Molecular alterations in endometrial cancer: implications for clinical management

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Promotores

Prof. dr. V.T.H.B.M. Smit
Prof. dr. C.L. Creutzberg

Co-promotor

dr. T. Bosse

Leden promotiecommissie

Prof. dr. C.A.M. Marijnen
Prof. dr. H. Hollema (University Medical Center Groningen)
Prof. dr. H.W. Nijman (University Medical Center Groningen)
Prof. dr. M.J. van de Vijver (Academic Medical Center Amsterdam)

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General introduction and thesis outline

General introduction

Epidemiology

Endometrial cancer is a cancer arising from the endometrium, the inner lining of the uterus. It is the most common gynecological cancer in developed countries, and the 5th most common cancer in women.[1] In the Netherlands, each year over 1 900 women are diagnosed with endometrial cancer and 490 women die from this cancer.[2] The majority of endometrial cancers develop after menopause, with the highest incidence between 65 and 75 years.[2,3] The incidence of endometrial cancer has grown over the last decade which can largely be ascribed to ageing of the population, increased life expectancy, increasing rates of obesity, and decreasing rate of hysterectomy for benign causes.[2,4-6] Due to early clinical symptoms of postmenopausal vaginal bleeding, most endometrial cancers (~70%) are detected in an early stage when the tumor is confined to the uterus.[7] A minority of endometrial cancers (2-5%) develop in women with Lynch syndrome, mainly before menopause.[8-10] Lynch syndrome is an hereditary disease with germline mutations in DNA mismatch repair genes and a 40-60% lifetime risk of colorectal and endometrial cancer.[11-13]

Pathology

Histological classification

Endometrial cancers can be histologically classified according to the World Health Organization (Table 1).[7] Endometrioid adenocarcinoma is the most common subtype, accounting for 75-80% of the cases, that usually develop in a background of hyperplasia of the endometrium (Figure 1A).[7,14] Most endometrioid endometrial cancers are well differentiated with preserved glandular architecture and lack of intervening stroma. The less common non-endometrioid subtypes, include serous and clear cell carcinomas, are often found in a background of atrophic endometrium, and can constitute ~20% of endometrial cancer diagnoses (Figure 1B-C).[7,14,15] Serous carcinoma can be distinctive by their architecture (hobnail appearance) and nuclear features (clumped chromatin, prominent nucleoli and mitotic activity).[16,17] Clear cell carcinomas can also be characterized by hobnail cells and a high mitotic activity, but also clear cells and hyalinized stroma.[16,17] Endometrial cancers are classified as mixed carcinoma if two histological subtypes with at least one non-endometrioid subtype is present in more than 10% of the lesion.[7,15] Mixed serous and endometrioid carcinomas and mixed clear cell and endometrioid carcinomas comprising more than 25% of the serous or clear cell component, respectively, are generally classified as serous or clear cell carcinomas. Carcinosarcomas, a mixture of epithelial and mesenchymal cells, are regarded as carcinomas with a mesenchymal component.[7] The significant difference in patient outcome between the histological subtypes stresses the importance of accurate histological assessment. Several studies have reported moderate to good reproducibility of subtype diagnosis by pathologists.[18-20]

FIGO grade

Endometrioid and mucinous endometrial cancers, but also other rare subtypes, are graded using a 3-tiered International Federation of Obstetricians and Gynecologists (FIGO) system based on architecture and cytologic atypia.[7] The architectural grading is as follows: grade 1 has ≤5% solid growth pattern, grade 2 has between 6-50% solid growth pattern and grade 3 has >50% solid growth pattern. Marked nuclear atypia could increase the architectural grade 1 to grade 2 or architectural grade 2 to grade 3. The non-endometrioid subtype is classified as grade 3, irrespective of growth pattern and cytologic atypia. The reproducibility of this grading system between pathologists was shown to be fair to moderate.[21-24] Two-tiered systems have been proposed to decrease interobserver variability, and has superior prognostic power.[21-24] Although, these binary systems are currently not used in clinical practice, grades 1-2 and grade 3 are often informally dichotomized into low grade and high grade, respectively.

Table 1. Histological subtypes of (epithelial) endometrial cancer.

Histological types (epithelial)	Frequency
Endometrioid adenocarcinoma	75-80%
Non-endometrioid adenocarcinoma	20-25%
Serous adenocarcinoma	5-10%
Mixed cell adenocarcinoma	3-5%
Clear cell adenocarcinoma	1-5%
Mucinous adenocarcinoma	1-2%
Undifferentiated carcinoma	1-2%
Squamous cell carcinoma	<1%
Transitional cell carcinoma	<1%
Small cell carcinoma	<1%
Others	<1%

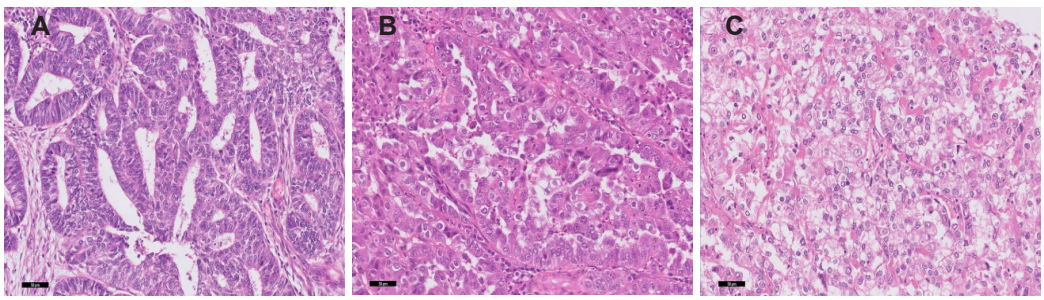

Figure 1. Histological classification of endometrial cancers. Common histological subtypes of epithelial endometrial cancer include endometrioid (A), serous (B) and clear cell (C). Scale bar represents 50 μM.

FIGO stage

The extent of tumor growth is divided into four stages using a surgical-pathological staging system from 1988.[25] A new version of the FIGO staging system was introduced in 2009 as more information became available with regard to risk factors associated with natural behavior of endometrial cancer and survival (Table 2).[26] Included risk factors are depth of myometrial invasion, extension into the cervical canal, pelvic node metastases, aortic node metastases, adnexal metastases, penetration of uterine serosa and positive peritoneal cytological findings.

Accurate pathological assessment of depth of myometrial invasion and cervical stromal involvement is crucial for FIGO staging. Stage IA tumors are those confined to the uterine corpus with less than 50% myometrial invasion, whereas stage IB tumors are those with greater than 50% myometrial invasion. Determination of myometrial invasion may be challenging due to different patterns of invasion.[27,28] Patterns of invasion that have been described include e.g. broad front invasion and invasion of irregular groups of glands with or without a stromal response. To diagnose stage II tumors, pathologists need to assess cervical stromal involvement in the absence of extra-uterine disease, which is easily recognized in most cases.[7,29] Difficulties can be to distinguish between cervical glandular or stromal involvement, and delimiting the uterine corpus from the cervix.

Table 2. FIGO 2009 staging system for endometrial cancer

Stage I	Tumor confined to the corpus uteri
	IA No or less than half myometrial invasion
	IB Invasion equal to or more than half of the myometrium
Stage II	Tumor invades the cervical stroma, but does not extend beyond the uterus
Stage III	Local and/or regional spread of tumor
	IIIA Tumor invades the serosa of the corpus uteri and/or adnexas
	IIIB Vaginal and/or parametrial involvement
	IIIC1 Positive pelvic lymph nodes
	IIIC2 Positive para-aortic lymph nodes with or without positive pelvic lymph nodes
Stage IV	Tumor invades bladder and/or bowel mucosa and/or distant metastases
	IVA Tumor invades bladder and/or bowel mucosa
	IVB Distant metastases, incl. intra-abdominal metastases and/or inguinal lymph nodes

Lymphovascular space invasion

Although, lymphovascular space invasion is not part of the FIGO staging system, it is an important prognostic factor in endometrial carcinoma.[30-34] A definition and optimal determination of this factor is still under investigation, especially regarding the clinical relevance of quantification. Lymphovascular space invasion can be defined as tumor cells present in a space lined by endothelial cells outside the immediate invasive border. Tumor spill, retraction artifacts or mimics (e.g. certain myometrial invasion growth patterns) may hamper correct assessment of lymphovascular space invasion.[35] Immunohistochemistry of markers for lymphatic channels (D2-40) and endothelial cells (CD31, CD34) may aid to recognize true lymphovascular space invasion.[36] In addition, quantification of lymphovascular space invasion may improve accurate evaluation. Substantial (diffuse or multifocal) lymphovascular space invasion strongly correlates with prognosis.[37-39]

Therapy

Surgery

Preoperative histopathological assessment is required to diagnose endometrial cancer and to guide treatment decisions.[14] Hysterectomy, usually in combination with bilateral salpingo-

oophorectomy, is the cornerstone of treatment. The procedure has been traditionally performed by laparotomy, but nowadays the laparoscopic procedure is preferred because of its reduced operative morbidity and hospital stay.[40] The role of a staging lymphadenectomy is still controversial. Two randomized trials did not show any benefit for survival or relapse-free survival, and the advantage of staging in grade 3 cancers remains to be elucidated.[41,42] In the Netherlands, complete surgical staging is considered for high-grade endometrioid cancers and recommended for serous and clear cell cancers.[43] An international randomized trial will address the role of lymphadenectomy in early stage, grade 3 cancers in determining the indication for adjuvant treatment.

