Molecular alterations in endometrial cancer: implications for clinical management

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Chapter 2

High concordance of molecular tumor alterations between pre-operative curettage and hysterectomy specimens in patients with endometrial cancer

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Abstract

Objective: Molecular alterations in endometrial cancer have been shown to be prognostically significant but have not yet been implemented in the current clinical risk assessment. Few studies have investigated the reliability of molecular alterations in pre-operative specimens. Therefore, the objective was to determine whether molecular analysis of pre-operative endometrial cancer samples accurately reflects those alterations in the subsequent hysterectomy specimens.

Methods: Paired pre-operative and hysterectomy specimens of 48 patients diagnosed with endometrial carcinoma, 42 endometrioid (EEC) and 6 non-endometrioid (NEEC) carcinomas, were analyzed for immunohistochemical expression of p53, PTEN and β-catenin. Tumor DNA was isolated and analyzed for microsatellite instability (MSI), *TP53* mutations and somatic hot spot mutations in 13 genes.

Results: In EEC patients, loss of PTEN, nuclear β-catenin and p53-mutant expression was found in 43%, 7% and 12%, respectively. No nuclear β-catenin was found in 5 of 6 NEEC patients, all serous cancers, whereas a p53-mutant expression was present in all serous cases. MSI was found in 19.5%, all EEC. Concordance for PTEN, β-catenin, p53 expression and MSI status was found in 79%, 92%, 79% and 93.5%, respectively. We detected 65 hot spot mutations in 39/48 (81%) tumors. Overall concordance of the GynCarta multigene analysis was 99.8%.

Conclusions: The results confirm the reliability of immunohistochemical and DNA-based techniques in the evaluation of molecular alterations in pre-operative endometrial specimens and high concordance rates with the definitive hysterectomy specimens. The resulting molecular signature provides initial pre-operative diagnostic information on the status of oncogenic pathways, which may contribute to individualized treatment strategies.

Introduction

Endometrial carcinoma is the most frequent malignancy of the female genital tract in developed countries. Due to early clinical symptoms of post-menopausal bleeding, most endometrial cancers (80%) are detected in an early stage (International Federation of Obstetricians and Gynecologists (FIGO) stage l). Hysterectomy and bilateral salpingo-oophorectomy is the cornerstone treatment and FIGO staging is assigned based on surgical and pathological findings.[1] Using both clinical (age) and pathologic factors (FIGO stage, tumor type, grade and LVSI) risk groups have been defined to tailor adjuvant treatment to the individual patient's risk of disease recurrence.[2-4]

The role of pelvic and para-aortic lymphadenectomy has been the subject of ongoing debate. Two randomized trials including predominantly intermediate risk patients found neither benefit in overall or disease free survival nor difference in site of recurrence, while lymphadenectomy was associated with higher rates of treatment related morbidity.[5,6] Current ongoing and planned trials are investigating the roles of lymphadenectomy and chemotherapy with or without radiation therapy in high-risk endometrial cancer. Reliable pre-operative risk assessment could be highly desirable to guide the patients' further (adjuvant) treatment.

Pre-operative tissue sampling methods used for the evaluation of endometrial pathology are conventional dilation & curettage, out-patient micro-curettage endometrial tissue sampling (such as Pipelle or Vabra) and hysteroscopy-guided tissue biopsy. The prognostic accuracy of typing and grading of endometrial cancer in such pre-operative samples is subject to considerable interobserver variation, especially since sometimes the scant biopsy material harbors the risk of misclassification and/or assigning a lower tumor grade based on tumor heterogeneity and therefore not always optimal.[7-9] Defining FIGO stage I and II endometrial carcinomas depends on the depth of myometrial invasion and endocervical involvement. Myometrial invasion will not be evident in the superficial sample of the pre-operative curettage material. Other methods of pre-operative risk assessment using ultrasound, computed tomography or magnetic resonance imaging have limited accuracy and high rates of variability.[10-12] Reliable pre-operative risk assessment based on the individual tumors' molecular signature would be valuable in tailoring the extent and route of surgery, patient counseling and adjuvant treatment to the patients' risk profile.

Several molecular alterations in pathways involved in endometrial carcinogenesis are independent prognostic factors, but are not yet used in the current system for risk assessment.[13-15] Recently, our group has shown that molecular alterations in the PI3K–AKT, p53 and Wnt/β-catenin signaling pathways and microsatellite instability may independently or in combination better predict an individual tumor's risk of early disease spread than the clinicopathologic features alone.[14] Most studies analyzing these molecular tumor alterations are performed on hysterectomy specimens. It is largely unknown whether such molecular alterations can be reliably identified in pre-operative samples and whether these correspond to the subsequent hysterectomy findings. In other cancer

types, risk analysis based on pre-operative material has been studied using endoscopic biopsies of colorectal cancer,[16] core biopsies in breast cancer,[17] biopsies of prostate cancer[18] and fine needle aspirates from non-small-cell lung cancer.[19] The main objective of this study was to analyze the presence and concordance of putative prognostic molecular alterations in endometrial cancer in pre-operative curettage samples and corresponding hysterectomy specimens.

Materials and methods

Patient and tissue selection

Fifty study subjects were randomly selected from the database of LUMC Department of Pathology in which both pre-operative curettage and hysterectomy specimens were available. We aimed for 50% of patients with superficial myometrial invasion and 50% of patients with deep myometrial invasion. The pre-operative sampling methods used for 15 of the 48 pre-operative samples include conventional dilation & curettage (n=5), out-patient micro-curettage endometrial tissue sampling (n=9) and hysteroscopy-guided tissue biopsy (n=1). Curettage samples of two patients contained insufficient material to perform all analysis, thus these were excluded, leaving 48 patients in the study. The study population consisted of 42 patients diagnosed with endometrioid endometrial cancer (EEC) and 6 patients with non-endometrioid endometrial cancer (NEEC, 5 serous and 1 clear cells) (Table 1). Formalin fixed paraffin-embedded (FFPE) blocks containing representative tumor and curettage material were selected with at least a 2 mm tumor fragment, unpaired and given a random number during the course of the experiments, so that it was unknown which hysterectomy and curettage specimens belonged together.

