**PIKing the type and pattern of PI3K pathway mutations in endometrioid endometrial carcinomas**

# UNIVERSITÀ DEGLI STUDI DI TORINO

# PIKing the type and pattern of PI3K pathway mutations in endometrioid endometrial carcinomas

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## Highlights

• The frequency of PI3K pathway mutations is similar between MSS and MSI-H EECs.

• The mutational signatures underpinning MSS and MSI-H EECs are distinct.

• PIK3CA hotspot mutations are significantly more frequent in MSS than in MSI-H EECs.

## Abstract

Objective. The vast majority of endometrioid endometrial carcinomas (EECs) harbor mutations in the PI3K path- way. Here we sought to determine whether the type and pattern of mutations targeting different components of the PI3K pathway are distinct between microsatellite stable (MSS) and high-level microsatellite instable (MSI-H) EECs.

Methods. Whole exome massively parallel sequencing-based mutation data from EECs of The Cancer Genome Atlas (TCGA) were used to define the number, type and pattern of mutations affecting PI3K pathway-related genes, including AKT1, INPP4B, MTOR, PIK3CA, PIK3R1 and PTEN. EECs were classified as MSI-H (n = 70) and MSS (n = 109) based on seven MSI markers assessed by TCGA. Ultramutated cases were excluded.

Results. Although the mutation rates and mutational signatures of MSS and MSI-H EECs were distinct, the prev- alence of PI3K pathway mutations was similar between these two groups (all p N 0.05), with the exception of PTEN mutations, which were more prevalent in MSI-H (61/70; 87%) than in MSS EECs (78/109; 72%; p = 0.017). The PIK3CA hotspot mutations E542K, E545K, and H1047R were found to be significantly more prevalent in PIK3CA- mutant MSS (21/58, 36%) compared to PIK3CA-mutant MSI-H EECs (5/37, 13.5%; p = 0.019).

Conclusion. Although the prevalence of mutations targeting PI3K pathway genes is similar between MSS and MSI-H EECs, PIK3CA hotspot mutations, which result in constitutive kinase activation, are significantly more preva- lent in MSS than in MSI-H EECs. Our findings warrant further investigation of the role of different types of PIK3CA mutations and their predictive impact on distinct subtypes of EECs.

**Keywords:** Endometrioid carcinoma PI3K pathway, Mutations, Microsatellite instability, Hotspot

## INTRODUCTION

Endometrial cancer (EC) is the most common gynecologic malignan- cy in women in North America and Europe [1]. The most frequent histo- logic types are endometrioid endometrial carcinoma (EEC), which accounts for 70–80% of all cases, and serous carcinoma (5–10%) [2]. In the past decade it has become increasingly clear that EC comprises a biologically, clinically, morphologically, and genetically heteroge- neous group of tumors [3]. The Cancer Genome Atlas (TCGA) project has recently shed light on the genome-wide genetic alterations in common-type ECs and proposed a genomic classification featuring four genomic subtypes [4]: 1) the POLE (ultramutated) subgroup repre- sents 10% of the EECs studied, has an excellent prognosis, and is charac- terized by hotspot mutations in the exonuclease domain of the POLE gene and ultrahigh somatic mutation rates; 2) the microsatellite instable (hypermutated) subgroup comprises EECs with microsatellite insta- bility (MSI) due to MLH1 promoter methylation, and is characterized by high mutation rates, few copy number alterations, and recurrent RPL22, KRAS and PTEN mutations; 3) the copy-number low (endometrioid) subgroup comprises microsatellite-stable (MSS) EECs with low muta- tion rates, few copynumber alterations, and frequent CTNNB1 muta- tions; and 4) the copy-number high (serous-like) subgroup comprises serous carcinomas and a subset of grade 3 EECs with extensive copy-number alterations and low mutation rates, recurrent TP53, FBXW7, and PPP2R1A mutations, infrequent PTEN and KRAS mutations, and the worst prognosis overall.

The TCGA study has also confirmed and expanded on previous observations in that more than 90% of EECs, of both MSI (hypermutated) and copy-number low (endometrioid) genomic subtypes, harbor genetic alterations in the phosphatidylinositol-3-OH kinase (PI3K) pathway, suggesting a potential for targeted therapy with PI3K pathway inhibitors in this large contingent of EEC patients [4–6]. Mutations in PIK3CA (the cat- alytic subunit p110α of PI3K) and PIK3R1 (the regulatory subunit p85α of PI3K) show a strong tendency for being mutually exclusive in EECs, but unlike in other tumor types such as breast cancer, they co-occur with PTEN mutations [4,5,7–9]. Also the spectrum of PIK3CA and PTEN mutations in EECs is different from that of other solid tumors, such as breast and colorectal cancers [10]. In addition to the well-documented somatic hotspot mutations in the helical and kinase domains of p110α, in ECs activating mutations in the p110α ABD domain, ABD-linker region and C2 domain are similarly frequent [6,9]. Furthermore, in the TCGA study, 22% of PTEN mutations in EECs occurred at the residue R130, at a rate four times higher than that observed in glioblastoma or in other types of solid tumors with frequent PTEN mutations [4].