Adjuvant therapy

The indication of adjuvant treatment is based on the patient's risk of disease recurrence using clinicopathological risk factors such as age, stage, and histological subtype.[44] The combination of clinicopathological factors is used to stratify a patient's risk of disease recurrence into three risk groups: low- (45-50% of all endometrial cancer patients), intermediate- (30-35%), and high-risk (15-20%).[45] In the Netherlands, PostOperative Radiation Therapy in Endometrial Cancer (PORTEC) criteria are used to define risk groups (Table 3), however, similar other definitions have been published, most recently those of an international consensus conference.[14,46,47] There is no indication for adjuvant radiation therapy for patients with low-risk features, as risk for recurrent disease is low. The PORTEC-1 trial, but also the Gynecology Oncology Group (GOG)-99 trial and A Study in the Treatment of Endometrial Cancer (ASTEC) trial, compared external beam radiotherapy with no additional treatment for patients with stage I endometrial cancer (Table 4).[46-48] Both the PORTEC-1 and GOG-99 trials defined a high-intermediate risk group that demonstrated a significant reduction in locoregional recurrence (4% vs. 14% and 1.6% vs. 7.4%) after external beam radiotherapy. In the PORTEC-1 trial, high-intermediate risk patients were defined as having two out of three of the following risk factors: age above sixty years, deep myometrial invasion and/or grade 3. In the subsequent PORTEC-2 trial, it was demonstrated that vaginal brachytherapy was equally effective in reduction of vaginal recurrence as external beam radiotherapy, with fewer gastro-intestinal toxic effects in women with high-intermediate risk disease (Table 4).[49]

Table 3. Definition of clinicopathological risk groups to guide adjuvant therapy.

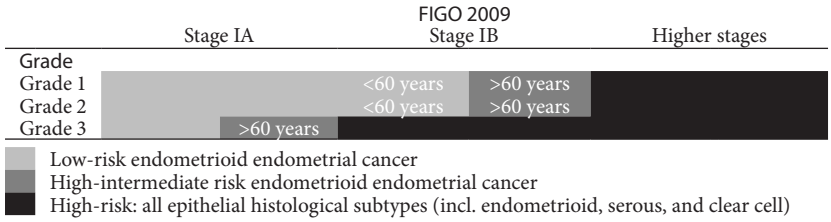

Table 4. Overview of randomized trials of postoperative adjuvant radiotherapy in endometrial cancers.

Study	No. of patients	Eligibility[1]	Study arms	LRR rate	PFS	OS	No. of EC-deaths
PORTEC-1	714	Stage I: G1, >50%MI; G2, any invasion; G3, <50%MI	EBRT vs. NAT	4% vs. 14%*	-	81% vs. 85%	23 vs. 18
GOG-99	392	Stage IB-C-II: G2/3, LVSI, >67%MI: ≥50yrs with 2 features; ≥70yrs any of the features	EBRT vs. NAT	2% vs. 7%*	-	92% vs. 86%	19 vs. 15
ASTEC	905	Stage I or IIA with either G3 (incl. serous) of >50%MI	EBRT vs. NAT	3% vs.6%*	-	84% vs. 84%	37 vs. 41
PORTEC-2	427	Stage I and G1/2, >60yrs, <50%MI or G3 with <50%MI; IIA, G1/2 or G3, <50%MI	EBRT vs. VBT	0.5% vs. 1.5%	83% vs. 78%	80% vs. 85%	10 vs. 15
JGOG	385	Stage IC–IIIC, <75yrs with >50%MI	EBRT vs. chemotherapy	7% vs. 7%	84% vs. 82%	85% vs. 87%	21 vs. 13
GICOG	345	Stage IC–II, G3; Stage III	EBRT vs. chemotherapy	12% vs. 16%	63% vs. 63%	69% vs. 66%	-
GOG-122	396	Stage III; Stage IV(<2cm residual disease)	Abdominal EBRT vs. chemotherapy	13% vs. 18%	38% vs. 42%*	42% vs. 53%*	100 vs. 78
NSGO/EORTC and Iliade-III	534	Stage I, ⩽80yrs or Stage II-III, serous/clear cell; Iliade: IIB-III	EBRT vs. EBRT + seq. chemotherapy	16% vs. 12%	69% vs. 78%*	75% vs. 82%	49 vs. 30*
GOG-249	601	Stage I-II with high-(intermediate) risk; serous/clear cell	EBRT vs. VBT + seq. chemotherapy	5 vs. 3 vagina, 2 vs. 19 pelvic	82% vs. 84%	93% vs. 92%	-
PORTEC-3	686	Stage I-III with high-risk features; serous/clear cell	EBRT vs. EBRT + seq. chemotherapy	Closed December 2013			
GOG-258	804	Stage III-IV endometrioid/serous/clear cell	EBRT + seq. chemotherapy vs. chemotherapy	Closed May 2014			

G=grade, MI=myometrial invasion, EBRT=external beam radiotherapy, VBT=vaginal brachytherapy, seq=sequential, NAT=no additional treatment. [1] FIGO stage (version 1988), * $P<0.05$

The optimal adjuvant therapy for endometrial cancer patients with high-risk features is still controversial due to lack of evidence of efficacy of adjuvant therapy (Table 4). External beam radiotherapy and vaginal brachytherapy provide optimal local control, however, distant metastases contribute to the inferior outcome of high-risk endometrial cancer patients. A meta-analysis of three randomized studies in which adjuvant radiotherapy was compared to chemotherapy demonstrated a small (~4%) survival benefit for chemotherapy.[50-53] There are indications that the combination of adjuvant external beam radiotherapy with chemotherapy improves the progression-free survival compared with either alone, but not for patients with non-endometrioid cancers (NSGO/EORTC/lliade-III).[54] The two-years outcomes of the GOG-249 randomized trial showed no evidence that progression-free survival with the combination of 3 cycles of adjuvant radiation therapy and chemotherapy with vaginal brachytherapy was better than pelvic radiation therapy alone.[55] The outcome of PORTEC-3 and GOG-258 trials, both evaluating the role of chemotherapy in combination with external beam radiotherapy will provide more evidence for the optimal therapy for high-risk endometrial cancer patients. Although the efficacy of adjuvant therapy for patients with high-risk features remains an area of controversy, external beam radiotherapy is currently recommended, and adjuvant platinum-based chemotherapy can be considered for stage III or IV, and non-endometrioid cancers.

Follow-up and recurrent disease

Risk of recurrence of endometrial cancer is related to the clinicopathological risk assessment. The recurrence rate is estimated to be 5-10%, 15-20% and >30% for patients with low-, intermediate- and high-risk features, respectively.[56-58] The use of adjuvant radiotherapy decreases vaginal and pelvic recurrences, but has no impact on distant metastasis or overall survival.[59] After treatment, all endometrial cancer patients undergo three to five years surveillance for early recurrence detection. The majority of recurrences are diagnosed within three years.[60-62] The salvage rate for early-stage endometrial cancer are high. Isolated vaginal recurrence occurs most commonly in patients who did not receive adjuvant radiotherapy. Radiotherapy is a curative treatment for vaginal recurrences. The frequent sites of recurrence in the intermediate- and high-risk endometrial cancer patients are pelvic and para-aortic nodal recurrences, peritoneal and lung metastases. These recurrences are treated with surgery, radiotherapy, hormonal therapy, chemotherapy, or combined modalities.[61,62]

Clinicopathological classification

Endometrial cancer was traditionally classified into two broad subtypes, type 1 and type 2, based on epidemiology, histopathology and clinical behavior by Bokhman in 1983.[63] Primarily, the histologic subtypes and molecular alterations were not part of the dualistic model.[64,65] Type 1 endometrial cancers are typically of endometrioid type, often low-grade and

develop from a background of endometrial hyperplasia. Risk factors for these cancers include unopposed estrogen exposure, obesity, nulliparity, late menopause and anovulation. Type 1 cancers generally show a indolent behavior and have in general a good prognosis (85% 5-years survival). Type 2 endometrial cancers, on the other hand, are often non-endometrioid, high-grade, arise in a background of atrophic endometrium, and occur in elderly women. These cancers are unrelated to estrogen exposure and are generally associated with an aggressive clinical course and poor prognosis (60% 5-years survival).

Subsequent molecular studies supported the dichotomous classification.[65-68] Type 1 carcinomas are associated with estrogen receptor (ER) and progesterone receptor (PR) expression, mutations in the PI3K-AKT (*PTEN*, *KRAS*, *PIK3CA*) and Wnt (*CTNNB1*) signaling pathways, and mutations in the chromatin remodeling gene *ARID1a*. In addition, type 1 carcinomas frequently show microsatellite instability either due to *MLH1* promoter hypermethylation (sporadic) or a germline mutation in DNA mismatch repair genes (*MLH1*, *PMS2*, *MSH2*, *MSH6*, Lynch-associated). In contrast, type 2 carcinomas exhibit loss of ER and PR protein expression, recurrent *TP53* mutations, and *HER2* gene amplification. However, this classification is too simplistic since not all endometrial carcinomas fit into these two pathways; e.g. some tumors show overlapping molecular features of both type 1 and -2 carcinomas.

Genomic classification

In 2013, The Cancer Genome Atlas has reported an integrated genomic, transcriptomic and proteomic characterization of endometrial cancers.[69] This analysis allowed reclassification of endometrial cancer into four molecular subgroups: *POLE* ultramutated, microsatellite instability hypermutated, copy-number low, and copy-number high (Table 5). *POLE*-mutant endometrial cancers, mainly endometrioid subtype, are characterized by hotspot mutations in exonuclease domain of *POLE* (subunit of DNA polymerase epsilon) and very high mutation rates, increased frequency of C>A transversions, few copy number alterations, mutations in *PTEN*, *PIK3R1*, *PIK3CA*, *FBXW7*, and *KRAS*, and favorable outcome. Microsatellite unstable endometrioid endometrial cancers are characterized by *MLH1* promoter hypermethylation, high mutation rates, few copy-number alterations and *PIK3CA* and *PTEN* mutations. The 'copy-number low' group comprises microsatellite stable grade 1 and 2 endometrioid endometrial cancers with low mutational rates, characterized by frequent *CTNNB1* mutations and chromosome 1q amplification. The copy-number high group consists primarily of serous and one-fourth of high-grade endometrioid endometrial cancers with low mutational rates, recurrent *TP53*, *FBXW7*, and *PPP2R1A* mutations and poor outcome. In view of these findings, Bokhman's dualistic model of endometrial cancer has been even further extended by the integration of molecular features both for prognostic and therapeutic purposes.