Immunohistochemical analysis

Immunohistochemistry for p53, β-catenin and PTEN was performed as described previously.[14] Antigen retrieval was achieved by microwave oven procedure in 10 mmol/L citrate buffer, pH 6.0 for p53 and β-catenin. For PTEN and MLH1 staining, antigen retrieval was performed in 10 mmol/L Tris–EDTA, pH 9.0. Sections were incubated overnight with primary monoclonal antibodies against p53 (clone DO-7, 1:1000; NeoMarkers), β-catenin (cat. 610154; 1:800; BD Transduction), PTEN (clone 6.H2.1, 1:800; DAKO) and MLH1 (clone ES05, 1:100; DAKO). Sections were incubated and stained for 30 min using a secondary antibody (Poly-HRP-GAM/R/R; DPV0110HRP; Immunologic). Diaminobenzidine tetrahydrochloride was used as a chromogen for p53 and β-catenin and DAB+ (DAKO, K3468) as chromogen for PTEN. The slides were counterstained with hematoxylin, dehydrated and mounted. Non-neoplastic endometrium and endometrial tumors with proven p53, β-catenin and PTEN were used as external negative and positive controls, respectively.

Evaluation of staining

Slides were evaluated by two independent pathologists (T.B. and V.S.), blinded for pairing between curettage and hysterectomy. Discrepancies were discussed and reviewed at a multihead microscope and until consensus was reached. p53 was scored "mutant-like" if more than 50% of the tumor cells showed strong positive nuclear staining, or when discrete geographical patterns showed more than 50% tumor cell positivity, or when no nuclear p53 staining was evident in the entire tumor.[14,20,21] Activated Wnt-signaling was defined as nuclear staining of β-catenin. MLH1 nuclear staining was scored as positive or negative, with stromal- and/or lymphocytic cells as internal controls. PTEN staining was evaluated in three categories as negative, positive and heterogeneous.[22] The cases scored heterogeneous were reclassified as positive when more than 10% of tumor cells were positive.

DNA analysis

Prior to DNA isolation, tumor DNA from hysterectomy specimens was enriched in the FFPE blocks by taking three 0.6 mm tissue punches from the tumor focus using a tissue microarrayer (Beecher Instruments), to reach tumor percentage >70%. DNA from curettage blocks was isolated depending on the volume of blood. When there was <50% blood, 2 whole sections (10 μM) were used for DNA isolation. When there was >50% blood, in 10 curettage specimens, then 10 sections (10 μM) were used to microdissect fragments of tumor, for the enrichment of tumor DNA. DNA isolation was performed fully-automated as described previously using the Tissue Preparation System (Siemens Healthcare Diagnostics).[23]

Microsatellite instability (MSI)

The microsatellite status of each tumor was determined using the Promega MSI analysis system (version 1.2), as described previously.[14] Tumors with instability in two or more of these markers were defined as being high-frequency MSI (MSI-H) whereas those with instability at one repeat or showing no instability were classified as being stable (MSS).[14]

TP53 mutation analysis

Sanger sequencing for exons 5–8 of *TP53* was performed on those samples that showed a 'mutant-like' p53 immunohistochemical staining pattern. Sanger sequencing was conducted following the exact protocol described previously.[14,24]

Mutation genotyping

The Sequenom MassARRAY system and the GynCarta multigene analysis 2.0 (Sequenom) were used to test for 159 hot spot mutations in 13 genes (*BRAF*, *CDKNA2*, *CTNNB1*, *FBXW7*, *FGFR2*, *FGFR3*, *FOXL2*, *HRAS*, *KRAS*, *NRAS*, *PIK3CA*, *PPP2R1A* and *PTEN*) as described previously by Spaans *et al.* (manuscript submitted, Supplementary Table 2). Briefly, isolated genomic DNA was amplified using the GynCarta PCR primer pools by multiplex PCR. Unincorporated nucleotides were inactivated by shrimp alkaline phosphatase followed

by a single base pair extension reaction using iPLEX Pro chemistry. Salts were removed using a cation exchange resin. Products were then spotted onto SpectroCHIP II arrays, and mutant and wildtype alleles were discriminated via mass spectrometry using the Sequenom Compact MassARRAY Analyzer. All tumor DNA samples were additionally analyzed using allele specific qPCR as described previously, to validate *KRAS* hot spot mutations in exon 2 and *PIK3CA* hot spot mutations in exons 9 and 20.[14,19]

Data analysis

Data analysis was performed using Sequenom MassARRAY Typer Analyzer software 4.0.22, which identifies mutants by comparing ratios of the wildtype peak to that of all suspected mutants and generates a report with specific mutations and the ratios of wildtype and mutation peaks. Two investigators manually reviewed mutations (≥ 5% mutant peak) to remove all artifact peaks due to salt peaks or other background peaks.

Results

Among the 42 endometrioid (EEC) and 6 non-endometrioid (NEEC) endometrial cancers included in this study, 26 tumors (54.2%) were diagnosed as grade 1, 10 (20.8%) as grade 2 and 12 (25.0%) as grade 3 in the definitive hysterectomy specimen (Table 1). The curettage diagnoses were compared to those from the hysterectomy. Among the 48 cases, 45 (93.8%) showed concordance in histological subtype and 32 cases (66.7%) showed concordance in grade between curettage and hysterectomy specimen (Table 1). In the curettage samples, 14.6% (7/48) of the tumors had been assigned a higher tumor grade and 16.7% (8/48) a lower tumor grade than those in the hysterectomy diagnoses. The accuracy of assigning tumor grade was higher for grade 2 (4 cases; 3 shift to grade 1, 1 shift to grade 3) and grade 3 (4 cases; 1 shifts to grade 1, 3 shift to grade 2) than for grade 1 (8 cases; 5 shift to grade 2, 3 shift to grade 3).

Table 1. Patient and tumor characteristics and concordance of histopathological features in hysterectomy and pre-operative curettage specimens.

