival

Next, we assessed whether the mutation status for any of the genes correlated with recurrence. When assessing recurrence as a categorical variable, none of the mutated genes correlated significantly with recurrence (Table 3). Likewise, when performing a Cox regression for time to recurrence (RFS), no correlation with any of the genes was found. Conversely, established prognostic factors (age, type, grade, FIGO and tumor type) correlated highly significantly with RFS (Fig. 1; Supplementary Figures S1–2).

Subsequently, we assessed whether mutations occurring in specific functional gene domains or affecting specific amino acids correlated with RFS. This has been previously described in endometrial cancer, where mutations in exon 20 of *PIK3CA* correlate with adverse prognostic markers such as myometrial invasion and positive lymph nodes [22,23]. When categorizing *PIK3CA* mutations into mutations occurring in exons 1–7, 9 and 20, only exon 20 *PIK3CA*-positive tumors were 2.80-fold more likely to be grade 3 positive (CI = 1.26–6.23; $P=0.011$ versus grade 1 tumors). However, there was no correlation with RFS after Cox regression (Fig. 2A–B). To assess whether any of the individual mutation hot spots in *PIK3CA* also correlated with RFS, mutations occurring with the highest frequency in *PIK3CA* were selected (i.e., E81K, E542K and H1047R mutations occurring respectively in 22, 25 and 36 tumors). Remarkably, H1047R mutations correlated with tumor grade (OR = 2.35; CI = 0.88–6.29; $P=0.087$ for grade 2 and OR = 4.55; CI = 1.64–12.65; $P=0.004$ for grade 3 versus grade 1 tumors) and RFS (HR = 2.18; CI = 1.09–4.39; $P=0.028$; independently of age, grade, FIGO, and tumor type; Fig. 2C–D).

Table 3
Presence of *PTEN*, *PIK3CA*, *TP53*, *FBXW7*, *KRAS*, and *NRAS* mutations according to different demographic and histopathologic characteristics. Percentages are given as row %. *P* values for age, grade, FIGO and type were calculated using a binary logistic regression for *PTEN*, *PIK3CA*, *TP53*, *FBXW7*, *KRAS* and *NRAS* mutation status while adjusting for each of the other variables. *P* values for lymph node and recurrence were calculated separately because there were many missing data for these variables, using binary logistic regression while considering age, grade, FIGO and type as co-variates. Clinical variables were missing for a subset of individuals: age ($n=2$), histopathological grade ($n=1$), FIGO ($n=9$), lymph node involvement ($n=650$), and recurrence ($n=100$).

Total cases analyzed = 1063	*PTEN* mutations			*PIK3CA* mutations			*TP53* mutations		
	Wild-type ($n=899$, 84.6%)	Mutant ($n=164$, 15.4%)	*P* value	Wild-type ($n=891$, 83.8%)	Mutant ($n=172$, 16.2%)	*P* value	Wild-type ($n=1033$, 97.2%)	Mutant ($n=30$, 2.8%)	*P* value
Age (66 ± 10)									
>=60 ($n=724$, 68.1%)	613 (84.7%)	111 (15.3%)	0.912	611 (84.4%)	113 (15.6%)	0.464	695 (96.0%)	29 (4.0%)	0.077
<60 ($n=337$, 31.7%)	284 (84.3%)	53 (15.7%)		279 (82.8%)	58 (17.2%)		336 (99.7%)	1 (0.3%)	
Type									
I ($n=970$, 91.3%)	807 (83.2%)	163 (16.8%)	0.003	812 (83.7%)	158 (16.3%)	0.616	955 (98.5%)	15 (1.5%)	<0.001
II ($n=93$, 8.7%)	92 (98.9%)	1 (1.1%)		79 (84.9%)	14 (15.1%)		78 (83.9%)	15 (16.1%)	
Histopathological grade (only Type I, $n=970$)									
Grade 1 ($n=393$, 37.0%)	327 (83.2%)	66 (16.8%)	0.721	349 (88.8%)	44 (11.2%)	0.004	389 (99.0%)	4 (1.0%)	0.169
Grade 2 ($n=378$, 35.6%)	318 (84.1%)	60 (15.9%)		303 (80.2%)	75 (19.8%)		373 (98.7%)	5 (1.3%)	
Grade 3 ($n=198$, 18.6%)	161 (81.3%)	37 (18.7%)		159 (80.3%)	39 (19.7%)		192 (97.0%)	6 (3.0%)	
FIGO 2009									
I ($n=798$, 75.1%)	685 (85.8%)	113 (14.2%)		673 (84.3%)	125 (15.7%)		779 (97.6%)	19 (2.4%)	
II ($n=78$, 7.3%)	59 (75.6%)	19 (24.4%)	0.036	63 (80.8%)	15 (19.2%)	0.520	77 (98.7%)	1 (1.3%)	
III ($n=144$, 13.5%)	116 (80.6%)	28 (19.4%)		121 (84.0%)	23 (16.0%)		137 (95.1%)	7 (4.9%)	0.622
IV ($n=34$, 3.2%)	31 (91.2%)	3 (8.8%)		26 (76.5%)	8 (23.5%)		32 (94.1%)	2 (5.9%)	
Lymph node involvement									
Positive nodes ($n=66$, 6.2%)	50 (75.8%)	16 (24.2%)	0.154	54 (81.8%)	12 (18.2%)	0.638	61 (92.4%)	5 (7.6%)	0.117
Negative nodes ($n=347$, 32.6%)	293 (84.4%)	54 (15.6%)		284 (81.8%)	63 (18.2%)		340 (98.0%)	7 (2.0%)	
Recurrence									
Yes ($n=151$, 14.2%)	128 (84.8%)	23 (15.2%)	0.652	118 (78.1%)	33 (21.9%)	0.361	145 (96.0%)	6 (4.0%)	0.281
No ($n=812$, 76.4%)	685 (84.4%)	127 (15.6%)		677 (83.4%)	135 (16.6%)		790 (97.3%)	22 (2.7%)	