The Cancer Genome Atlas further revealed clusters of endometrial cancers based on messenger RNA expression, protein expression, and DNA methylation, which significantly correlated with the four molecular subgroups. The gene transcriptional activity was consistent with the copy-number alteration. In addition, the loss- and gain-of-function mutations correlated well with the protein expression data. The subgroup with microsatellite instability was associated with an heavily methylated subtype, whereas the copy-number high subgroup showed minimal DNA methylation changes.

The publicly availability of these data have led to subanalyses and further studies by independent researchers focusing on their specific topic of interest. Protein expression of L1 cell adhesion molecule (L1CAM) has been found as promising prognostic factor.[70,71] L1CAM-positive cancers demonstrated remarkably high hazard ratios for distant recurrences in a large series of stage I endometrial cancer patients.[71] Further studies have shown that L1CAM is an independent predictor of poor survival in endometrial cancer, and is associated with advanced stage, high-risk endometrial cancer using RNA expression data of The Cancer Genome Atlas.[72,73]

Table 5. Characteristics of molecular subgroups in endometrial cancer.

	POLE	MSI	CNA low	CNA high
TCGA population (%)	n=17 (7%)	n=65 (28%)	n=90 (39%)	n=60 (26%)
CNA	Very low	Low	Low (1q gain)	High
MSI status	MSI-high, MSS	MSI-high	MSS	MSS
Mutation rate (mut/Mb)	Very high (232×10^{-6})	High (18×10^{-6})	Low ($2{\cdot}9\times10^{-6}$)	Low ($2{\cdot}3\times10^{-6}$)
Frequently mutated genes (%)	*POLE* (100%) *PTEN* (94%) *FBXW7* (82%) *PIK3CA* (71%) *PIK3R1* (65%) *KRAS* (53%)	*PTEN* (88%) *PIK3CA* (54%)	*PTEN* (77%) *CTNNB1* (52%) *PIK3CA* (53%)	*TP53* (92%) *FBXW7* (47%) *PPP2R1A* (22%)
Clinical outcome	Good	Intermediate	Intermediate	Poor
Histological type	Endometrioid	Endometrioid	Endometrioid	Endometrioid, Serous
Grade	Grades 1–3	Grades 1–3	Grades 1-2	Grade 3

Adapted from The Cancer Genome Atlas (TCGA) and Murali *et al.*
MSI=microsatellite instability, CNA=copy-number low, mut=mutations

Thesis outline

Over the last decades, advances have been made in the treatment of endometrial cancer. The clinicopathological risk stratification for postoperative therapy has considerably reduced overtreatment by refining indications and introducing treatment with fewer side effects. Despite refinement in the use of postoperative radiation therapy in EC, over- and under-treatment remain a clinical problem: seven patients with stage I high-intermediate risk EC need to receive vaginal brachytherapy to prevent one recurrence, while 8% of patients develop distant metastases, a risk that might have been reduced with tailored adjuvant chemotherapy. This may be caused by the limited accuracy of the clinicopathological risk stratification to select patients of higher risk of recurrence.[45] The lack of reproducibility of pathologists to diagnose tumor type and grade may also limit the accuracy of the clinicopathological risk stratification. Expert gyneco-pathology review and a two-tiered grading system will lead to more accurate and reproducible diagnoses.[21-24,74,75] Nonetheless, there is pressing need to understand tumor behavior and design tailored treatments to further improve risk stratification. The identification of molecular markers predictive of recurrence risk or treatment benefit beyond current clinicopathological factors would represent a major advance. The aims of this thesis were to gain insight in the molecular alterations of endometrial cancer and to identify prognostic markers in endometrial cancer to refine clinicopathological risk assessment and direct adjuvant therapy.

Chapter 2 reports on the concordance of molecular tumor alterations between pre-operative curettage specimen and the hysterectomy specimen in patients with endometrial cancer. **Chapter 3** shows the prognostic value of *POLE* exonuclease domain mutations in early-stage endometrial cancer tissues from patients enrolled in the PORTEC-1 and -2 clinical trials and in three additional smaller endometrial cancer series. **Chapter 4** describes an integrated analysis of clinicopathological risk factors, The Cancer Genome Atlas proposed molecular subgroups, a multi-gene mutation analysis and established biomarkers such as L1CAM, ER/PR and lymphovascular space invasion in two large early-stage endometrial cancer trial populations. **Chapter 5** shows prognostic molecular subgroups and potentially targetable alterations in high-risk endometrial cancer. **Chapter 6** focuses on the optimal approach for mismatch repair deficiency testing in routine clinical pathology for endometrial cancer. **Chapter 7** reports on the remarkably high frequency of *JAK1* mutations in microsatellite unstable endometrial cancers and its association with tumor immune evasion. Finally, **Chapter 8** provides a general discussion of this thesis, focusing on implications for clinical practice and future research.

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Chapter 2

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

Stelloo E, Nout RA, Naves LC, Ter Haar NT, Creutzberg CL, Smit VT, Bosse T

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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

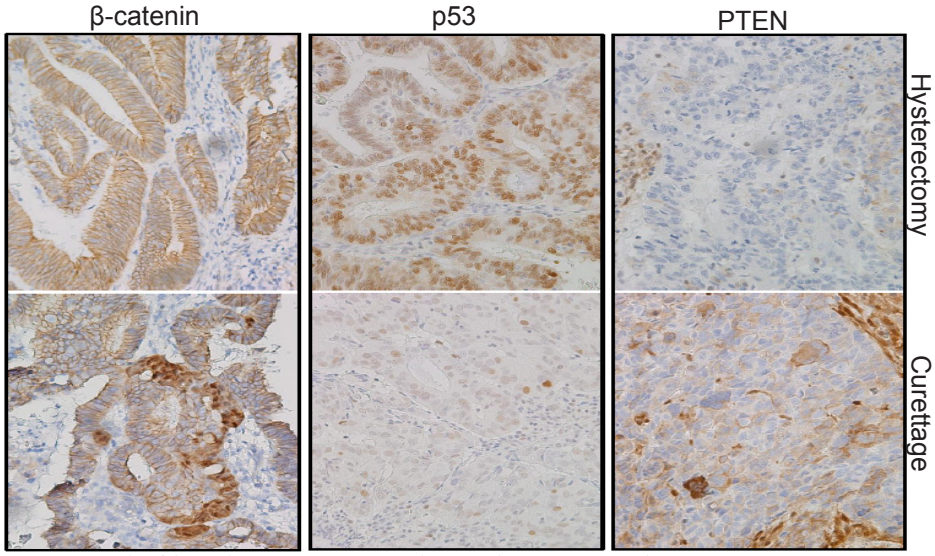

Figure 1. Example of discordant immunostainings between hysterectomy and pre-operative curettage specimens. For PTEN, the hysterectomy showed loss of expression whereas the paired pre-operative curettage showed a positive staining. A p53 'mutant-like' expression was observed in the hysterectomy while 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).

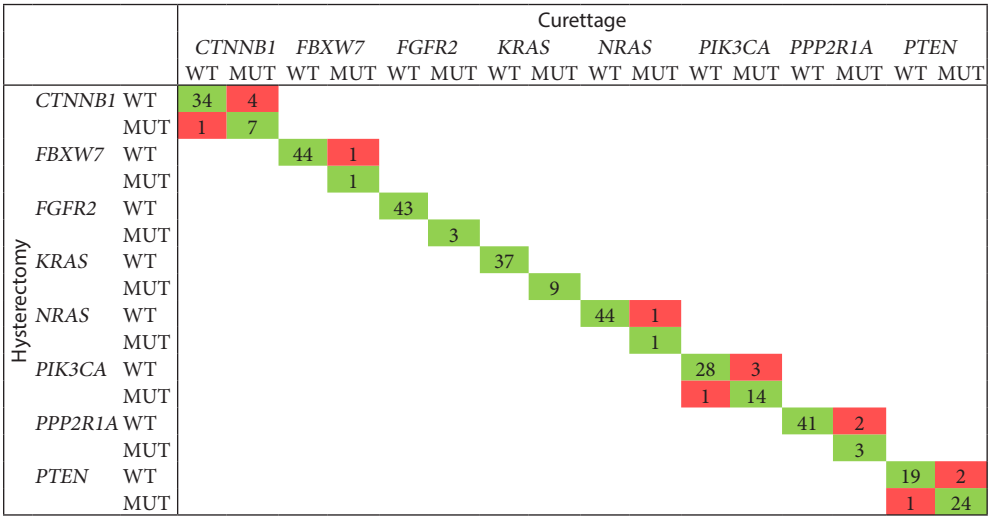

Figure 2. Concordance of molecular alterations in endometrial hysterectomy and pre-operative specimens using GynCarta multigene analysis. Concordant cases between the paired hysterectomy and curettage specimen wildtype (WT) and mutant (MUT) cases are indicated in green and discordant cases in red. No 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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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

Chapter 3

Prognostic significance of *POLE* proofreading mutations in endometrial cancer

Church DN, Stelloo E, Nout RA, Valtcheva N, Depreeuw J, ter Haar N, Noske A, Amant F, Tomlinson IP, Wild PJ, Lambrechts D, Jürgenliemk-Schulz IM, Jobsen JJ, Smit VT, Creutzberg CL, Bosse T

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Abstract

Background: Current risk stratification in endometrial cancer (EC) results in frequent over- and underuse of adjuvant therapy, and may be improved by novel biomarkers. We examined whether *POLE* proofreading mutations, recently reported in about 7% of ECs, predict prognosis.