The association between MSI and PTEN mutations has been controversial [11], and it is unknown whether the pattern of mutations affecting other genes in the PI3K pathway within the group of EECs is different depending on the MSI status. The aims of this study were to investigate the mutational signatures present in MSI-H and MSS EECs and whether the type and pattern of somatic genetic alterations targeting different components of the PI3K pathway are distinct between MSI-H and MSS EECs. The availability of publicly available massively parallel sequencing data of a large series of sporadic EECs provides an unprecedented opportunity to assess the associations between mutational pat- terns, mutational signatures and MSI status in these cancers.

## METHODS

### Case selection and definitions

Clinico-pathologic data and whole exome sequencing-derived mutational data were retrieved from the TCGA data portal (https://tcga-data. nci.nih.gov/docs/publications/ucec_2013/; files “Key Clinical Data”, “UCEC Somatic Mutations”, “Cumulative Data Freeze List”) [4]. As this study focuses on the pattern of mutations in the PI3K pathway according to the presence of MSI, we

selected only EECs (n = 200) from the cases with available sequencing data. In addition, EECs of POLE (ultramutated) genomic subtype (n = 17) were excluded (total of 183 EECs).
The MSI status of ECs was defined by the TCGA employing a seven- marker call. Using a panel of four mononucleotide markers (BAT25, BAT26, BAT40, and TGFBRII) and three dinucleotide markers (D2S123, D5S346, and D17S250), tumors were classified as microsatellite-stable (MSS) if no markers were altered, low level MSI (MSI-L) if one or two markers (b40%) were altered, and high-level MSI (MSI-H) if three or more markers (N40%) were altered [4]. For all 183 EECs, information about the MSI status was available and 70 were classified as MSI-H, 109 as MSS and 4 as MSI-L. Due to the limited number of MSI-L cases, we focused our analysis only on the MSI-H and MSS groups. In addition, given that in sporadic EECs, MSI, the expansion or contraction of the length of microsatellite tracts, is mainly due to MLH1 promoter methylation [12,13], we obtained the information on MLH1 silencing by promoter methylation from cBioPortal (www.cBioPortal.org) [14].

**Protein domain information**
The information on protein domains was retrieved from UniProt (www.uniprot.org). For the generation of mutation diagrams ("lolli- pop plots"), MutationMapper on cBioPortal was employed (www. cBioPortal.org) [14].

**Mutational signature**
To infer the number of elementary mutational processes and their spectra from the sequencing data of MSS and MSI-H EECs, we employed EMu, a method based upon the expectation–maximization (EM) algorithm, as previously described [15].

**Statistical analysis**
Comparisons between groups were generally performed using a two-tailed Fisher's exact test. When a Chi-squared, Mann–Whitney U test or Student's t test was employed, it is indicated in the text according- ly. Event-free survival was expressed as the number of months from diagnosis to the occurrence of distant or local relapse or death (disease- related death). Cumulative survival probabilities were calculated using the Kaplan–Meier method. Differences between survival rates were tested with the log-rank test (SPSS version 20.0; IBM). Survival data were censored at 5 years. A p b 0.05 was considered statistically significant.

**DNA extraction from EEC cell lines**
The human EEC cell lines ECC-1, EFE-184, EN, HEC-108, HEC-116, HEC-1-B, HEC-265, HEC-50B, HEC-59, HHUA, Ishikawa, NOU-1, RL95-2, SNG-II and SNG-M were cultured as previously described [16]. Genomic DNA from each of the EEC cell lines was extracted using the DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's instructions.

**MSI analysis of EEC cell lines**
MSI was assessed in each of the EEC cell lines using a panel of five mononucleotide repeat markers (BAT25, BAT26, NR-21, NR-24 and NR- 27) as described previously [17]. PCR products were analyzed on an ABI 3100 Genetic Analyzer using GeneMapper v4.0 software (Life Technologies), which allowed for the identification of novel alleles (expansions or contractions) within the repetitive tract of a given marker. A cell line was considered MSI-H when ≥2 of the 5 loci assessed showed MSI.