	Hysterectomy n=48 (%)	Curettage n=48 (%)	Total discordant cases	Concordance rate
Age at Diagnosis				
Mean	68.4			
Range	51-84			
Histopathological Type				
Endometrioid	42 (87.5)	41 (85.4)	3	93.8
Non-endometrioid	6 (12.5)	7 (14.6)		
Clear cell	5	6		
Serous	1	1		
Myometrial Invasion				
<50%	25 (52.1)	-	-	-
>50%	23 (47.9)			
Grade				
1	26 (54.2)	30 (62.5)		
2	10 (20.8)	6 (12.5)	16	66.7
3	12 (25.0)	12 (25.0)		

Immunohistochemical analysis of PTEN, β-catenin and p53 succeeded in paired curettage and hysterectomy samples of all patients, while DNA analysis completely failed for two curettage samples due to low DNA concentration (0.6 and 1.0 ng/μL) and poor DNA quality and therefore excluded from further analysis. The average yield of DNA recovered from the 46 hysterectomy and 46 curettage specimens was 12.5 ± 5.8 ng/μL and 7.0 ± 4.2 ng/μL, respectively (Supplementary Table 1). The DNA quality assessed by qualitative multiplex PCR assay showed that most samples contained moderate or good quality DNA as seen by the amplification of PCR fragments of different lengths (Supplementary Table 1). No significant differences were observed in the yield of DNA and DNA quality obtained from the pre-operative curettage and hysterectomy specimens. *TP53* sequencing of exons 5–8 was performed on those samples that showed either mutant or no immunohistochemical staining and paired analysis was successful in 18 cases (85.7%; 20 hysterectomy/18 curettage). MSI analysis was successful in 40 cases (87.0%; 41 hysterectomy/40 curettage). Furthermore, GynCarta multigene analysis was successful in 98.5% of all assays (13 multiplexes for 159 hot spot mutations) and paired hot spot mutation analysis of *KRAS* and *PI3KCA* was successful in 42 cases (89.5%; 45 hysterectomy/ 42 curettage). The reason for failure was either running out of material or poor DNA quality due to suboptimal fixation.

Molecular alterations found in the tumor of both hysterectomy and pre-operative specimens are depicted in Table 2. In the hysterectomy specimens diagnosed as EEC, 42.9% showed loss of PTEN, 7.1% showed nuclear β-catenin staining and 20.0% were microsatellite unstable. In contrast, NEEC showed only in 16.6% loss of PTEN, showed no nuclear β-catenin staining and were all microsatellite stable. Through analysis of mutations of fourteen genes, we could detect at least one mutation in 42 of the 46 hysterectomy specimens. The distribution of mutations is shown in Supplementary Table 3. We identified 11.9% *TP53* mutations, 17.5% *CTNNB1* (β-catenin), 2.5% *FBXW7*, 7.5% *FGFR2*, 22.5% *KRAS*, 2.5% *NRAS*, 37.5% *PIK3CA* and 60.0% *PTEN* hot spot mutations in EEC. In NEEC, mutations were found in *TP53* (83.3%), *CTNNB1* (16.6%), *PTEN* (16.6%) and *PPP2R1A* (50%). Notably, the only NEEC tumor without a p53 mutation was a clear-cell carcinoma with a *PTEN* and *CTNNB1* mutation.

Mutations in *PPP2R1A* in combination with *TP53* mutations were specific for non-endometrioid endometrial tumors whereas *FBXW7*, *FGFR2*, *KRAS*, *NRAS*, and *PIK3CA* mutations were subtype-specific for endometrioid endometrial tumors. The frequency of *PIK3CA* exon 9 mutations was higher in grade 1 endometrioid carcinomas (16.7%) than in grade 2 (10%) or grade 3 (0%) tumors. Conversely, mutations in *PIK3CA* exon 20 were more common in grade 3 (33.3%) than in grade 2 (30%) or grade 1 (8.3%) endometrioid carcinomas. In addition, a slightly higher number of molecular alterations per case were seen in endometrioid tumors with deep myometrial invasion compared to tumors with less than 50% myometrial invasion (*P*-value=0.062, parametric t-test, equal variances). Additionally, the depth of myometrial invasion was not related to a specific mutated gene or gene mutation.

Table 2. Concordance of molecular alterations in endometrial hysterectomy and pre-operative specimens using immunohistochemistry and DNA analysis.

	Hysterectomy n (%)	Curettage n (%)	Total discordant cases	Concordance Rate
Immunohistochemistry (n=48)				
PTEN				
Positive	29 (60.4)	28 (58.3)	5	89.6
Negative	19 (39.6)	20 (41.7)		
p53				
Wildtype	38 (79.2)	39 (81.3)	1	97.9
Mutant-like	10 (20.8)	9 (18.8)		
Nuclear β-catenin				
Absent	45 (93.8)	43 (89.6)	2	95.8
Present	3 (6.3)	5 (10.4)		
DNA analysis (n=46)				
Microsatellite instability				
MSS	33 (80.5)	36 (90.0)	3	93.5
MSI	8 (19.5)	4 (10.0)		
BRAF				
Wildtype	46 (100)	46 (100)	0	100
Mutant	0 (0)	0 (0)		
CDKN2A				
Wildtype	46 (100)	46 (100)	0	100
Mutant	0 (0)	0 (0)		
CTNNB1				
Wildtype	38 (82.6)	35 (76.1)	5	99.8
Mutant	8 (17.4)	11 (23.9)		
FBXW7				
Wildtype	45 (97.8)	44 (95.7)	1	99.8
Mutant	1 (2.2)	2 (4.3)		
FGFR2				
Wildtype	43 (93.5)	43 (93.5)	0	100
Mutant	3 (6.5)	3 (6.5)		
FGFR3				
Wildtype	46 (100)	46 (100)	0	100
Mutant	0 (0)	0 (0)		
FOXL2				
Wildtype	46 (100)	46 (100)	0	100
Mutant	0 (0)	0 (0)		
HRAS				
Wildtype	46 (100)	46 (100)	0	100
Mutant	0 (0)	0 (0)		
KRAS				
Wildtype	37 (80.4)	37 (80.4)	0	100
Mutant	9 (19.6)	9 (19.6)		
NRAS				
Wildtype	45 (97.8)	44 (95.7)	1	99.9
Mutant	1 (2.2)	2 (4.3)		
PIK3CA				
Wildtype	31 (67.4)	29 (63.0)	4	99.8
Mutant	15 (32.5)	17 (37.0)		
PPP2R1A				
Wildtype	43 (93.5)	41 (89.1)	2	99.8
Mutant	3 (6.5)	5 (10.9)		
PTEN				
Wildtype	22 (47.8)	21 (45.7)	3	99.9
Mutant	24 (52.2)	25 (54.3)		