Methods: We performed targeted *POLE* sequencing in ECs from the PORTEC-1 and -2 trials (n=788), and analyzed clinical outcome according to *POLE* status. We combined these results with those from three additional series (n=628) by meta-analysis to generate multivariable-adjusted, pooled hazard ratios (HRs) for recurrence-free survival (RFS) and cancer-specific survival (CSS) of *POLE*-mutant ECs. All statistical tests were two-sided.

Results: *POLE* mutations were detected in 48 of 788 (6.1%) ECs from PORTEC-1 and -2 and were associated with high tumor grade ($P<0.001$). Women with *POLE*-mutant ECs had fewer recurrences (6.2% vs. 14.1%) and EC deaths (2.3% vs. 9.7%), though, in the total PORTEC cohort, differences in RFS and CSS were not statistically significant (multivariable-adjusted HR=0.43, 95% CI=0.13 to 1.37, $P=0.15$; HR=0.19, 95% CI=0.03 to 1.44, $P=0.11$ respectively). However, of 109 grade 3 tumors, 0 of 15 *POLE*-mutant ECs recurred, compared with 29 of 94 (30.9%) *POLE* wild-type cancers; reflected in statistically significantly greater RFS (multivariable-adjusted HR=0.11, 95% CI=0.001 to 0.84, $P=0.03$). In the additional series, there were no EC-related events in any of 33 *POLE*-mutant ECs, resulting in a multivariable-adjusted, pooled HR of 0.33 for RFS (95% CI=0.12 to 0.91, $P=0.03$) and 0.26 for CSS (95% CI=0.06 to 1.08, $P=0.06$).

Conclusion: *POLE* proofreading mutations predict favorable EC prognosis, independently of other clinicopathological variables, with the greatest effect seen in high-grade tumors. This novel biomarker may help to reduce overtreatment in EC.

Introduction

Endometrial cancer (EC) is the commonest gynecological malignancy in the Western world, and is rising in incidence because of increasing obesity and ageing of the population.[1] Most cases (80%) are detected at an early stage (FIGO stage I)[2] because of early symptoms. The standard management of EC consists of hysterectomy and bilateral salpingo-oophorectomy with or without postoperative vaginal brachytherapy (VBT) or pelvic external beam radiotherapy (EBRT) depending on recurrence risk.[3,4] Women with grade 3 cancers or advanced disease are increasingly treated with adjuvant chemotherapy,[5,6] the role of which will be further defined by ongoing studies. Current risk stratification is based on both clinical (age) and pathologic factors (FIGO stage, tumor type, grade, and lymphovascular space invasion [LVSI]). Despite refinement in the use of postoperative treatment in EC over the last two decades, over- and underusage of adjuvant therapy remains a clinical problem. Approximately seven patients with stage I EC with risk factors need to receive VBT to prevent one recurrence, while 8% to 10% patients develop distant metastases that may have been prevented with adjuvant chemotherapy.[3-6] Consequently, the identification of molecular markers predictive of recurrence risk or treatment benefit beyond current clinicopathological factors would represent a major advance.[7] While studies have investigated the prognostic significance of several molecular alterations involved in endometrial carcinogenesis, including microsatellite instability (MSI), *PIK3CA* and *TP53* mutation,[8,9] to date none have been incorporated into routine clinical practice.

We recently showed that germline variants in the exonuclease domain of the DNA polymerases *POLE* and *POLD1* predispose to cancer, including EC, by impairing polymerase proofreading and greatly increasing the rate of base substitution mutations.[10] We subsequently demonstrated that somatic *POLE* proofreading mutations are found in about 7% of sporadic ECs, where they strongly associate with high tumor grade.[11] Similar findings have been reported in parallel by The Cancer Genome Atlas (TCGA), which also demonstrated that while *POLE* mutations were only found in endometrioid tumors, they were not inversely associated with *TP53* mutation.[12] These pathogenic *POLE* proofreading mutations, about 90% of which cluster in exons 9 and 13, localize to amino acids within, or close to, conserved motifs essential for proofreading function.[11,12] In keeping with this, *POLE* proofreading-mutant ECs are ultramutated, with a base substitution mutation frequency among the highest in human tumors.[13]

Different forms of genomic instability in cancers are known to be associated with clinicopathological features, including prognosis.[14-16] Favorable outcome of women with *POLE*-mutant ECs has been suggested,[12] but only reached statistical significance when limited to analysis of grade 3 tumors in a recent report,[17] and current evidence is insufficient to inform practice.[18] In this study, we have analyzed associations between *POLE* proofreading mutations, recurrence-free and cancer-specific survival in two large, randomized controlled

trials (PORTEC-1 and -2)[3,4] of early-stage (FIGO stage I), (high-) intermediate risk EC, with central pathology review and mature follow-up data, and in three additional smaller EC series.[9,12,19]

Methods

PORTEC study details

Details of the PORTEC-1 and PORTEC-2 studies have been published previously (see the Supplementary Materials).[3,4] PORTEC-1 compared pelvic EBRT with no additional treatment (NAT) in 715 women with intermediate risk, stage I EC recruited between June 1990 and December 1997.[3] PORTEC-2 randomly assigned 427 women with high-intermediate risk stage I/IIA EC between May 2002 and September 2006 to either EBRT or VBT following surgery.[4] The median (range) duration of follow-up was 159.6 (33.6–222) months in PORTEC-1 and 89 (18–122) months in PORTEC-2. The PORTEC study protocols were approved by the Dutch Cancer Society and by the medical ethics committees at participating centers. All patients provided written informed consent to study participation and treatment.

Additional EC Series

The Leuven (n=187)[9], Zurich/Basel (n=267)[19], and TCGA (n=373)[12] series have also been reported previously (Supplementary Materials). The Leuven and TCGA cohorts were collected prospectively, while Zurich/Basel cases were identified retrospectively. These sets included both endometrioid and nonendometrioid ECs (EECs and NEECs), and also included patients with stage III/IV disease (21.8–31.2%). Central pathology review was mandated in both TCGA and the Zurich/Basel cohorts, while the Leuven cases were reviewed by a single academic pathologist. Patients in the Leuven and Zurich/Basel cohorts were managed according to standard protocols, while treatment in the TCGA series was at the discretion of the attending physician: median (range) follow-up in each was 29 (1–184) months, 46 (1–173) months, and 28.7 (0.6–185.6) months, respectively. Follow-up data varied between series. RFS data were available for the Leuven and TCGA series, but not the Zurich/Basel set, while CSS data were available for the Leuven and the Zurich/Basel series, but absent from the TCGA study. Collection and analysis of the Leuven and Zurich/Basel series were approved by the scientific ethics committee from all centers (UZ Leuven Medical ethics committee and KEK-ZH-NR: 2010-0358, respectively). Ethical approval for anonymized tumor molecular analysis was granted by Oxfordshire Research Ethics Committee B (Approval No. 05\Q1605\66).

Demographic and Clinicopathological Variables

Baseline demographic and clinicopathological variables were treated as either categorical (e.g., grade, stage, EEC vs. NEEC) or continuous (age) as appropriate. All analyses were based on data from central pathology review.

Molecular Analysis

Tumor DNA was extracted from formalin-fixed, paraffin-embedded (FFPE) blocks from 434 (60.1%) and 398 (93.2%) ECs from the PORTEC-1 and -2 studies, respectively. There were no statistically significant differences between the biomarker and the total study populations in demographic/ clinicopathological characteristics, treatment or survival. DNA was extracted from 187 fresh-frozen ECs in the Leuven set, 373 fresh-frozen ECs in the TCGA set, and 260 FFPE ECs in the Zurich/Basel set (Supplementary Methods). The Leuven, Zurich/ Basel, and TCGA biomarker cohorts were similar to the total series population in each case (Supplementary Methods).

Sanger sequencing of *POLE* exons 9 and 13 (Supplementary Table 1) was successful in over 94% of cases. For TCGA, we extracted *POLE* mutation data from publicly available whole-exome sequencing data (https://tcga-data.nci.nih.gov/tcga/).[12] We defined pathogenic *POLE* proofreading mutations as variants absent from public germline sequence databases (http://evsgs.washington.edu/EVS/; http://www.1000genomes.org/) and previously confirmed as somatic variants associated with tumor ultramutation,[11,12,20,21] with the exception of one novel variant predicted to perturb protein function by SIFT (http://sift.jcvi.org) and Mutation Assessor (http://mutationassessor.org). All variants were confirmed in at least duplicate independent polymerase chain reaction and sequencing reactions.