Total cases analyzed = 1063	*FBXW7* mutations			*KRAS* mutations			*NRAS* mutations		
	Wild-type ($n=1031$, 97.0%)	Mutant ($n=32$, 3.0%)	*P* value	Wild-type ($n=902$, 84.9%)	Mutant ($n=161$, 15.1%)	*P* value	Wild-type ($n=1044$, 98.2%)	Mutant ($n=19$, 1.8%)	*P* value
Age (66 ± 10)									
>=60 ($n=724$, 68.1%)	708 (97.8%)	16 (2.2%)	0.237	619 (85.5%)	105 (14.5%)	0.742	708 (97.8%)	16 (2.2%)	0.194
<60 ($n=337$, 31.7%)	322 (95.5%)	15 (4.5%)		281 (83.4%)	56 (16.6%)		334 (99.1%)	3 (0.9%)	
Type									
I ($n=970$, 91.3%)	940 (96.9%)	30 (3.1%)	0.780	817 (84.2%)	153 (15.8%)	0.036	952 (98.1%)	18 (1.9%)	0.631
II ($n=93$, 8.7%)	91 (97.8%)	2 (2.2%)		85 (91.4%)	8 (8.6%)		92 (98.9%)	1 (1.1%)	
Histopathological grade (only Type I, $n=970$)									
Grade 1 ($n=393$, 37.0%)	379 (96.4%)	14 (3.6%)	0.056	331 (84.2%)	62 (15.8%)	0.699	386 (98.2%)	7 (1.8%)	0.871
Grade 2 ($n=378$, 35.6%)	373 (98.7%)	5 (1.3%)		316 (83.6%)	62 (16.4%)		370 (97.9%)	8 (2.1%)	
Grade 3 ($n=198$, 18.6%)	187 (94.4%)	11 (5.6%)		169 (85.4%)	29 (14.6%)		195 (98.5%)	3 (1.5%)	
FIGO 2009									
I ($n=798$, 75.1%)	776 (97.2%)	22 (2.8%)		682 (85.5%)	116 (14.5%)		785 (98.4%)	13 (1.6%)	0.732
II ($n=78$, 7.3%)	75 (96.2%)	3 (3.8%)	0.845	66 (84.6%)	12 (15.8%)	0.399	77 (98.7%)	1 (1.3%)	
III ($n=144$, 13.5%)	140 (97.2%)	4 (2.8%)		119 (82.6%)	25 (17.4%)		139 (96.5%)	5 (3.5%)	
IV ($n=34$, 3.2%)	33 (97.1%)	1 (2.9%)		29 (85.3%)	5 (14.7%)		34 (100%)	–	
Lymph node involvement									
Positive nodes ($n=66$, 6.2%)	60 (90.9%)	6 (9.7%)	0.045	53 (80.3%)	13 (19.7%)	0.354	66 (100%)	–	0.999
Negative nodes ($n=347$, 32.6%)	336 (96.8%)	11 (3.2%)		287 (82.7%)	60 (17.3%)		341 (98.3%)	6 (1.7%)	
Recurrence									
Yes ($n=151$, 14.2%)	145 (96.0%)	6 (4.0%)	0.534	133 (88.1%)	18 (11.9%)	0.610	148 (98.0%)	3 (2.0%)	0.953
No ($n=812$, 76.4%)	790 (97.3%)	22 (2.7%)		682 (84.0%)	130 (16.0%)		799 (98.4%)	13 (1.6%)	