**RESULTS**
From the TCGA dataset, 70 MSI-H and 109 MSS EECs were included in the current study (Table 1). As expected, MSI-H EECs were classified as of MSI (hypermutated) genomic subtype (65/70, 92.9%; 5 cases not assigned), while MSS EECs were of copy-number low (endometrioid) (85/109,

78%) or copy-number high (serous-like) (16/109; 14.7%; 8 cases not assigned) genomic subtypes. We further noted that in this series, MSS EECs were preferentially of grades 1 and 2, whereas MSI-H EECs were almost evenly distributed between grades 1, 2 and 3 (Table 1). In addition, we observed that MSI-H EECs had a worse out- come than MSS EECs, however this difference was not statistically significant ($p = 0.068$; Supplementary Fig. 1).

MSI-H EECs displayed a mutator phenotype with a median mutation rate of 10.55 mutations/Mb [18], which is attributable to MLH1 promoter hypermethylation found in all but four MSI-H EECs (66/70, 94.3%) (Table 1). By contrast, the mutation rate of MSS EECs was significantly lower (median 1.44 mutations/Mb [18]; Student's t-test with Welch's correction p b 0.001), and the vast majority did not show MLH1 silencing (105/109, 96.3%). The sequencing data were fur- ther analyzed for mutational processes and their spectra [15], which revealed four mutational signatures present in MSI-H vs two mutational signatures present in MSS EECs (Fig. 1A). In both MSI-H and MSS EECs a mutation signature related to age was identified, characterized by prominent C N T transitions at NpCpG trinucleotides (Fig. 1A, blue) [19]. In MSS tumors also a mutational signature attributed to the APOBEC family of cytidine deaminases was identified, which is primarily characterized by C N T and C N G mutations at TpCpN trinucleotides [19] (Fig. 1A, orange), whereas in MSI-H tumors, a DNA- mismatch repair (MMR) deficiency-like signature was found, with increased T N C transitions [19,20] (Fig. 1A, red). These results sug- gest that the mutational processes underlying MSI-H EECs are more diverse and, with exception of the age-related mutation signature, are distinct from those of MSS EECs.

Deficiency in mismatch repair leads to uncorrected small insertions and deletions (indels), particularly in microsatellite regions. As expected [20], the number of indels was found to be significantly higher in MSI-H EECs compared to MSS EECs (p b 0.001, Mann–Whitney U-test), where- as the mean indel size was significantly smaller in MSI-H EECs (3.2 bp) than in MSS EECs (6.2 bp; p b 0.001, Mann–Whitney U-test; Table 2). Interestingly, however, the mean number of indels per case affecting the PI3K pathway genes studied here (i.e. PIK3CA, PTEN, PIK3R1, INPP4B, AKT1, MTOR) and the mean indel size found in these six PI3K pathway genes did not differ between MSI-H and MSS EECs (p N 0.1, Mann–Whitney U-test; Table 2). To investigate whether these findings could be attributable to chance, we selected 10,000 random regions in the exome of the same size as the six PI3K pathway genes (i.e. 29.8 kb regions) and assessed the number of indels found in MSS and MSI-H EECs. The median number of indels in 10,000 random 29.8 kb regions was significantly higher in MSI-H EECs (n = 1, range 0–13) than in MSS EECs (n = 0, range 0–28) (p b 0.001, Mann–Whitney U-test; data not shown). Given the relatively high number of indels in MSS EECs targeting the PI3K pathway genes investigated, when adjusted for the frequency of indels, these data provide evidence to suggest that PI3K pathway related genes may undergo positive selection in the development and progression of MSS EECs.

In similar lines, despite the distinct mutation rates observed be- tween MSI-H and MSS EECs, the prevalence of mutations targeting the PI3K pathway genes AKT1, INPP4B, MTOR, PIK3CA and PIK3R1 were similar between these groups and no statistically significant differences were found (p N 0.05, Table 3). The tumor suppressor gene PTEN, how- ever, was more frequently mutated in MSI-H EECs than in MSS EECs (61/70 MSI-H, 87.1% vs 78/109 MSS, 71.6%; $p = 0.017$). Of the MSI-H and MSS tumors, 95.7% and 94.5% had at least one mutation in the six PI3K pathway genes studied, respectively; 81.4% and 66.1% harbored at least two mutations in these PI3K pathway genes, respectively; and 20% and 6.4% harbored at least three mutations, respectively.