MSS=microsatellite stable; MSI=microsatellite unstable

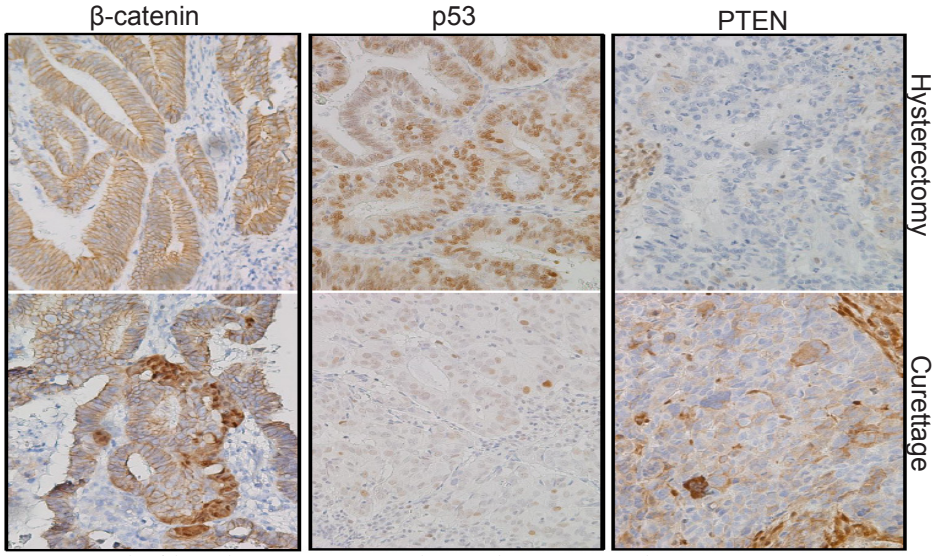

Figure 1. Example of discordant immunostainings between hysterectomy and pre-operative curettage specimens. For PTEN, the hysterectomy showed loss of expression whereas the paired pre-operative curettage showed a positive staining. A p53 'mutant-like' expression was observed in the hysterectomy while focal, weak and heterogeneous staining was observed in the paired pre-operative curettage. Nuclear β-catenin staining was observerd in the curettage but not in the paired hysterectomy.

Concordance rates between hysterectomy and pre-operative curettage specimens were high (88–100%) for molecular alterations using immunohistochemical and DNA analysis. For immunohistochemical analysis, loss of PTEN expression showed the lowest concordance (89.6%). For nuclear β-catenin staining, discordant cases were those where nuclear staining was observed in the curettage but not in the hysterectomy. In all except one curettage, p53 immunostaining corresponded to the staining observed in the hysterectomy. p53 'mutant-like' immunostained concordant cases showed concordance of *TP53* mutation analyses using Sanger sequencing in both the curettage and hysterectomy specimens. In the discordant case, in which immunostaining of the curettage was scored wildtype and the hysterectomy showed a clonal mutant staining, no mutation was identified in *TP53* using Sanger sequencing in either curettage or hysterectomy specimen. Sequencing of tumors that showed entirely negative staining did not reveal any mutations. Figure 1 shows an example of a discordant PTEN, p53 and β-catenin immunostaining between hysterectomy and pre-operative curettage specimens.

Concordance for microsatellite status between curettage and hysterectomy specimens was 93.5%. MSI assay was successfully performed on 40 paired cases, and 9 MSI cases were all endometrioid endometrial tumors. In the three discordant cases, the curettage specimen was microsatellite stable while the tumor in the hysterectomy was microsatellite instability. MSI endometrial cancers are most often sporadic and caused by promoter hypermethylation of *MLH1*. Using immunohistochemistry, loss of MLH1 expression was observed in curettage and hysterectomy specimens of all three discordant cases from which one curettage specimen showed a heterogeneous staining pattern.

Finally, overall concordance for GynCarta multigene analysis between curettage and corresponding hysterectomy specimens was 99.8% (16 discordant cases / (159 mutation * 46 paired curettage and hysterectomy specimens minus failed reactions)). Thirteen of the discordant cases showed a mutation in the pre-operative curettage specimen while the mutation was not found in the paired hysterectomy specimen (Figure 2). *KRAS* and *PIK3CA* mutations were validated for all samples using allele specific qPCR. Upon validation, concordance of 99.7% was observed between GynCarta multigene analysis and allele specific qPCR. A *KRAS* G13D mutation was not detected in one paired hysterectomy and curettage specimen using GynCarta multigene analysis. Furthermore, one curettage showed a *PIK3CA* E545K mutation which was only detected with GynCarta multigene analysis (Supplementary Table 4).

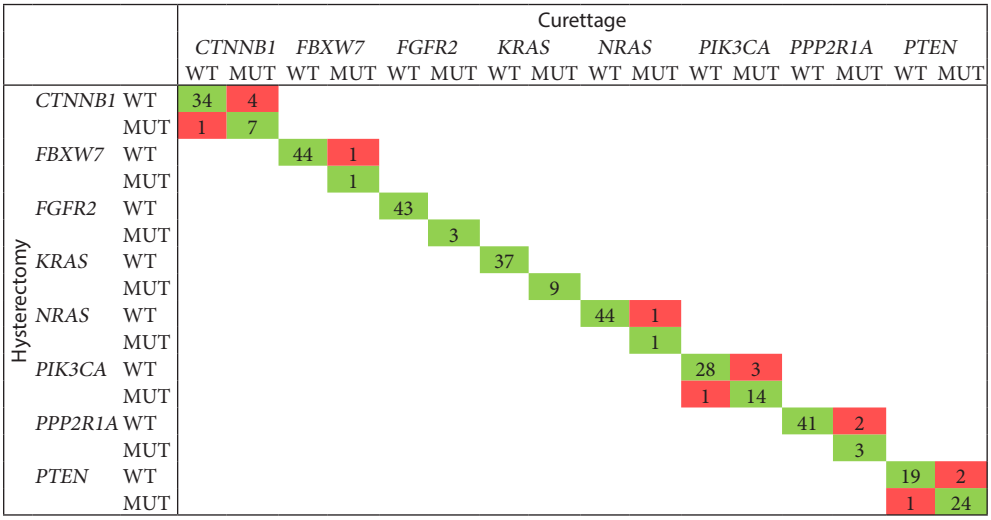

Figure 2. Concordance of molecular alterations in endometrial hysterectomy and pre-operative specimens using GynCarta multigene analysis. Concordant cases between the paired hysterectomy and curettage specimen wildtype (WT) and mutant (MUT) cases are indicated in green and discordant cases in red. No mutations in *BRAF*, *CDKNA2*, *FGFR3*, *FOXL2* and *HRAS* were found in paired curettage and hysterectomy-specimens. The concordance was calculated as discordant cases 16/ ((total samples 46 * 159 assays)−137 failed reactions)=0.998.