Statistical Analysis

Analyses performed and reported in this biomarker study are listed in Supplementary Table 2 in accordance with published guidelines.[22,23] For analysis of the association of *POLE* mutation with outcome, our primary endpoint was recurrence-free survival (RFS), with secondary endpoints of cancer-specific survival (CSS), and overall survival (OS). In the PORTEC studies, RFS was defined as the time from random assignment to relapse, with censoring at last contact or death in case of no recurrence. CSS was measured as the time from random assignment to death from EC, with censoring at date of last contact or noncancer death. OS was measured as the time from random assignment to death from any cause, with censoring at date of last contact in patients still alive. The same criteria were used in the additional series, with the exception that survival measurements were from time of diagnosis. Survival curves were plotted using the Kaplan-Meier method and compared by the log-rank test. We used Cox proportional hazards models to calculate hazard ratios (HRs) for RFS, CSS, and OS of *POLE*-mutant ECs relative to *POLE* wild-type tumors by univariable analyses and, following adjustment for baseline characteristics and prognostic factors (age, tumor type, grade, LVSI, depth of myometrial invasion, and treatment), by multivariable analyses (see Supplementary Methods and Supplementary Tables 3–6). Proportionality of hazards in Cox models was confirmed by visual inspection of complementary log plots or by interaction terms of covariables and (log)time. In view of the similar patient populations, the limited number of cancer-related events in both PORTEC trials, and the modest frequency of *POLE*

mutations, the PORTEC studies were combined for most analyses. For Cox regression analysis of the PORTEC grade 3 subset and the Leuven, Zurich/Basel, and TCGA series, we applied Firth's correction,[24] owing to the absence of events in the *POLE*-mutant groups. For multivariable analysis of the additional series, we included disease stage as a covariable; although myometrial invasion, LVSI, and treatment were not included because of lack of data, we confirmed that omission of these variables from the PORTEC multivariable analyses did not alter estimates of RFS or CSS with *POLE* mutation (*P*=0.93 and *P*=0.87 respectively) (see Supplementary Methods). Statistical analyses were performed using SPSS (SPSS Inc., Chicago, IL), Stata (StataCorp, College Station, TX) and R (http://www.r-project.org/). All *P*-values were two-sided. A *P*-value under 0.05 was considered statistically significant.

Results

Patient Characteristics and POLE Proofreading Mutations in PORTEC Studies

Demographic and clinicopathological characteristics of the PORTEC study participants in whom tumor *POLE* sequencing was successful are shown in Table 1. The majority (about 98%) of cancers in both studies were EECs. *POLE* exon 9 and 13 proofreading mutations were detected in 48 of 788 (6.1%) tumors from the combined PORTEC studies (Table 1), with similar distribution across study arms.[11,12] With the exception of two tumors harboring a germline polymorphism of uncertain pathogenicity (rs150032060; c.1282G>A, p.Ala428Thr), detected in tumor-free myometrium in both cases and excluded from subsequent analyses, all mutations were recurrent substitutions at somatic mutational hotspot codons (Supplementary Table 7) known to cause ultramutation.[11,12] Apart from one neuroendocrine tumor, all *POLE* mutations occurred in endometrioid ECs. Analysis of 48 available preoperative curettings identified *POLE* mutations in all five cases in which they were detected in the subsequent hysterectomy, resulting in 100% concordance. Compared with *POLE* wild-type ECs, *POLE*-mutant tumors occurred in younger women (median age 63.5 vs. 68.5 years, *P*<0.001, t test), and were more commonly grade 3 (31.3% vs. 12.7%, *P*<0.001, $\chi 2$ test), though LVSI and deep (>50%) myometrial invasion were less frequent (0% vs. 9.5%, *P*=0.03, and 58.2% vs. 71.9%, *P*=0.045, respectively, $\chi 2$ test) (Table 1).

Clinical Outcome by POLE Proofreading Mutation in PORTEC Studies

We first examined the association of *POLE* proofreading mutation with EC recurrence. Three of 48 (6.2%) women with *POLE*-mutant tumors developed local or distant recurrence during study follow-up, compared with 104 of the other 740 (14.1%) patients (Figure 1A, Table 2). All three recurrences in the *POLE*-mutant cohort were distant metastases without locoregional relapse and occurred in women with grade 1 EECs and deep myometrial invasion, managed by observation, EBRT, and VBT, respectively. The univariable HR for recurrence-free survival (RFS) with tumor *POLE* proofreading mutation was 0.41 (95% CI=0.13 to 1.28, *P*=0.13),

Table 1. Clinicopathological characteristics of patients in PORTEC studies according to *POLE* proofreading mutation.

	PORTEC-1 (n=412)		PORTEC-2 (n=376)		Combined (n=788)		
	POLE wild-type n=386 (%)	*POLE* mutant* n=26 (%)	*POLE* wild-type n=354 (%)	*POLE* mutant* n=22 (%)	*POLE* wild-type n=740 (%)	*POLE* mutant* n=48 (%)	*P*-value**
Age, y							
Mean	67.0	59.8	70.1	67.9	68.5	63.5	<0.001
Range	41–90	46–76	46–88	61–81	41–90	46–81	
<60	91 (23.6)	16 (61.5)	15 (4.2)	0 (0)	106 (14.3)	16 (33.3)	0.002
60–70	152 (39.4)	4 (15.4)	173 (48.9)	12 (54.5)	325 (43.9)	16 (33.3)	
>70	143 (37.0)	6 (23.1)	166 (46.9)	10 (45.5)	309 (41.8)	16 (33.3)	
Tumor type							
EEC	380 (98.4)	26 (100)	343 (96.9)	21 (95.5)	723 (97.7)	47 (97.9)	0.920
NEEC	6 (1.6)	0 (0)	11 (3.1)	1 (4.5)	17 (2.3)	1 (2.1)	
FIGO stage (1988)							
IB	150 (38.9)	13 (50)	27 (7.6)	4 (18.2)	177 (23.9)	17 (35.4)	0.190
IC	236 (61.1)	13 (50)	283 (79.9)	16 (72.7)	519 (70.1)	29 (60.4)	
IIA	NA	NA	44 (12.4)	2 (9.1)	44 (5.9)	2 (4.2)	
Depth of invasion							
<50%	150 (38.9)	13 (50)	58 (16.4)	7 (31.8)	208 (28.1)	20 (41.7)	0.045
>50%	236 (61.1)	13 (50)	296 (83.6)	15 (68.2)	532 (71.9)	28 (58.3)	
Grade							
1	264 (68.4)	16 (61.5)	279 (78.8)	12 (54.5)	543 (73.4)	28 (58.3)	0.001
2	63 (16.3)	3 (11.5)	40 (11.3)	2 (9.1)	103 (13.9)	5 (10.4)	
3	59 (15.3)	7 (26.9)	35 (9.9)	8 (36.4)	94 (12.7)	15 (31.3)	
LVSI							
Absent	364 (94.3)	26 (100)	306 (86.4)	22 (100)	670 (90.5)	48 (100)	0.029
Present	22 (5.7)	0 (0)	48 (13.6)	0 (0)	70 (9.5)	0 (0)	
Radiotherapy							
NAT	196 (50.8)	13 (50)	3 (0.8)	0 (0)	199 (26·9)	13 (27.1)	0.980
EBRT	190 (49.2)	13 (50)	171 (48.3)	11 (50)	361 (48.8)	24 (50)	
VBT	NA	NA	180 (50.8)	11 (50)	180 (24.3)	11 (22.9)	

* *POLE* exon 9 or 13 exonuclease domain mutation (excludes two cases of confirmed germline variant rs150032060 [c.1282G>A, p.Ala428Thr], confirmed in tumor-free myometrium and previously shown not to associate with tumor ultramutation). EBRT=(pelvic) external beam radiotherapy; EEC=endometrioid endometrial cancer; LVSI=lymphovascular space invasion; NAT=no additional treatment; NEEC=nonendometrioid endometrial cancer; VBT=vault brachytherapy. ** *P*-values represent comparison of *POLE* wild-type and *POLE* proofreading mutant cohorts in the combined cohorts calculated by unpaired *t* test (age) or $\chi 2$ test (all other comparisons). All statistical tests were two-sided.

with little change following adjustment for known prognostic variables by Cox regression (HR=0.43, 95% CI=0.13 to 1.37, *P*=0.15) (Figure 1A, Table 2; Supplementary Tables 3 and 5). As many local EC recurrences are salvageable with therapy, we next examined whether cancer-specific survival (CSS) varied according to tumor *POLE* mutation. In women with *POLE*-mutant EC, there was one (2.3%) EC death during follow-up, compared with 72 (9.7%) in the rest of the study population. The unadjusted HR for CSS was 0.20 (95% CI=0.03 to 1.46, *P*=0.11), with minimal alteration following multivariable analysis (HR=0.19, 95% CI=0.03 to 1.44, *P*=0.11) (Figure 1B, Table 2; Supplementary Tables 4 and 6). Overall survival of women with *POLE*-mutant ECs was not statistically significantly greater than that of other patients by univariable or multivariable analysis (10-year OS=76.2% vs. 70.4%) (Table 2).

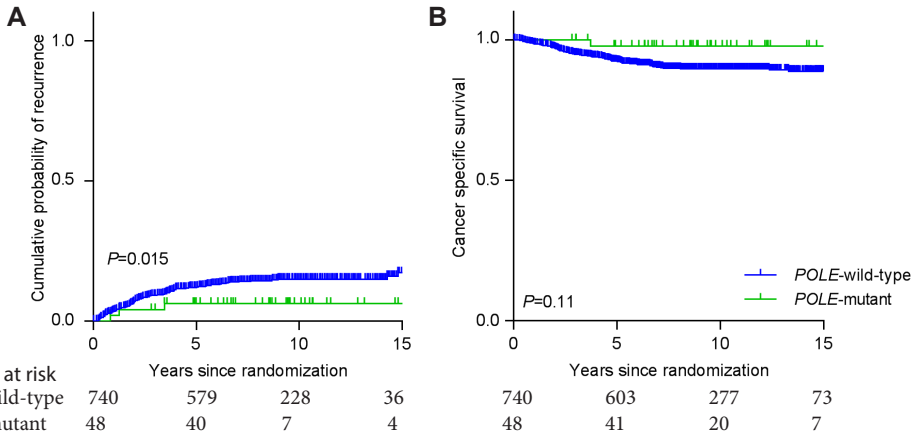


Figure 1. Cumulative probability of recurrence (A) and cancer-specific survival (B) according to *POLE* proofreading mutation in the combined PORTEC studies. *P*-values are obtained by two-sided log-rank test.