Given that i) despite the differences in mutational rates and number of indels between MSI-H and MSS EECs, the prevalence of alterations affecting PI3K pathway genes was similar in both groups, and that ii) the mutational signatures of MSS and MSI-H were found to be distinct, we assessed whether the type and pattern of mutations targeting these PI3K pathway genes would differ between EECs of different MSI status.

**PIK3CA**

The mutation rate was comparable between MSS and MSI-H, and PIK3CA mutations were found in 53.2% (58/109) and 52.9% (37/70) of the samples, respectively (p = 1.0) (Table 3). The total number of PIK3CA mutations was 66 in MSS and 49 in MSI-H EECs, with 32.4% and 13.7% of MSI-H and MSS cases harboring two distinct PIK3CA mutations, respectively (p = 0.0397).
The type of PIK3CA mutation (i.e. missense vs insertions and deletions) was similar between MSI-H and MSS EECs (data not shown). Interestingly, however, we observed that PIK3CA-mutant MSS EECs significantly more frequently harbored mutations in the hotspots E542K, E545K and H1047R (21/58, 36.2%) than PIK3CA-mutant MSI-H EECs (5/37, 13.5%; p = 0.019, Fisher's exact test; Fig. 1B, Table 4). In fact, while E545K hotspot mutations were present in 13.8% of MSS EECs (8/58), none of the MSI-H EECs harbored this mutation (p = 0.021). The p110α helical domain mutations E542K and E545K as well as the H1047R kinase domain mutation have been shown to have the highest oncogenic potency (hotspot), but other mutations with strong oncogenic potency including N345K, C420R, E545G, and H1047L, or intermediate oncogenic potency, including E545A, M1043I/V and H1047Y, have also been described [21]. Here we found that the PIK3CA hotspot, strong and intermediate mutations were significantly more prevalent in PIK3CA-mutant MSS than in PIK3CA-mutant MSI-H EECs (36/58, 62.1% MSS vs 11/37, 29.7% MSI-H; p = 0.003, Table 4), whereas PIK3CA mutations outside the helical and kinase domains were more frequent in PIK3CA-mutant MSI-H EECs (20/58, 34.5% MSS vs 27/37, 73% MSI-H; p b 0.001; Fig. 1A, Table 4). These data provide evidence to suggest that the prevalence of mutations targeting PIK3CA is similar between MSS and MSI-H EECs, however the type of mutations is distinct with hotspot PIK3CA mutations being more prevalent in MSS EECs.
To assess the associations between MSI status and the type of PIK3CA mutations, we defined the MSI status of 15 EEC cell lines. Analysis of a panel of 5 mononucleotide markers revealed that 87% (13/15) of the EEC cell lines studied were MSI-H (Supplementary Fig. 2A). In agreement with the observations made in MSI-H primary tumors from the TCGA dataset, none of these cell lines harbored a PIK3CA hotspot mutation (Supplementary Fig. 2A). The MSS cell lines were PIK3CA-wild-type. We next sought to define whether these PIK3CA non- hotpot mutations would be associated with response to a p110α inhib- itor. Reanalysis of our previous work [16] demonstrated no differences in response to the p110α inhibitor A66 between MSI-H and MSS EEC cell lines, and between PIK3CA-mutant (i.e. non-hotspot) and PIK3CA- wild-type EEC cell lines (Supplementary Fig. 2B).

**PIK3R1**

The p85α regulatory subunit of PI3K has been suggested to have tumor suppressor properties, and mutations in PIK3R1 may lead to activation of the PI3K pathway through activation of p110α or decrease of PTEN activity [6,9,22,23]. Recently, neomorphic PIK3R1 mutations have also been described, leading to MAPK and JNK pathway activation [24]. The PIK3R1 mutation rates were comparable between MSS and MSI-H cases, and were found in 36.7% (40/109) and 41.4% (29/70) of the EECs, respectively (p = 0.534; Table 3). The total number of mutations was 58 in MSS and 38 in MSI-H EECs. A subset of cases harbored multiple PIK3R1 mutations, and this occurred unexpectedly more frequently in the PIK3R1-mutant MSS (14/40, 35%) compared to the PIK3R1-mutant MSI-H EECs (6/29, 20.7%), however this difference was not statistically significant (p = 0.283). In addition, mutations at position Q579 were identified in 5/40 PIK3R1-mutant MSS EECs (12.5%), whereas none of the 29 PIK3R1-mutant MSI-H EECs harbored a mutation at this position, however this finding was not statistically significant (p = 0.069; Fig. 1B).
No difference in terms of type (e.g. missense, nonsense, frameshift) and pattern (i.e. domains affected) of PIK3R1 mutations was observed between the MSI-H and MSS subgroups of EEC. The distribution of mutations across the entire PIK3R1 coding sequence was similar be- tween MSI-H and MSS EECs (Fig. 1B), and none of the neomorphic PIK3R1 mutations (i.e. R348*, L370fs) were identified in this set of EECs [24]. As described in the TCGA study [4], mutations in PIK3R1 were mutually exclusive with PIK3CA mutations in both MSS EECs (odds ratio 0.095; 95%

confidence interval (CI), 0.0372–0.2426; p b0.001) and MSI-H EECs (odds ratio 0.0679; 95% CI, 0.0205–0.2255; p b 0.001; cBioPortal).