We also assessed whether other frequent hot spot mutations besides *PIK3CA* such as those occurring in *KRAS*, *PTEN* and *FBXW7* correlated with RFS. No significant correlations were noticed (data not shown).

Discussion

The most significant finding of our study is that *PIK3CA* mutations correlate with high tumor grade and the specific PIK3CA mutation (H1047R) is correlated to reduced RFS. Furthermore, we observed that mutations in *FBXW7*, a p53-dependent tumor suppressor gene, also correlate with a positive lymph node status. Importantly, our observations are based on a cohort of 1063 endometrial tumors, representing – to the best of our knowledge – the largest endometrial cancer cohort ever investigated.

PIK3CA mutations were detected in 16.2% of endometrial tumors, a frequency that is slightly lower compared to previous studies [24,25]. Nevertheless, since in our hotspot mutation approach only 81.4% of all known *PIK3CA* mutations were covered, these data are comparable. *PIK3CA* mutations have previously been linked with endometrioid subtype, but correlations with disease grade or stage have been lacking, possibly due to the limited sample size [6,25]. Our observations that *PIK3CA* mutations correlate with increased tumor grading, independently of the tumor type, are therefore unique. Recently, a weak correlation between *PIK3CA* mutations and tumor grade was demonstrated, though only in endometrioid carcinomas [24]. Likewise, a *PIK3CA* activating expression signature correlates with aggressive disease [26]. Our data, together with these previous findings, thus firmly establish *PIK3CA* mutations as a genetic marker for high-grade endometrial carcinoma. Our data also indicate that exon 20 mutations were more frequently