Discussion

This study shows that molecular alterations detected in pre-operative curettage samples reliably predict the alterations found in the subsequent hysterectomy specimens. We showed concordance rates ranging from 88% for immunohistochemical techniques to 99% for DNA techniques in paired samples of 48 patients with endometrial cancer. Various studies have shown an inconsistency of tumor typing and histological grading between pre-operative and hysterectomy specimens.[25-27] In accordance with these previous reports, we found that especially pre-operative tumor grade 1 does not accurately predict final histological results.

Pre-operative assignment of a lower grade is not an unusual finding due to grading of more superficially located parts of the tumor, but may lead to underestimating the risk of disease recurrence. Early recognition of high-risk status would be desirable for accurate patient counseling and determining further treatment, tailoring the extent of surgery and/or adjuvant therapies to the individual patient's risk profile. The present study showed very high concordance rates for DNA-based techniques: 99.8% for GynCarta multigene analysis, 93.5% for microsatellite analysis and 100% for *TP53* sequencing. Immunostaining showed more discrepancies, however it still resulted in an acceptable concordance for PTEN (89.6%), β-catenin (95.8%) and p53 (97.9%). These data indicate that molecular alterations found in pre-operative tissue samples correspond better to the subsequent hysterectomy specimens than the classic histopathologic features. Finally, specific molecular alterations may help to predict the tumor's propensity for early invasion and disease spread.

So far, most studies of potential prognostic biomarkers on endometrial curettage materials have been limited to immunostaining approaches, and the results have rarely been correlated with the hysterectomy specimens.[15,28-30] Concordance-studies have been inconsistent regarding the reliability of p53 scoring, with a concordance ranging from 86 to 96%.[28,29] We found a concordance of 97.9% and the different observations in other studies are likely explained by differences in scoring method for evaluating p53 expression. Concordance of other potential prognostic biomarkers such as stathmin and p16 has also been tested previously, resulting in a discordance of approximately 30%.[15,30] Taken together, the results of these and our studies indicate that immunohistochemical evaluation of candidate prognostic factors in pre-operative specimens only moderately predicts the expression pattern in a hysterectomy specimen. Clinical implementation of immunohistochemistry based prognostic factors should therefore be approached with caution.

The majority of discrepancies in immunostaining in our study could be explained by heterogeneous topographical staining within one tumor. Nuclear staining of β-catenin is frequently found at the surface of endometrial cancers, which could explain why in some cases nuclear staining of β-catenin was found in the curettage but not in the hysterectomy specimen. In particular, squamous differentiated cells and morules exhibit nuclear staining of β-catenin.[31] PTEN is known to display substantial topographic heterogeneity as well in tumors.[22] Scant and fragmented tumor material obtained by curettage may give a distorted staining pattern not reflective of the whole tumor seen in hysterectomy specimen. These findings exemplify limitations for the use of immunohistochemistry to predict the expression in the tumor on pre-operative material. However, immunohistochemistry is probably the best method to assess functional PTEN loss in endometrial cancer, since loss of PTEN has been attributed to a variety of causes including gene mutations, gene methylation, *PTEN* post-transcriptional regulation and actions of microRNAs.[32] Pallares *et al.* have shown that only the PTEN 6H2.1 antibody exhibited a good correlation with the presence of molecular

alterations in PTEN in endometrial tumors.[33] Nonetheless, we found twelve cases with *PTEN* mutations, substitutions and frameshifts that showed normal PTEN expression. Possibly the absence of loss of heterozygosity explains the discrepancy in these cases. Additionally, 6 of 8 cases with *CTNNB1* mutations did not show a nuclear β-catenin staining. This difference can be explained by alternative Wnt-signaling activating events independent of mutations in β-catenin.[31] Together these findings suggest that PTEN and β-catenin status is best assessed by combining immunohistochemistry with mutation status in endometrial specimen.

The limitation of DNA-based assays is presented by a failure rate which in this study was the highest for the microsatellite assay (13%). The failure to identify microsatellite instability in curettage specimens is explained by running out of material and by decreased sensitivity of the assay with relatively low concentrations of tumor DNA in these samples. When loss of MLH1 immunostaining is used as a surrogate marker for MSI, the combination of MLH1 staining with the MSI data results in 100% concordance. Therefore, reliable MSI analysis on curettage samples can be achieved when combined with MLH1 staining. The DNA-based analysis for mutation genotyping used in this study showed a high concordance and an acceptable failure rate (<10%). A limitation of our study is that we could not specify the type of pre-operative sampling for all included patients. However, molecular alterations were successfully identified in endometrial pre-operative specimens obtained by hysteroscopy-guided tissue biopsy, out-patient micro-curettage and the classical dilation & curettage sampling. The pre-operative samples with poor DNA quality (21.7%) were not related to the volume of blood or to one specific type of pre-operative sampling. Recently, Perez-Sanchez *et al.* showed a limited success (81%) of molecular diagnosis using quantitative reverse transcriptase-polymerase chain reaction on RNA from uterine aspirates samples for diagnosis of endometrial cancer.[34] However, a combination of the molecular and histological diagnosis could diagnose 17% more uterine aspirates in comparison to histological diagnosis alone. Furthermore, Kinde *et al.* recently compared somatic mutations found in pre-operative liquid-based pap smears to subsequent endometrial and ovarian tumors.[35] Similar, to the present study, they were able to identify the same mutations in the DNA from pre-operative specimens as in the subsequent endometrial tumors, stressing the high potential of DNA-based analysis. The findings of our study and published literature regarding the concordance of molecular alterations between hysterectomy specimens and pre-operative specimens are shown in Supplementary Table 5.

Future studies are required to investigate whether the addition of a molecular profile results in a better risk assessment as compared to clinicopathological parameters alone. The implementation of molecular diagnostics on endometrial pre-operative specimens has major challenges such as turnaround time, costs per patient, logistics and analytical test validity.[36] For clinical decision-making, data from molecular diagnostics should ideally be available within a few days of sampling. Similarly to next generation sequencing, a turnaround time under 14 days is

expected for sample preparation, protein and DNA analysis and data analysis.[36] However, it will be a challenge to collect tumor tissue at individual hospitals for molecular testing within set time limits. Regulations, standard protocols, trained personnel, laboratory accreditation and validation including external quality assessments should improve the adoption of molecular diagnostics. Future studies investigating the feasibility of applying this molecular approach in the workup of patients with endometrial cancer will be required to address these issues.