**PTEN**

As mentioned above, the number of cases harboring a PTEN mutation was significantly higher in the MSI-H group (61/70, 87.1%) than in the MSS group (78/109, 71.6%; p = 0.017) of EECs (Table 3). Akin to PIK3R1 mutations, however, we again observed a significantly higher number of PTEN-mutant MSS EECs harboring multiple PTEN mutations (42/78, 53.8%) compared to MSI-H EECs (20/61, 32.8%; p = 0.016; Table 5).

Regarding the type of PTEN mutations, nonsense mutations were more frequently found in PTEN-mutant MSI-H (21/61, 34.4%) than in MSS EECs (14/78, 17.9%; p = 0.031), whereas the prevalence of PTEN frameshift insertions and deletions was higher in MSS EECs (42/78, 53.8%) than in MSI-H EECs (21/61, 34.4%; p = 0.026; Table 5).

The distribution of PTEN mutations in MSS and MSI-H EECs was similar, and representative of that of a tumor suppressor gene with multiple nonsense and frameshift mutations across the entire coding sequence. Finally, mutations affecting the hotspot residue R130 were significantly more frequent in PTEN-mutant MSS EECs (33/78, 42.3%) than in MSI-H EECs (14/61, 23%; p = 0.019), whereas the R233* mutation was more prevalent in PTEN-mutant MSI-H EECs (9/61, 14.8%) than in MSS EECs (4/78; 5.1%), however this difference was not statistically significant (p = 0.077, Fig. 1C).

**AKT1, INPP4B, MTOR**

The number of EECs harboring mutations in AKT1, INPP4B and MTOR was found to be low (0.9–11.4%; Table 3). No differences in terms of number, type, distribution of mutations and affected domains were observed between MSS and MSI-H EECs for these genes. Given the low prevalence of AKT1, INPP4B and MTOR mutations, however, assessment of a larger sample set is warranted.

**DISCUSSION**

The recent massively parallel sequencing studies have broadened our understanding of serous and EECs [3]. In this re-analysis of the EC TCGA dataset [4], we expanded on the finding that the vast majority of EECs harbor mutations in the PI3K pathway, and showed that there are differences when the presence of MSI is taken into account. Consistent with the results from a recent report [25], we observed that MSI-H EECs are associated with a less favorable outcome than MSS EECs, pro- viding evidence to suggest that MSI-H and MSS EECs not only have distinct mutational profiles but may also have distinct clinical behaviors.

MSI is the result of impaired DNA MMR, and as a consequence, DNA MMR-deficient cells tend to accumulate errors, such as single base mis- matches and short deletions and insertions. Here we show that al- though the mutation rate of MSI-H EECs is significantly higher than that of MSS EECs, the prevalence of mutations targeting the distinct components of the PI3K pathway was comparable between these groups with the exception of PTEN mutations, which were more frequent in MSI-H than in MSS tumors (p = 0.017). Furthermore, we observed that the mutational processes underlying MSI-H EECs are more diverse and, with the exception of the age-related mutation signature, distinct from those of MSS EECs. MSI-H EECs were characterized by four mutational processes, including a DNA MMR-deficiency-like signature, whereas MSS EECs displayed two mutational signatures, including an APOBEC signature. Our findings provide evidence to imply that the high number of mutations found in the PI3K pathway in EECs is not solely caused by the MSI-associated hypermutator phenotype.