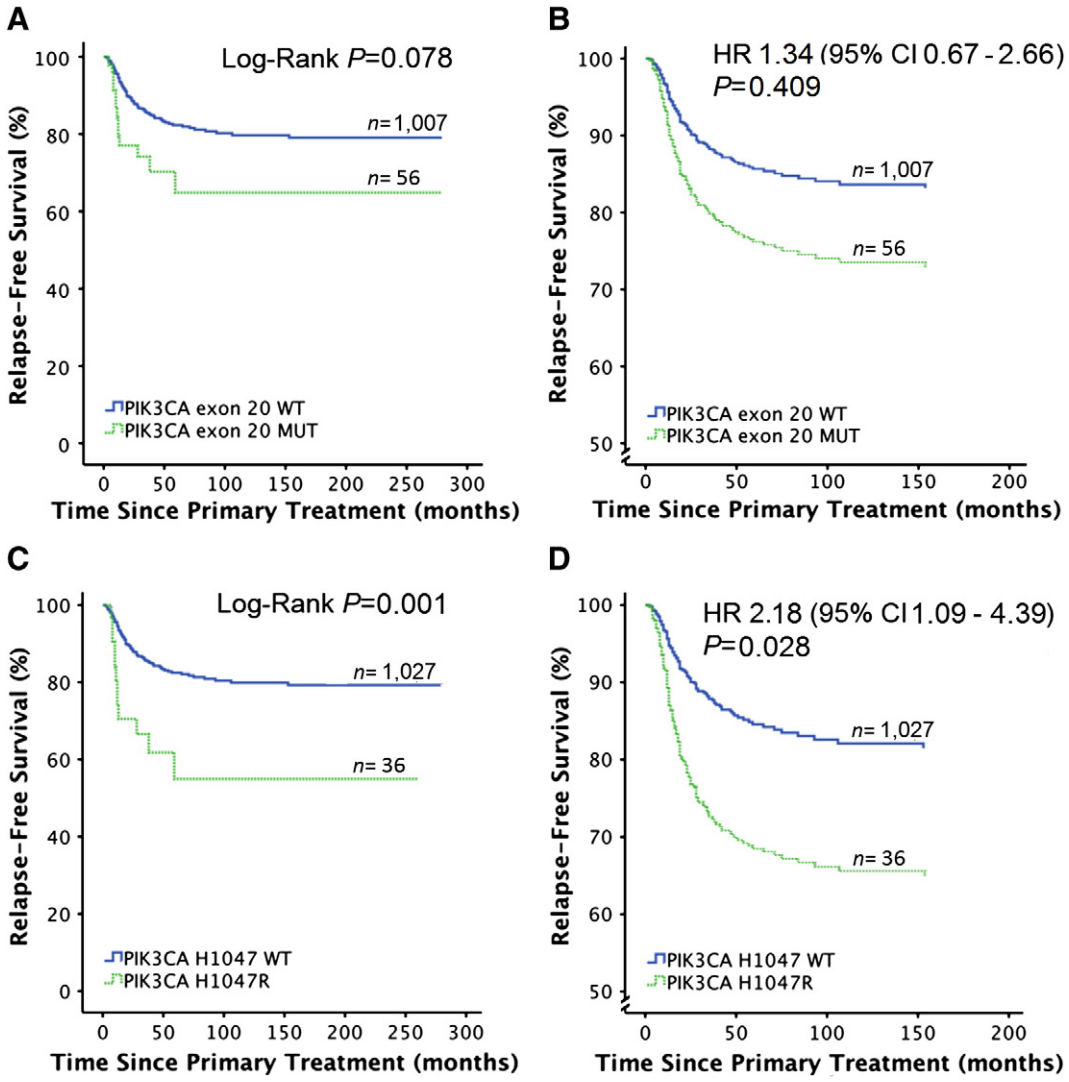

Fig. 2. Effect of *PIK3CA* mutations on relapse-free survival in endometrial cancer patients. Kaplan–Meier survival (A, C) and Cox regression (B, D) analyses show significantly reduced relapse-free survival in patients carrying a *PIK3CA* H1047R mutation as compared to *PIK3CA* H1047R wild-type tumors (C, D), whereas the difference in patients with a *PIK3CA* exon 20 mutation was not significant as compared to *PIK3CA* exon 20 wild-type tumors (A; B). Cox regression analysis was performed independently of age, tumor grade, FIGO stage and cancer type (types I–II). WT: wild-type; MUT: mutant.

observed in grade 3 than in grade 1 tumors. These observations are similar with previous studies showing that *PIK3CA* exon 20 mutations are associated with adverse prognostic markers, including myometrial and lymphovascular invasion [22,23]. More specifically, H1047R *PIK3CA* mutations correlated with tumor grade and patients with this specific mutation showed a significantly reduced RFS. Altogether, this suggests that – at least in endometrial cancer – exon 20 *PIK3CA* mutations are more oncogenic than exon 1–7 or 9 mutations. Since exon 20 encodes the *PIK3CA* catalytic domain and mutations herein constitutively activate its activity [27], this hypothesis may indeed be correct. Additionally, previous studies have shown how different mutations in the *PIK3CA* gene present different oncogenic potential [28–30].

A second activator of the PI3K pathway is somatic loss of *PTEN* function. *PTEN* mutations are frequently observed in type I endometrial cancer and may define a subgroup, as suggested by correlation with low FIGO stage, endometrioid subtype, low grade and favorable prognosis in previous studies [31,32]. In our study, *PTEN* mutation frequency was lower as compared to other studies in endometrial cancer [25,33] and mutations were not associated with RFS, but correlated independently with FIGO and endometrioid subtype. This discrepancy might be due to the fact that we miss a number of *PTEN* mutations using our hotspot mutation profiling approach compared to targeted sequencing of *PTEN*.