In conclusion, this molecular profiling concordance-study using 48 endometrial cancers with their corresponding curettage specimens provides evidence that pre-operative curettage samples can reliably predict the molecular alterations of the endometrial cancers as found in the definitive hysterectomy specimens. These findings may impact future studies that determine the prognostic value of hysterectomy-based molecular profiling, as the results can safely be translated towards the pre-operative tissue samples. We have shown that the concordance of DNA-based techniques is superior to the concordance of classic histology and immunohistochemical approaches. Whether these molecular alterations can have superior prognostic and predictive power than the classical clinicopathological risk features still remains to be determined and will require analysis of large study cohorts, preferably from randomized controlled trials.

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References

1. Wright JD, Barrena Medel NI, Sehouli J, Fujiwara K, Herzog TJ. Contemporary management of endometrial cancer. Lancet. 2012;379(9823):1352-1360.
2. Nout RA, Smit VT, Putter H, et al. Vaginal brachytherapy versus pelvic external beam radiotherapy for patients with endometrial cancer of high-intermediate risk (PORTEC-2): an open-label, non-inferiority, randomised trial. Lancet. 2010;375(9717):816-823.
3. Keys HM, Roberts JA, Brunetto VL, et al. A phase III trial of surgery with or without adjunctive external pelvic radiation therapy in intermediate risk endometrial adenocarcinoma: a Gynecologic Oncology Group study. Gynecol.Oncol. 2004;92(3):744-751.
4. Creutzberg CL, van Putten WL, Koper PC, et al. Surgery and postoperative radiotherapy versus surgery alone for patients with stage-1 endometrial carcinoma: multicentre randomised trial. PORTEC Study Group. Post Operative Radiation Therapy in Endometrial Carcinoma. Lancet. 2000;355(9213):1404-1411.
5. Benedetti Panici P, Basile S, Maneschi F, et al. Systematic pelvic lymphadenectomy vs. no lymphadenectomy in early-stage endometrial carcinoma: randomized clinical trial. Journal of the National Cancer Institute. 2008;100(23):1707-1716.

6. group As, Kitchener H, Swart AM, Qian Q, Amos C, Parmar MK. Efficacy of systematic pelvic lymphadenectomy in endometrial cancer (MRC ASTEC trial): a randomised study. Lancet. 2009;373(9658):125-136.
7. McCluggage WG, Hirschowitz L, Wilson GE, Oliva E, Soslow RA, Zaino RJ. Significant variation in the assessment of cervical involvement in endometrial carcinoma: an interobserver variation study. The American journal of surgical pathology. 2011;35(2):289-294.
8. Karateke A, Tug N, Cam C, Selcuk S, Asoglu MR, Cakir S. Discrepancy of pre- and postoperative grades of patients with endometrial carcinoma. European journal of gynaecological oncology. 2011;32(3):283-285.
9. Wang XY, Pan ZM, Chen XD, Lu WG, Xie X. Accuracy of tumor grade by preoperative curettage and associated clinicopathologic factors in clinical stage I endometriod adenocarcinoma. Chinese medical journal. 2009;122(16):1843-1846.
10. Mavromatis ID, Antonopoulos CN, Matsoukis IL, et al. Validity of intraoperative gross examination of myometrial invasion in patients with endometrial cancer: a meta-analysis. Acta obstetricia et gynecologica Scandinavica. 2012;91(7):779-793.
11. Zerbe MJ, Bristow R, Grumbine FC, Montz FJ. Inability of preoperative computed tomography scans to accurately predict the extent of myometrial invasion and extracorporal spread in endometrial cancer. Gynecologic oncology. 2000;78(1):67-70.
12. Saarelainen SK, Koobi L, Jarvenpaa R, Laurila M, Maenpaa JU. The preoperative assessment of deep myometrial invasion by three-dimensional ultrasound versus MRI in endometrial carcinoma. Acta obstetricia et gynecologica Scandinavica. 2012;91(8):983-990.
13. Akiyama-Abe A, Minaguchi T, Nakamura Y, et al. Loss of PTEN expression is an independent predictor of favorable survival in endometrial carcinomas. British journal of cancer. 2013;109(6):1703-1710.
14. Nout RA, Bosse T, Creutzberg CL, et al. Improved risk assessment of endometrial cancer by combined analysis of MSI, PI3K-AKT, Wnt/beta-catenin and P53 pathway activation. Gynecologic oncology. 2012;126(3):466-473.
15. Engelsen IB, Stefansson I, Akslen LA, Salvesen HB. Pathologic expression of p53 or p16 in preoperative curettage specimens identifies high-risk endometrial carcinomas. American journal of obstetrics and gynecology. 2006;195(4):979-986.
16. Krol LC, t Hart NA, Methorst N, Knol AJ, Prinsen C, Boers JE. Concordance in KRAS and BRAF mutations in endoscopic biopsy samples and resection specimens of colorectal adenocarcinoma. European journal of cancer. 2012;48(7):1108-1115.
17. Al Sarakbi W, Salhab M, Thomas V, Mokbel K. Is preoperative core biopsy accurate in determining the hormone receptor status in women with invasive breast cancer? International seminars in surgical oncology : ISSO. 2005;2:15.
18. Irshad S, Bansal M, Castillo-Martin M, et al. A molecular signature predictive of indolent prostate cancer. Science translational medicine. 2013;5(202):202ra122.
19. van Eijk R, Licht J, Schrumpf M, et al. Rapid KRAS, EGFR, BRAF and PIK3CA mutation analysis of fine needle aspirates from non-small-cell lung cancer using allele-specific qPCR. PloS one. 2011;6(3):e17791.
20. McCluggage WG, Soslow RA, Gilks CB. Patterns of p53 immunoreactivity in endometrial carcinomas: 'all or nothing' staining is of importance. Histopathology. 2011;59(4):786-788.
21. Seeber LM, Zweemer RP, Marchionni L, et al. Methylation profiles of endometrioid and serous endometrial cancers. Endocrine-related cancer. 2010;17(3):663-673.
22. Garg K, Broaddus RR, Soslow RA, Urbauer DL, Levine DA, Djordjevic B. Pathologic scoring of PTEN immunohistochemistry in endometrial carcinoma is highly reproducible. International journal of gynecological pathology: official journal of the International Society of Gynecological Pathologists. 2012;31(1):48-56.
23. van Eijk R, Stevens L, Morreau H, van Wezel T. Assessment of a fully automated high-throughput DNA extraction method from formalin-fixed, paraffin-embedded tissue for KRAS, and BRAF somatic mutation analysis. Experimental and molecular pathology. 2013;94(1):121-125.
24. da Costa CE, Szuhai K, van Eijk R, et al. No genomic aberrations in Langerhans cell histiocytosis as assessed by diverse molecular technologies. Genes, chromosomes & cancer. 2009;48(3):239-249.
25. Thanachaiviwat A, Thirapakawong C, Leelaphatanadit C, Chuangsuwanich T. Accuracy of preoperative curettage in determining tumor type and grade in endometrial cancer. Journal of the Medical Association of Thailand. 2011;94(7):766-771.
26. Mitchard J, Hirschowitz L. Concordance of FIGO grade of endometrial adenocarcinomas in biopsy and hysterectomy specimens. Histopathology. 2003;42(4):372-378.
27. Francis JA, Weir MM, Ettler HC, Qiu F, Kwon JS. Should preoperative pathology be used to select patients for surgical staging in endometrial cancer? International journal of gynecological cancer: official journal of the International Gynecological Cancer Society. 2009;19(3):380-384.
28. Vandenput I, Trovik J, Leunen K, et al. Evolution in endometrial cancer: evidence from an immunohistochemical study. International journal of gynecological cancer: official journal of the International Gynecological Cancer Society. 2011;21(2):316-322.
29. Oreskovic S, Babic D, Kalafatic D, Barisic D, Beketic-Oreskovic L. A significance of immunohistochemical