We identified significant differences affecting the pattern of mutations targeting PTEN and PIK3CA. PTEN loss-of-function mutations are common and have been suggested to be an early even in the neoplastic development of EECs [3,26]. We now provide evidence that the type of PTEN mutations may be distinct between MSS and MSI-H EECs, with the former more frequently

harboring frameshift insertion and deletions, and the latter more frequently harboring nonsense mutations. One may hypothesize that EECs with a mutator phenotype would be more prone to harbor multiple mutations affecting the same gene. Unexpect- edly, however, in the cases studied here, we observed that PTEN-mutant MSS EECs significantly more frequently harbored multiple PTEN muta- tions compared to MSI-H EECs, providing another line of evidence that the high frequency of genetic hits in the PI3K pathway in EECs may not be the result of MSI alone. In addition, we found that the number of indels was significantly higher in MSI-H EECs compared to MSS EECs when all genes were assessed, however, the mean numbers of indels per case affecting the PI3K pathway genes studied here (i.e. PIK3CA, PTEN, PIK3R1, INPP4B, AKT1, MTOR) were similar between MSI-H and MSS EECs. These data suggest that MSS EECs may be enriched for indels targeting PI3K pathway-related genes, however further validation in independent datasets are certainly warranted for this unexpected finding.

By comparing MSS and MSI-H EECs we further observed that MSS tumors were significantly enriched for PIK3CA hotspot mutations in the helical and kinase domains of p110α (E542K, E545K, H1047R). In fact, the E545K mutation was not present in the MSI-H EECs studied here. Furthermore, other PIK3CA mutations, which have been shown to have a strong or intermediate oncogenic potency [21], were significantly more frequently present in MSS than in MSI-H EECs. By contrast, MSI-H EECs were significantly enriched for mutations outside the kinase and helicase domains of p110α, some of which are activating but to a lesser extent than the hotspot mutations [6,21,27–30]. This is of importance, given that the PIK3CA hotspot mutations confer constitutive kinase activity [31], and exon 20 PIK3CA mutations have been associated with adverse prognosis in EC [32]. Furthermore, in other cancer types, the presence of PIK3CA hotspot or helicase and kinase mutations has been associated with response to therapy [33–35]. Using EEC cell line models, we have previously shown that the genetic predictors of response may be distinct between different PI3K pathway inhibitors [16]. In this study we showed that the vast majority of EEC cell lines are MSI-H and are reflective of primary MSI-H EECs as they do not harbor PIK3CA hotspot mutations. We further observed that the PIK3CA status in these cell lines was not associated with response to a p110α inhibitor. The development of more representative MSS cell line models, in particular EEC cell line models harboring PIK3CA hotspot mutations, to investigate the effect of the type of PIK3CA mutation on PI3K pathway inhibition is warranted.

To date, only few clinical trials of PI3K pathway inhibitors in EEC patients have reported on the associations between therapeutic response and PIK3CA mutation status [7]. Janku et al. showed that of three patients with PIK3CA-mutant ECs, two of whom harbored c1047 References
mutations and one had a c1049 mutation, showed partial response and progressive disease, respectively, when treated with PI3K pathway inhibitors [36]. In two other studies, the presence of PIK3CA mutations was assessed in trials testing rapamycin analogs in patients with EC, however the data are not sufficiently detailed to define the type of PIK3CA mutation present on a per case basis [37,38]. It should be noted that, despite the high frequency of genetic alterations in the PI3K pathway in ECs, clinical trials of single agents targeting different components of this pathway have neither shown substantial therapeutic benefits nor resulted in the identification of robust biomarkers of therapeutic response [7,39,40]. Our data suggest, however, that defining not only the presence of a PIK3CA and/or PTEN mutation in tumor material obtained from clinical trials but also the type of PIK3CA mutation would be of importance for the development of optimal biomarkers of response to PI3K pathway inhibitors. In particular, it would be of great interest to investigate whether EECs harboring hotspot vs non-hotspot PIK3CA mutations would respond differently to pan- or isoform-specific PI3K inhibitors.

The genomic subtypes, as described by TCGA, have not been incorporated in clinical practice as yet, given that the classification of tumors into each subtype requires genome-wide gene copy number and mutational data. Importantly, the vast majority of MSI-H EECs are of MSI (hypermutated) genomic subtype, however MSS EECs have been shown to be heterogeneous at the molecular level and may be classified as copy-number low (endometrioid), copy-number high

(serous-like) or POLE (ultramutated) genomic subtypes. The genomic heterogeneity of MSS ECs is mirrored by the heterogeneity of their clinical behavior; copy-number high (serous-like) MSS ECs have been shown to have a worse outcome than POLE (ultramutated) MSS ECs [4]. Taken together, although the genomic subtype classification of EECs has yet to be translated into a clinically useful taxonomy, the classification of MSS EECs into genomic subtypes may provide prognostic and/or predictive information in addition to that provided by MSI status alone.