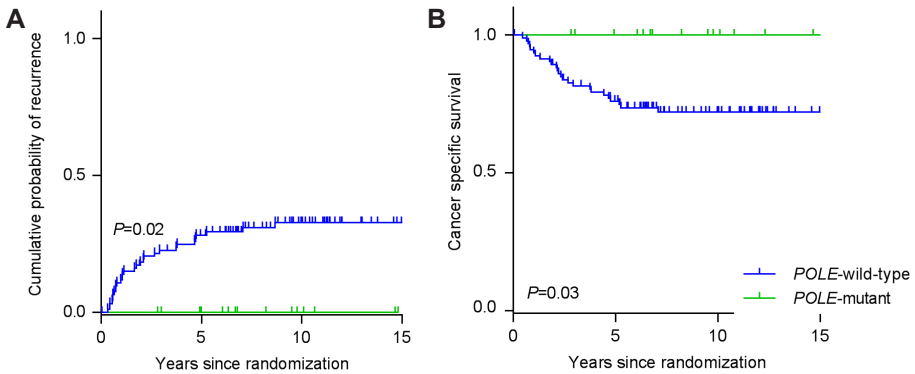
Table 2. Clinical outcome in PORTEC studies according to *POLE* proofreading mutation determined by univariable and multivariable analysis.

			Univariable analysis		Multivariable analysis*	
Outcome	Events/total (%)	10-year, %	HR (95% CI)	*P*†	HR (95% CI)	*P*†
Recurrence						
POLE wild-type	104/740 (14.1)	15.2	0.41 (0.13-1.28)	0.130	0.43 (0.13-1.37)	0.150
POLE mutant‡	3/48 (6.2)	5.5				
Cancer-specific survival						
POLE wild-type	72/740 (9.7)	89.7	0.20 (0.03-1.46)	0.110	0.19 (0.03-1.44)	0.110
POLE mutant‡	1/48 (2.3)	97.7				
Overall survival						
POLE wild-type	248/740 (33.5)	70.4	0.69 (0.38-1.22)	0.200	1.06 (0.59-1.92)	0.850
POLE mutant‡	12/48 (25.0)	76.2				

* Multivariable Cox models include *POLE* mutation, age, nonendometrioid histology, tumor grade, and lymphovascular invasion. CI=confidence interval; HR=hazard ratio. †Calculated using Cox proportional hazards test. All statistical tests were two-sided. ‡*POLE* exon 9 or 13 proofreading mutation.

The strong association of *POLE* mutations with high tumor grade (11) caused us to hypothesize that their apparent prognostic effect would be most evident in this group, who are commonly considered for treatment intensification. Of 109 patients with grade 3 tumors, there were no recurrences or cancer deaths in the 15 (13.7%) *POLE*-mutant case, compared with 29 (30.9%)

relapses and 25 (26.6%) EC deaths in the remaining 94 women (Figure 2, A and B), reflected in statistically significantly improved RFS in univariable analysis (HR=0.09, 95% CI=0.001 to 0.66, *P*=0.01) and, following adjustment for other prognostic variables in multivariable analysis (HR=0.11, 95% CI=0.001 to 0.84, *P*=0.03) (Table 3; Supplementary Tables 8 and 9). These results were essentially unchanged after limiting RFS analysis to the 97 grade 3 endometrioid ECs (univariable HR=0.11, 95% CI=0.001 to 0.78, *P*=0.02; multivariable-adjusted HR=0.12, 95% CI=0.001 to 0.87, *P*=0.03) (Supplementary Table 8). Notably, *POLE* proofreading mutation was a stronger predictor of recurrence and EC death than all other prognostic variables examined in these analyses (Supplementary Tables 8 and 9).



Number at risk

POLE wild-type	94	63	28	2	94	66	33	8
POLE-mutant	15	11	4	0	15	12	5	0

Figure 2. Cumulative probability of recurrence (A) and cancer-specific survival (B) according to *POLE* proofreading mutation in patients with grade 3 tumors in the combined PORTEC studies. *P*-values are obtained by two-sided log-rank test.

Pooled Analysis With Additional EC Series

While the PORTEC analyses demonstrated a tendency for *POLE* proofreading-mutant ECs of all grades to improved outcome, the generally good prognosis of patients limited our ability to confirm this beyond the grade 3 subgroup. We therefore sought to support our results by analysis of two EC series from Leuven and Zurich/Basel, together with the published TCGA set, collectively comprising an additional 628 patients. *POLE* proofreading mutations were detected in 33 (5.3%) case patients in the additional series (Supplementary Table 7). As in the PORTEC studies, *POLE*-mutant tumors were more frequently high-grade (54.5% vs. 32.4%, *P*=0.013, $\chi 2$ test) and were generally of endometrioid histology, though three *POLE*-mutant mixed endometrioid/ serous and one serous cancer were detected in the Leuven series (Supplementary Tables 10–12).

In the two series with RFS data—Leuven and TCGA—no *POLE*-mutant EC recurred with median follow-up of 28 months, compared with 31.6% and 19.0% of other tumors, respectively (Supplementary Figures 1A and 2, Supplementary Tables 13 and 14). Similarly, for the two series

in which CSS was documented—Leuven and Zurich/Basel (median follow-up 46 months)—there were no EC deaths in women with *POLE*-mutant tumors, compared with 18.6% and 10.8% of the remaining patients (Supplementary Figures 1B and 3, Supplementary Tables 13 and 15). As anticipated given their sizes, no individual series demonstrated statistically significant differences in the RFS/CSS of *POLE*-mutant ECs by either univariable or multivariable analyses (Supplementary Tables 13–15). We therefore combined multivariable-adjusted HRs from each series with those from the PORTEC studies by meta-analysis to generate pooled estimates for RFS and CSS according to *POLE* proofreading mutation (Figure 3, A and B). *POLE*-mutant ECs were associated with statistically significantly greater RFS compared with other tumors (HR=0.33, 95% CI=0.12 to 0.91, *P*=0.03), with no evidence of heterogeneity among studies (*P*=0.66), though the difference in CSS was not statistically significant using a two-tailed test (HR=0.26, 95% CI=0.06 to 1.08, *P*=0.06). Interestingly, the only *POLE*-mutant tumors that recurred were early-stage, grade I ECs from the PORTEC cohorts (Supplementary Table 16). To date, most *POLE* proofreading mutations have been detected in EECs,[11,12,17] although *POLE*-mutant NEECs have been reported.[11,25,26] Given the difficulty in histotyping high-grade ECs, we considered the possibility that the four *POLE*-mutant tumors reported as NEECs in the Leuven series may have been misclassified, thus biasing our results because of the poor prognosis of NEEC. We therefore confirmed that the estimate of RFS was essentially unchanged by repeating the meta-analysis after excluding *POLE* wild-type NEECs from all cohorts (HR=0.34, 95% CI=0.12 to 0.93, *P*=0.04) (Supplementary Tables 5, 6, 13, and 14).

Table 3. Clinical outcome of patients with grade 3 tumors in PORTEC studies according to *POLE* proofreading mutation by univariable and multivariable analysis.

			Univariable analysis		Multivariable analysis*	
	Events/total (%)	10-year (%)	HR (95% CI)	*P*†	HR (95% CI)	*P*†
Recurrence						
POLE wild-type	29/94	30.8	0.09 (0.001-0.66)	0.010	0.11 (0.001–0.84)	0.028
POLE mutant‡	0/15	0				
Cancer specific survival						
POLE wild-type	25/94	73.4	0.11 (0.001-0.78)	0.020	0.14 (0.001-1.01)	0.051
POLE mutant‡	0/15	100				
Overall survival						
POLE wild-type	46/94	60	0.49 (0.18-1.36)	0.170	0.78 (0.27-2.21)	0.630
POLE mutant‡	4/15	73.3				

* Multivariable Cox models include *POLE* mutation, age, non-endometrioid histology and lymphovascular invasion. †Calculated using Cox proportional hazards two-sided test with Firth's correction for analysis of recurrence and cancer-specific survival (due to absence of events in the *POLE*-mutant groups). ‡*POLE* exon 9 or 13 proofreading mutation.