determination of steroid receptors, cell proliferation factor Ki-67 and protein p53 in endometrial carcinoma. Gynecologic oncology. 2004;93(1):34-40.
30. Trovik J, Wik E, Stefansson IM, et al. Stathmin overexpression identifies high-risk patients and lymph node metastasis in endometrial cancer. Clinical cancer research: an official journal of the American Association for Cancer Research. 2011;17(10):3368-3377.
31. Moreno-Bueno G, Hardisson D, Sanchez C, et al. Abnormalities of the APC/beta-catenin pathway in endometrial cancer. Oncogene. 2002;21(52):7981-7990.
32. Fata JE, Debnath S, Jenkins EC, Jr., Fournier MV. Nongenomic Mechanisms of PTEN Regulation. International journal of cell biology. 2012;2012:379685.
33. Pallares J, Bussaglia E, Martinez-Guitarte JL, et al. Immunohistochemical analysis of PTEN in endometrial carcinoma: a tissue microarray study with a comparison of four commercial antibodies in correlation with molecular abnormalities. Modern pathology: an official journal of the United States and Canadian Academy of Pathology, Inc. 2005;18(5):719-727.
34. Perez-Sanchez C, Colas E, Cabrera S, et al. Molecular diagnosis of endometrial cancer from uterine aspirates. International journal of cancer. 2013;133(10):2383-2391.
35. Kinde I, Bettegowda C, Wang Y, et al. Evaluation of DNA from the Papanicolaou test to detect ovarian and endometrial cancers. Science translational medicine. 2013;5(167):167ra164.
36. Rhea JM, Singh HV, Molinaro RJ. Next generation sequencing in the clinical molecular diagnosis of cancer. Med Lab Obs 2011.

Supplementary files

Supplementary Table 1. DNA quality of endometrial hysterectomy and pre-operative specimens and success rate of DNA analysis.

			Hysterectomy n=46 (%)	Curettage n=46 (%)
DNA concentration (ng/µl)				
		mean	12.5	7.0
		range	0.3-22.5	0.9-20.3
DNA quality				
	poor	0 bp	3 (6.5)	10 (21.7)
	moderate	150 bp	6 (13.0)	6 (13.0)
		255 bp	4 (6.5)	2 (6.5)
	good	343 bp	29 (63.0)	14 (30.4)
		511 bp	4 (6.5)	14 (30.4)

Total n=46	Hysterectomy	Curettage
Microsatellite instability assay	89.1	87.0
GynCarta	98.7	91.8
allele specific qPCR	97.9	91.3

Supplementary Table 2. Design of the GynCarta multigene analysis. The panel (GynCarta, Sequenom®, Hamburg) consists of 13 multiplexes containing 100 assays to detect 159 mutations in 13 genes that are most frequently described to be involved in gynecological malignancies according to a COSMIC meta-analysis.