In conclusion, in this re-analysis of the massively parallel sequencing data of ECs by the TCGA we observed that MSS and MSI-H EECs are characterized by distinct PIK3CA and PTEN mutational patterns. MSS EECs more frequently harbor PIK3CA hotspot mutations as well as PTEN frameshift insertions and deletions. Conversely, in MSI-H EECs the majority of PIK3CA mutations were outside the p110α helical and kinase domains, and an enrichment for PTEN nonsense mutations was found. Future studies are warranted to define whether these distinct mutational patterns of the PI3K pathway affect response to therapies targeting this pathway. Furthermore, we have found that the mutational processes operating in the development of MSI-H and MSS EECs are distinct, and that the landscape of mutations affecting PI3K pathway related genes may be shaped by multiple mutational processes in these cancers.

**Conflict of interest statement**

The authors have no conflicts of interest to declare.

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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.2015.02.010.

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# TABLES

**TABLES**

**Table 1** Clinico-pathologic features and mutation rates of MSS and MSI-H endometrioid endome- trial cancers included in this study.

| | MSI-H | MSS | *p*-Value |
|---|---|---|---|
| Patients (n) | 70 | 109 | |
| Histologic type | Endometrioid | Endometrioid | |
| Age (y) | | | |
| Median (range) | 63 (35-88) | 60 (34-90) | 0.110* |
| Grade (n) | | | |
| Grade 1 | 20 | 49 | <0.001** |
| Grade 2 | 24 | 46 | |
| Grade 3 | 26 | 14 | |
| Stage (n) | | | |
| Stage I | 55 | 90 | 0.764*** |
| Stage II | 3 | 6 | |
| Stage III | 9 | 8 | |
| Stage IV | 2 | 4 | |
| Unknow | 1 | 1 | |
| Genomic subtype (n) | | | |
| MSI (hypermutated) | 65 | 0 | <0.001** |
| Copy-number low (endometrioid) | 0 | 85 | |
| Copy-number high (serous-like) | 0 | 16 | |
| Not assigned | 5 | 8 | |
| Mutation rate (n/Mb) | | | |
| Median (range) | 10.55 | 1.44 | <0.001* |
| | (0.06-153.1) | (0.56-18.71) | |
| *MLH1* hypermethylation | 66 | 4 | <0.001*** |

Mb, megabase: MSI-H, high-level microsatellite instable; MSS, microsatellite stable; n, number; y, years. Information on mutation rate obtained from Kandoth et al. [18].

*Student's t-test with Welch's correction.

**Chi-squared.

***Fisher'sexact test, two-tailed.

**Table 2** Insertions and deletions in MSS and MSI-H endometrioid endometrial cancers.

| | MSS EECs (n = 109) | MSI-H EECs (n = 70) | Mann-Whitney *U* test |
|---|---|---|---|
| **Median/ mean number indels per case** (range) | 5/5.5 (1-17) | 21.5/23.8 (4-72) | <0.001 |
| **Median/ mean indel size (bp)** (range) | 3/6.2 (1-96) | 2/3.2 (1-91) | <0.001 |
| **Median/ mean number of indels per case in PI3K pathway genes** *(PIK3CA, PTEN, PIK3R1, INPP4B, AKT1, MTOR)* | 1/1 (0-3) | 1/0.74 (0-4) | 0.126 |
| **Median/ mean indel size in PI3K pathway genes** *(PIK3CA, PTEN, PIK3R1, INPP4B, AKT1, MTOR)* | 3/5.4 (1-33) | 3/5.5 (1-28) | 0.654 |

Bp, base pair; EEC, endometrioid endometrial cancer; indel, insertion and deletion; MSI-H, high-level microsarellite instable; MSS, microsatellite stable; n, number.

**Table 3** Mutations in PI3K pathway genes in MSS and MSI-H endometrioid endometrial carcinomas.

| Gene | MSS EECs (n = 190) | | | MSI-H EECs (n = 70) | | | *p*-Value mutated samples |
|---|---|---|---|---|---|---|---|
| | Mutated samples (%) | Mutation rate | Total mutations (n) | Mutated samples (n) | Mutation rate | Total mutations (n) | |
| AKT1 | 1 | 0.9% | 1 | 2 | 2.9% | 2 | 0.562 |
| INPP4B | 1 | 0.9% | 1 | 3 | 4.3% | 3 | 0.301 |
| MTOR | 4 | 3.7% | 4 | 8 | 11.4% | 9 | 0.064 |
| PIK3CA | 58 | 53.2% | 66 | 37 | 52.9% | 49 | 1.000 |
| PIK3R1 | 40 | 36.7% | 58 | 29 | 41.4% | 38 | 0.534 |
| PTEN | 78 | 71.6% | 128 | 61 | 87.1% | 82 | 0.017 |

EEC, endometrioid endometrial carcinoma; MSI-H, high-level microsarellite instable; MSS, microsatellite stable; n, number.
*Fisher's exact test, two-tailed.