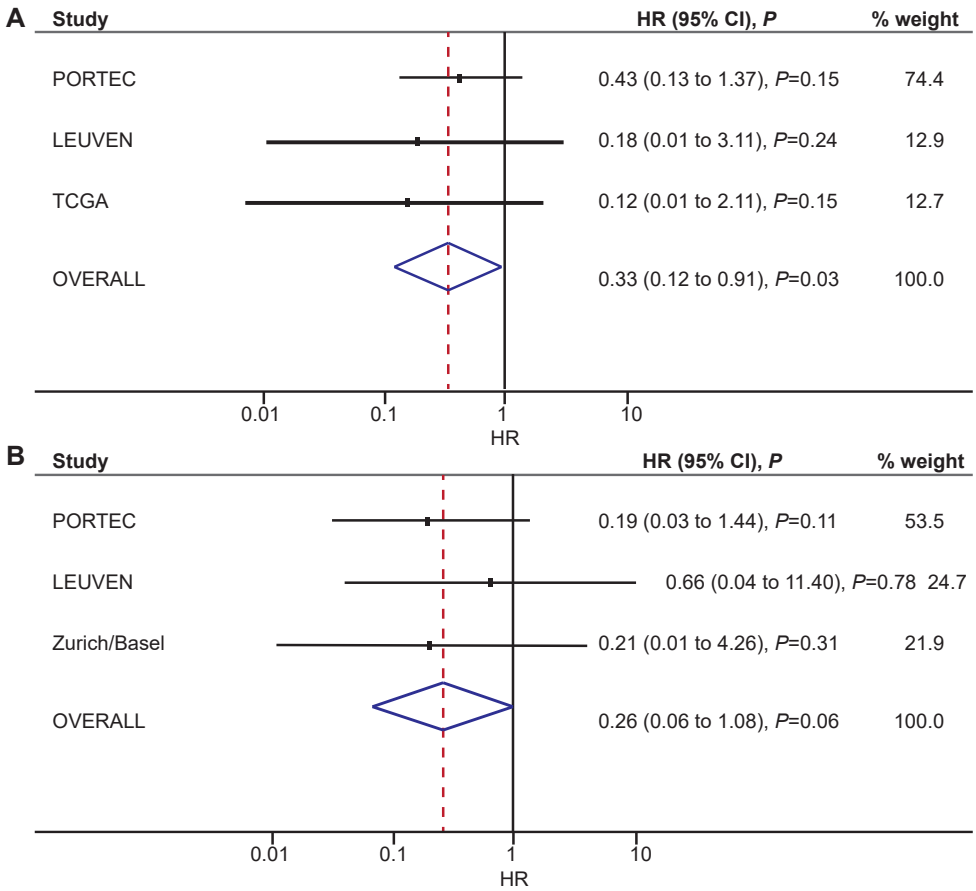


Figure 3. Pooled HRs for recurrence-free survival (A) and cancer-specific survival (B) of *POLE* proofreading-mutant endometrial cancer derived from the PORTEC studies and additional series. Results from multivariable analyses were combined by meta-analysis using Mantel-Haenszel weights to generate pooled, adjusted HRs and 95% confidence intervals. All statistical tests were two-sided. CI=confidence interval; HR=hazard ratio; TCGA=The Cancer Genome Atlas.

Discussion

By analyzing tumors from nearly 800 women in two large, randomly assigned controlled trials and pooling data with three independent series comprising over 600 additional patients, we have demonstrated that *POLE* proofreading-mutant ECs of all grades display excellent prognosis, independent of other known prognostic factors. *POLE*-mutant tumors have a risk of recurrence approximately one third that of other ECs, and a relative risk of cancer death that appears even lower, though the latter finding was not statistically significant in our analysis. This is despite a strong association of *POLE* proofreading mutations with high tumor grade—a characteristic that predicts a high risk of metastases in early EC.[27] Indeed, in the PORTEC studies, no *POLE*-mutant grade 3 EC recurred, compared with 30.9% of

grade 3 tumors in the rest of this subgroup, consistent with a recent report, which combined high-grade ECs from the TCGA series with an additional, small retrospective series.[17] The role of adjuvant chemotherapy in patients with high-risk grade 3 tumors is currently under investigation, and as systemic therapy was not used in PORTEC-1 or -2 these data suggest that patients with *POLE* proofreading-mutant EC may be unlikely to benefit from such treatment. Similarly, the absence of locoregional recurrence of *POLE*-mutant ECs was observed across all arms of the PORTEC studies, including the NAT arm of PORTEC-1. While further studies are required before firm conclusions can be drawn on the implications of *POLE* proofreading mutations for postoperative treatment in EC, our results suggest that minimization of adjuvant therapy for *POLE*-mutant ECs localized to the uterus may be worthy of investigation. They also suggest *POLE* proofreading mutation should be considered for inclusion in cancer gene panels used in EC, as it may improve prognostication, particularly for grade 3 tumors.

While highlighting the strengths of using high-quality clinical trial sample banks for biomarker research,[18] our study has limitations. The challenge of confirming even a strong effect of a marker with modest (<10%) frequency in a population with relatively favorable prognosis meant that we used additional EC series to confirm the improved RFS of *POLE*-mutant ECs. While acknowledging the limitations of sets containing a mixture of histological subtypes and stages, with limited follow-up and lacking comprehensive treatment data[18] the similar results from each following multivariable analysis suggests that our results are unlikely to be because of chance or confounding by inclusion of NEECs in our analyses. However, as highlighted above, it will be important to confirm the favorable prognosis of *POLE*-mutant ECs in further independent series, and particularly in tumors of advanced stages. It should also be noted that as a single biomarker survey of *POLE* hotspot exons in ECs of predominantly endometrioid histology, we are presently unable to determine the effect of the about 10% of pathogenic variants outside exons 9 and 13,[11,12] or whether the effect of *POLE* mutation varies according to tumor molecular subtypes. Both questions are likely to be addressed by future studies.

POLE proofreading-mutant cancers are a molecularly distinct group of tumors with a striking mutation burden and distinctive mutation signature.[11-13] Whether these characteristics contribute to their favorable prognosis awaits confirmation. Study of mutator polymerases in yeast has confirmed the existence of a mutational threshold, which, if exceeded, results in decreased viability because of lethal mutations in essential genes prior to cell division.[28] It will be of interest to determine whether the dramatic increase in mutation rate in *S. cerevisiae* caused by the corresponding substitution to human *POLE* p.Pro286Arg[29] approaches this error threshold. Similarly, analysis of the burden of deleterious mutations accumulated in *POLE* proofreading-mutant cancers may provide insights into their behavior.[30]

Over the last two years, we and others have shown that somatic mutations in the proofreading domain of *POLE* occur in several human tumors.[10-12,20,31] We now demonstrate that, despite

a strong association with high grade, *POLE* proofreading-mutant ECs have a favorable prognosis. While the frequency of *POLE* mutation in EC is modest, it is worth noting that it is broadly similar to that of many novel molecular aberrations recently discovered by TCGA and other sequencing efforts. As most common cancer variants are currently not actionable, similar analyses of these modest-frequency (5% to 10%) molecular subgroups are likely to be essential if we are to realize the ambition of personalized cancer medicine during the next decade.

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Notes

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Supplementary files

Supplementary Methods

PORTEC studies

Full details of the PORTEC-1 and PORTEC-2 clinical trials are provided in the original publications.[1,2] Inclusion criteria for PORTEC-1 were histologically proven endometrial adenocarcinoma (including adenocarcinoma with squamous features, adenosquamous carcinoma, papillary serous carcinoma, and clear-cell carcinoma), of intermediate risk, defined as postoperative FIGO stage I, grade 1 with deep (>50%) myometrial invasion, grade 2 with any depth of invasion, or grade 3 with superficial (<50%) invasion. 662/714 (92.7%) of tumors were endometrioid endometrial cancers (EECs). Tumor subtype and grade were confirmed by central pathology review in 567 (79.4%) cases.

Eligibility criteria for PORTEC-2 were endometrial adenocarcinoma of high-intermediate risk, defined as: (i) age greater than 60 years and stage IC grade 1 or 2 disease, or stage IB grade 3 disease; or (ii) patients of any age with stage IIA disease (apart from grade 3 with greater than 50% myometrial invasion). Patients whose cancers had serous or clear cell histology were excluded. Central pathology review was performed in 86% cases, following which 12 tumors were reclassified as of non-endometrioid histology.

Leuven series

Details of the Leuven prospective series have been previously reported.[3] Pathological analysis and grading were performed by a single, experienced gynecological pathologist according to the WHO classification of Tumors of Female Reproductive Organs. Clinical information was collected by trained researchers. Patients were managed by TAH-BSO, with pelvic

lymphadenectomy in cases of grade 1-2 tumors with diameter > 2 cm or 50% myometrial invasion, or grade 3 EECs and NEECs. Patients were followed up every 3 months for the first 2 years, every 6 months from 2-5 years and yearly afterwards. Follow-up data including in details of RFS, CSS and OS were extracted from electronic patient files.

Zurich/Basel series

Details of the Basel/Zurich series have been previously reported.[4-6] Tumor blocks were collected retrospectively from patients treated in academic centers and outpatient clinics. Staging was according to the AJCC TNM system (7th edition), and classified by two independent histopathologists with the aid of IHC. Patients with localized disease were treated by TAH-BSO (with or without pelvic and para-aortic lymphadenectomy). Adjuvant intra-vaginal radiation therapy was given postoperatively in cases of myometrial invasion or grade 3 tumor. Follow-up information was obtained from the participating centers and from the Cancer Registries of Basel and Zurich.

TCGA series

Details of the TCGA EC series have been previously reported.[7] Patients were recruited from participating centres, and tumors staged according to the AJCC system. Whole exome sequencing was performed on 248 cases. Treatment and follow up were at the discretion of the treating clinician; of the overall population, 19% received adjuvant RT, 10% adjuvant chemotherapy, and 14% adjuvant chemoradiotherapy; in 51% of cases, the postoperative treatment delivered was not known.

DNA extraction

In the PORTEC studies, DNA was extracted from 0.6mm tissue punches taken from areas containing ≥70% tumor in FFPE blocks, with exception of 14 cases with tumor size of <0.6mm, or insufficient fraction of tumor cells, where DNA was extracted following manual microdissection by standard methods. Similar methodology was used to extract tumor DNA in the Zurich/Basel series, while in Leuven series, DNA was extracted from fresh-frozen tissue using DNeasy Blood & Tissue Kit (Qiagen), following confirmation of adequate tumor cellularity using H&E slides on FFPE material.