We also analyzed the mutation frequency of *FBXW7*, a p53-dependent tumor suppressor gene [10]. *FBXW7* mutations have been reported in several types of human cancers, including endometrial cancer with a mutation frequency varying between 2 and 16% [11,34]. In our study, *FBXW7* mutants correlated with a positive lymph node status, previously also hypothesized in a set of 51 endometrial tumors [11]. Notably, low *FBXW7* expression has previously been correlated with lymph node invasion in colorectal and gastric cancer [35,36]. Loss of *FBXW7* function has also been implicated in increased genomic instability and carcinogenesis [37], but it is not yet clear how it exactly contributes to lymph node invasion.

Intriguingly, our study also highlights the delicacy of the binary classification system of endometrial cancer, whereby endometrioid cancers are classified as type I, and serous or clear cell tumors as type II. The precise allocation of tumors according to this dualistic model has been highly debated, since, based on clinical findings, grade 3 endometrioid carcinomas have been classified as type II by some groups [2,19]. However, our observed mutation profiles do not support this classification. We indeed observed that grade 3 endometrioid carcinoma patients have a significantly shorter RFS than patients with grade 1 or 2 tumors, whereas the mutation profile of grade 3 endometrioid tumors clearly differs from that of type II tumors and is more similar to that of grade

1 or 2 endometrioid tumors. As such, grade 3 type I and type II tumors are characterized by different sets of somatic changes, but could share a common aggressive clinical behavior. Our current observations suggest that *PIK3CA* mutations could represent one such marker leading to aggressive clinical behavior of type I tumors. A more accurate classification of endometrial tumors should thus take into account the molecular profile of the tumor as well as its pathological features and the patient's clinical parameters. As such, endometrioid grade 3 tumors may represent a separate group of endometrial tumors, which is distinct from type I (only endometrioid grades 1–2) and type II tumors.

Classification of mixed endometrial tumors is also debatable and these tumors are often classified according to the component with the worst prognosis. Our observations suggest, however, that mixed subtypes represent a distinct entity, with mutation profiles characteristic to both type I and type II tumors and RFS times intermediate to both tumor types. Our observations in the mixed tumors were similar to previous findings [38].

A limitation of our study is the lack of a central pathology review. However, a large number of samples from only a few experienced clinical centers were included, thereby minimizing the risk of errors. Furthermore, no obvious differences in mutation frequencies across the centers were detected. Another limitation is that we only genotyped hotspot mutations. For a number of genes, such as oncogenes *KRAS*, *BRAF* and *PIK3CA*, it is sufficient to cover hotspot mutations only, but for other genes, such as tumor suppressor genes *PTEN*, *TP53* and *FBXW7*, data should be interpreted more cautiously, as a considerable fraction of mutations cannot be genotyped using hotspot mutations alone. This could also explain why the frequency of *PTEN* and *TP53* mutations were less frequently observed in our patient cohort as compared to previously published data [9,25] and why in our study *TP53* mutations were not associated with poor clinical outcome. However, since sequencing such large number of genes using very small quantities of FFPE-derived tumor DNA is currently not possible, our study provides the best possible alternative to study large series of tumors collected in a routine clinical setting.

Possibly, the inclusion of 90 fresh-frozen tissue in our cohort of 1063 primary endometrial tumors could also have influenced our results. It has indeed been shown that mutation frequencies are underestimated in FFPE samples [18]. In our study, mutations in *PIK3CA*, but not in other genes, were also more frequent in fresh-frozen compared to FFPE tissue. Nevertheless, we are convinced that this difference did not influence the association between *PIK3CA* mutations and high grade. First of all, because we performed an additional analysis, in which we adjusted the association for tissue type. Secondly, because in the replication cohorts, which consisted of tumor samples derived from FFPE only, *PIK3CA* mutations were also associated with high grade ($P = 0.033$). Furthermore, in a final sub-analysis, after we excluded fresh frozen tumors (FFPE only $n = 973$), *PIK3CA* mutations still correlated with high grade.

In conclusion, our study supports a dualistic molecular classification system consisting of endometrioid and non-endometrioid subtypes. We established that *PIK3CA*, *TP53*, *PTEN* and *FBXW7* mutations, respectively, correlate with high grade, endometrial cancer type and lymph node status, whereas a gene-specific mutation in *PIK3CA* (H1047R) correlates with poor prognosis. Overall, these markers may be used for selection of high-risk endometrial cancer patients or to predict the benefit of systemic adjuvant chemotherapy in combination with specific targeted therapies.

Conflict of interest statement

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ygyno.2012.11.037.

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