**Table 4** Type of PIK3CA mutations affecting MSS and MSI-H endometrioid endometrial carcinomas.

| PIK3CA mutation type* or domain** | PIK3CA-mutant MSS EECs (n = 58) | PIK3CA-mutant MSI-H EECs (n = 37) | *p*-Value mutated samples |
|---|---|---|---|
| **Hotspot** (E542K, E545K, H1047R) | 21 | 5 | 0.019 |
| **Hotspot and strong** (E542K, E545K, H1047R, N345K, C420R, E545G, Q546K/P, H1047L) | 31 | 9 | 0.006 |
| **Hotspot, strong and intermediate** (E542K, E545K, H1047R, N345K, C420R, E545G, Q546K/P, H1047L, E545A, M1043I/V, H1047Y) | 36 | 11 | 0.003 |
| **Helical domain** (AA 517-694) | 24 | 8 | 0.074 |
| **Kinase domain** (AA 797-1068) | 20 | 8 | 0.249 |
| **Outside helical and kinase domains** | 20 | 27 | <0.001 |

AA, amino acid; EEC, endometrioid endometrial carcinoma; MSI-H, high-level microsarellite instable; MSS, microsatellite stable
*Oncogenic potency as described in Gymnopoulos et al. [21].
**Information from UniProt (P42336). p-Value, Fisher's exact test, two-tailed

**Table 5** Distribution and type of PTEN mutations in MSS and MSI-H endometrioid endometrial carcinomas.

| PTEN mutation type or domain* | Cases harboring PTEN mutation | | | Total number of PTEN mutations | | |
|---|---|---|---|---|---|---|
| | MSS EECs (n = 78) | MSI-H EECs (n = 61) | *p*-Value | MSS EECs (n = 128) | MSI-H EECs (n = 82) | *p*-Value |
| **Missense** | 41 | 28 | 0.496 | 52 | 29 | 0.471 |
| **Nonsense** | 14 | 21 | 0.031 | 16 | 23 | 0.006 |
| **In-frame insertions and deletions** | 5 | 4 | 1.0 | 5 | 4 | 0.739 |
| **Frameshift insertions and deletions** | 42 | 21 | 0.026 | 48 | 21 | 0.097 |
| **Slice site** | 7 | 5 | 1.0 | 7 | 5 | 1.0 |
| **Phosphatase domain** (AA 14-185) | 57 | 42 | 0.706 | 80 | 45 | 0.314 |
| **C2 domain** (AA 190-350) | 32 | 28 | 0.607 | 39 | 31 | 0.296 |

AA, amino acid; EEC, endometrioid endometrial carcinoma; MSI-H, high-level microsarellite instable; MSS, microsatellite stable
* Information from UniProt (P60484). p-Value, Fisher's exact test, two-tailed.

**Figure 1.** Mutational signatures and patterns of PIK3CA, PIK3R1 and PTEN mutations in MSS and MSI-H endometrioid endometrial carcinomas. (A), Mutational signatures identified in MSS (left) and MSI-H (right) endometrioid endometrial cancers (top), the contribution of the mutation signatures and mutation rates of each case (bottom). In MSS, an APOBEC signature (Signature 1) and an age-related mutation signature (Signature 2) were identified. In MSI-H endometrioid endometrial cancers, four mutational signatures were identified, including an age-related signature (Signature 1) and a DNA mismatch repair deficiency-related signature (Signature 3). The underlying processes of the other signatures are not known. The con- tribution of each mutational signature towards the total mutational load in each sample is expressed as a percentage of the total number of mutations in that sample, color-coded according to the mutational signatures. The corresponding mutation rate of each sample is shown, and the samples are colored based on their dominant mutational signature. (B), Diagrams representing the protein domains of p110α encoded by PIK3CA (top), of p85α encoded by PIK3R1 (middle), and of PTEN (bottom). The mutation in each of the genes in MSS (left) and MSI-H (right) endometrioid endometrial cancers are shown on the x-axis. The frequency of mutations is shown on the y-axis. Missense mutations are presented as green circles, truncating mutations (i.e. nonsense, frameshift deletions, frameshift insertions and splice site) are depicted in red circles, in-frame insertions and deletions are shown in black circles, and circles colored in purple indicate residues affected by different types of mutation at the same proportion. Plots were generated using MutationMapper on cBioPortal (www.cBioPortal.org) and manually curated. Information on protein domains was retrieved from UniProt (www.uniprot.org).