Genes (13)	*BRAF*	*CDKN2A*	*CTNNB1*	*FBXW7*	*FGFR2*	*FGFR3*	*FOXL2*	*HRAS*	*KRAS*	*NRAS*	*PIK3CA*	*PTEN*	*PPP2R1A*
Mutations	p.V600E	p.R58*	p.D32A	p.R465C	p.S252W	p.R248C	p.C134W	p.G12A	p.G12A	p.G12A	p.R88Q	p.K6fs*4	p.P179L
	p.V600K	p.R58X	p.D32G	p.R465H	p.Y375C	p.S249C		p.G12C	p.G12C	p.G12C	p.E542K	p.E7*	p.P179R
	p.V600R	p.R80*	p.D32H	p.R479Q	p.C382R	p.G370C		p.G12D	p.G12D	p.G12D	p.E545A	p.F37S	p.R183G
	p.V600L	p.D108Y	p.D32N	p.R479L	p.N549K	p.S371C		p.G12R	p.G12F	p.G12R	p.E545D	p.R84G	p.R183Q
		p.D108A	p.D32V	p.R505C	(T>A)	p.Y373C		p.G12V	p.G12R	p.G12S	p.E545G	p.R130*	p.R183W
		p.D108C	p.D32Y		p.N549K	p.A391E		p.G13C	p.G12S	p.G12V	p.E545K	p.R130fs*4	p.R256F
		p.W110*	p.S33A		(T>G)	p.K650E		p.G13D	p.G12V	p.G13A	p.Q546E	p.R130G	p.R256Y
		p.W110X	p.S33C		p.K659E	p.K650Q		p.G13R	p.G13A	p.G13C	p.Q546K	p.R130L	p.W257C
		p.P114L	p.S33F			p.G697C		p.G13S	p.G13C	p.G13D	p.Q546L	p.R130P	p.R258H
		p.P114X	p.S33P					p.G13V	p.G13D	p.G13R	p.Q546P	p.R130Q	
			p.S33Y					p.G13X	p.G13R	p.G13S	p.Q546R	p.R173C	
			p.G34E					p.Q61H	p.G13V	p.G13V	p.Y1021C	p.R173H	
			p.G34R					(C>A)	p.Q61E	p.Q61E	p.T1025A	p.Q214*	
			p.G34V					p.Q61H	p.Q61H	p.Q61K	p.T1025X	p.R233*	
			p.S37A					(C>G)	(T>A)	p.Q61L	p.M1043I	p.R234W	
			p.S37C					p.Q61K	p.Q61H	p.Q61P	(G>A)	p.P248fs*5	
			p.S37F					p.Q61L	(T>G)	p.Q61R	p.M1043I	p.C250fs*2	
			p.S37P					p.Q61P	p.Q61K		(G>T)	p.K267fs*9	
			p.S37T					p.Q61R	p.Q61L		p.M1043V	p.K267fs*31	
			p.S37Y						p.Q61P		p.H1047L	p.V290fs*1	
			p.T41A						p.Q61R		p.H1047R	p.L318fs*2	
			p.T41I								p.H1047Y	p.T321fs*3	
			p.T41N									p.T321fs*23	
			p.T41S									p.N323fs*2	
			p.S45C									p.N323fs*21	
			p.S45F									p.R335*	
			p.S45P										
			p.S45Y										
Total (159)	4	10	28	5	5	9	1	18	18	17	20	26	9
Assays(100)	2	5	12	4	5	8	1	8	7	6	13	23	6

Supplementary Table 3. Mutations detected in hysterectomy and pre-operative curettage specimens.

	Hysterectomy n	Curettage n
CTNNB1		
D32H	3	3
G43E	0	1
S33C	1	1
S33Y	0	1
S33F	2	1
S37C	1	1
S37F	1	3
S45F	0	1
FBXW7		
R479Q	0	1
R505C	1	1
FGFR2		
S252W	2	2
KRAS		
G12C	2	2
G12D	3	3
G12A	1	1
G12V	2	2
G13S	1	1
NRAS		
G12S	1	1
G12D	0	1
PIK3CA		
R88Q	3	4
E542K	0	1
E545A	1	1
E545G	0	1
E545K	1	1
Q546K	2	2
Q546R	1	1
Y1021	3	2
M1042I	1	1
H1047R	3	3

	Hysterectomy n	Curettage n
PPP2R1A		
P179L	2	2
R183W	1	1
S256F	0	1
R258H	0	1
PTEN		
E7Star	1	1
R130fs*4	4	4
R130P	1	1
R130*	3	3
R130G	4	4
R173C	2	1
R173H	1	1
R233*	3	3
R234W	0	1
V290fs*1	1	2
L318fs*2	2	2
T321fs*23	1	1
N323fs*21	1	1

Supplementary Table 5. Comparison of the current study with published literature regarding the concordance of molecular alterations between hysterectomy specimens and pre-operative specimens.

Study	n	Pre-operative specimen	Method	Marker	Concordance
Protein analysis					
Oreskovic *et al.*[29]	136	Fractional curettage	IHC	p53	96%
Oreskovic *et al.*[29]	136	Fractional curettage	IHC	ER	99%
Oreskovic *et al.*[29]	136	Fractional curettage	IHC	PR	95%
Oreskovic *et al.*[29]	136	Fractional curettage	IHC	Ki67	95%
Engelsen *et al.*[15]	140	Curettage	IHC	p53	86%
Engelsen *et al.*[15]	200	Curettage	IHC	p16	73%
Trovik *et al.*[30]	477	Curettage	IHC	Stathmin	67%
Stelloo *et al.* (current)	48	Curettage	IHC	p53	97.9%
Stelloo *et al.* (current)	48	Curettage	IHC	PTEN	89.6%
Stelloo *et al.* (current)	48	Curettage	IHC	β-catenin	95.8%
DNA analysis					
Pradhan *et al.*[37]	111	Curettage	DNA image cytometry	DNA ploidy	72.7%
Kinde *et al.*[35]	24	Pap smears	Sequencing	12 genes	100%
Stelloo *et al.* (current)	46	Curettage	MSI assay	5 markers	93.5%
Stelloo *et al.* (current)	46	Curettage	Multigene assay	13 genes	99.8%

Supplementary Table 4. Concordance between GynCarta multigene analysis and allele specific qPCR in the hysterectomy (HYST) and pre-operative curettage specimens (CUR). Concordance was determined to validate the results for 7 *KRAS* and 3 *PIK3CA* mutations. Failed reactions were excluded as comparison was not possible (6 for *PIK3CA* and 5 for *KRAS*; 53/920 in total). This led to a concordance of (3/(920-53))=0.997.

KRAS		GynCarta multigene analysis															
		G12S		G12R		G12C		G12D		G12A		G12V		G13D		Wildtype	
		HYST	CUR	HYST	CUR	HYST	CUR	HYST	CUR	HYST	CUR	HYST	CUR	HYST	CUR	HYST	CUR
Allele specific qPCR	G12S	HYST	0														
		CUR		0													
	G12R	HYST			0												
		CUR				0											
	G12C	HYST					2										
		CUR						2									
	G12D	HYST							2								
		CUR								2							
	G12A	HYST									1						
		CUR										1					
	G12V	HYST											2				
		CUR												2			
	G13D	HYST													1		1
		CUR														1	1
	Wildtype	HYST														36	
		CUR															33
	Failed	HYST														1	
		CUR															4

PIK3CA		GynCarta multigene analysis							
		H1047R		E542K		E545K		Wildtype	
		HYST	CUR	HYST	CUR	HYST	CUR	HYST	CUR
Allele specific qPCR	H1047R	HYST	3						
		CUR		3					
	E542K	HYST			0				
		CUR				0			
	E545K	HYST					1		
		CUR						1	
	Wildtype	HYST						40	
		CUR			1				38
	Failed	HYST						2	
		CUR							4