PCR and sequencing reactions

PCR primers for sequencing of *POLE* exons 9 and 13 are shown in Table S1. PCR was performed using 10ng of template DNA with Qiagen multiplex PCR kit with Q solution in a 15μL reaction volume, with primers at 0.4μM final concentration and proportions of other reagents according to the manufacturer's recommendations. Reaction conditions were as follows; 95°C denaturation for 15 minutes; 94°C melt for 45 seconds, 55°C annealing for 90 seconds and 72°C extension for 45 seconds repeated for 38 cycles; and final 72°C extension for 10 minutes. PCR products were confirmed by gel electrophoresis and cleaned up with Exosap-

IT (Affymetrix) as per manufacturer's instructions. BDT (Life technologies) sequencing was performed according to manufacturer's recommendations using the forward primer for sequencing of *POLE* exon 9 and reverse primer for *POLE* exon 13.

PORTEC. PCR screening of *POLE* exons 9 and 13 was successful in 412/434 (94.9%) and 376/398 (95.4%) cases respectively from the PORTEC-1 and -2 studies. There was no significant difference between the biomarker study and overall populations in the proportion of cases with grade 3 tumors (13.7% vs. 13.3%, *P*=0.84) or disease stage (*P*=0.07).

Leuven. Of 187 fresh frozen samples analyzed in the Leuven set, PCR screening was successful in 183 (97.9%) cases. Of these, 13 (7.1%) were excluded due to non-endometrial histology (n=7, 3.8%), missing data (n=2, 1.1%), and insufficient follow up (n=4, 2.2%). There was no significant difference between the biomarker and overall populations in the proportion of cases with grade 3 tumors (54.3% vs. 55.2%, *P*=0.92) or stage III/IV disease (30.4% vs. 31.2%, *P*=0.91).

TCGA. Data were downloaded from cBioportal (http://www.cbioportal.org/public-portal/). Of 248 cases with whole-exome sequence data, 19 were excluded due to absence of outcome data (n=17, 6.9%) or lack of tumor grading (n=2, 0.8%). There was no significant difference between the analyzed and overall populations in the proportion of cases with grade 3 tumors (39.1% vs. 37.1%, *P*=0.71) or stage III/IV disease (23.0% vs. 21.0%, *P*=0.66).

Zurich/Basel. Of 267 cases from the Zurich/Basel set in whom outcome data and tumor DNA were available, PCR screening was successful in 260 cases (97.4%). 31 (11.9%) cases were excluded from multivariable analysis due to absence of data on tumor grade (n=30, 11.5%) or grade and stage (n=1, 0.4%). There was no significant difference between the biomarker and overall populations in the proportion of cases with grade 3 tumors (18.0% vs. 14.4%, *P*=0.24) or stage III/IV disease (18.6% vs. 18.5%, *P*=1.0).

Statistical Analyses

All informative subjects were used for statistical analyses, and subjects in whom data were absent were excluded. Baseline clinicopathological and molecular variables were compared using Fisher's exact or Chi-square test for categorical variables, and Student's t-test for continuous variables. Analyses performed in this biomarker study are listed in Table S2 in accordance with published guidelines. All statistical tests were two-sided.

For analysis of clinical outcome, we used multivariable Cox proportional hazards models to minimize confounding from prognostic factor imbalance between *POLE* proofreading-mutant and wild-type groups in all datasets. For the PORTEC studies, 784 patients had complete data and were informative for Cox regression analysis. In accordance with published

guidelines,[8,9] for the PORTEC analyses, we first examined the effect of addition of *POLE* proofreading mutation to a Cox model containing all standard prognostic factors for which data were available (Table S3, S4). For the final Cox models reported, variables associated with recurrence at a significance level of $P<0.1$ by univariable analysis (age, non-endometrioid histology, grade and lymphovascular invasion) were included in the initial model. With the exception of non-endometrioid histology, all four variables retained independent significance at $P<0.1$ following the addition of *POLE* EDM status to the model and were used for the final analyses (Tables S5, S6). For the grade 3 subgroup in PORTEC, and the non-PORTEC series, we used the Firth correction[10,11] owing to the absence of events in the *POLE* proofreading-mutant cohorts. Although we were unable to include myometrial invasion, LVSI and treatment in multivariable analyses of the additional series, we confirmed that their omission from the 'standardized' PORTEC Cox models containing all prognostic variables (Tables S3,S4) did not significantly alter the estimates of the effect of *POLE* mutation on either RFS (HR=0.39, 95% CI 0.12-1.23 vs. HR=0.46, 95% CI 0.14-1.47, $P=0.88$) or CSS (HR=0.18, 95% CI 0.03-1.29 vs. HR=0.20, 95% CI 0.03-1.46, $P=0.93$).[12] For these analyses, confidence intervals and *P*-values were calculated by the Wald method using the Coxphf function in R (http://www.r-project.org/). Multivariable-adjusted hazard ratios were pooled by meta-analysis using the metan command in Stata.

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Supplementary Table 1. *POLE* exon 9 and 13 sequencing PCR primer details.

Target (residues)	Primer name	Primer sequence	Primer used for sequencing
Exon 9 (268-303)	*POLE*-Ex9Fw	5'- tgcttattttgtccccacag-3'	Forward
	POLE-Ex9Rv	5'- tacttcccagaagccacctg-3'	
Exon 13 (410-445)	*POLE*-Ex13Fw	5'- tctgttctcattctccttccag-3'	Reverse
	POLE-Ex13Rv	5'- cgggatgtggcttacgtg-3'	

Supplementary Table 2. Analyses performed and reported in this study.

Comparison	Population	Primary endpoint	Secondary endpoints	Analysis	Reported
POLE proofreading-mutant vs. wild-type	Combined PORTEC 1/2 studies, all patients*	RFS	CSS, OS	Log rank *P*, univariable and multivariable adjusted HR	Main text, Tables 2, S3-S6, Figure 1
POLE proofreading-mutant vs. wild-type	Combined PORTEC 1/2 studies, grade 3 subgroup*	RFS	CSS	Log rank *P*, univariable and multivariable adjusted HR	Main text, Figure 2, Tables 3,S8,S9
POLE proofreading-mutant vs. wild-type	Leuven series, all patients*	RFS	CSS	Log rank *P*, Univariable and multivariable adjusted HR	Table S13, Figure S1
POLE proofreading-mutant vs. wild-type	TCGA series, all patients*	RFS	CSS	Log rank *P*, Univariable and multivariable adjusted HR	Table S14, Figure S2
POLE proofreading-mutant vs. wild-type	Zurich/Basel series, all patients*	CSS	NA	Log rank *P*, Univariable and multivariable adjusted HR	Table S15, Figure S3
POLE proofreading-mutant vs. wild-type	PORTEC, Leuven & TCGA series	RFS	NA	Weighted HR from pooled multivariable analyses	Main text, Figure 3A
POLE proofreading-mutant vs. wild-type	PORTEC, Leuven, Zurich/ Basel series	CSS	NA	Weighted HR from pooled multivariable analyses	Main text, Figure 3B

*Analyses include both tumors of all histologies and endometrioid endometrial cancers (EECs) only.

Supplementary Table 3. Cox regression model for recurrence-free survival in PORTEC-1 and -2 demonstrating effect of addition of *POLE* proofreading mutation to model of standard prognostic variables.

Variable	HR (95% CI)	*P**
Base model		
NEEC vs. EEC	1.82 (0.76–4.33)	0.18
Tumor grade		
Grade 2 vs. grade 1	2.30 (1.42–3.71)	0.001
Grade 3 vs. grade 1	3.41 (2.11–5.52)	<0.0001
Myometrial invasion (>50% vs. <50%)	1.48 (0.92–2.37)	0.11
LVSI (present vs. absent)	2.28 (1.40–3.69)	0.001
Age (continuous)	1.04 (1.01–1.06)	0.004
Treatment		
EBRT vs. NAT	0.61 (0.39–0.95)	0.03
VBT vs. NAT	0.84 (0.50–1.40)	0.50
Base model with addition of *POLE* mutation		
POLE mutation†	0.46 (0.14–1.47)	0.19

**P*-value calculated by two-sided Cox proportional hazards test. †*POLE* proofreading exonuclease domain mutations detected from sequencing exons 9 and 13 only (contain ~80% pathogenic variants)
EEC=endometrioid endometrial cancer, NEEC=non-endometrioid endometrial cancer. LVSI=lymphovascular space invasion, EBRT=external beam radiotherapy, VBT=vaginal brachytherapy, NAT= no additional treatment.

Supplementary Table 4. Cox regression model for cancer-specific survival in PORTEC-1 and -2 demonstrating effect of addition of *POLE* proofreading mutation to model of standard prognostic variables.

Variable	HR (95% CI)	*P**
Base model		
NEEC vs. EEC	1.70 (0.65–4.44)	0.28
Tumor grade		
Grade 2 vs. grade 1	2.43 (1.34–4.40)	0.003
Grade 3 vs. grade 1	5.43 (3.11–9.49)	<0.001
Myometrial invasion (>50% vs. <50%)	1.80 (1.01–3.19)	0.045
LVSI (present vs. absent)	1.93 (1.08–3.45)	0.03
Age (continuous)	1.04 (1.01–1.07)	0.02
Treatment		0.90
EBRT vs. NAT	1.14 (0.65–1.20)	0.65
VBT vs. NAT	1.14 (0.59–2.28)	0.70
Base model with addition of *POLE* mutation		
POLE mutation†	0.2 (0.03–1.46)	0.11

**P*-value calculated by two-sided Cox proportional hazards test. †*POLE* proofreading exonuclease domain mutations detected from sequencing exons 9 and 13 only (contain ~80% pathogenic variants)
EEC=endometrioid endometrial cancer, NEEC=non-endometrioid endometrial cancer. LVSI= lymphovascular space invasion, EBRT= external beam radiotherapy, VBT =vaginal brachytherapy, NAT= no additional treatment.

Supplementary Table 5. Final multivariable Cox models for recurrence-free survival including *POLE* proofreading mutation using all PORTEC cases and limited to endometrioid ECs only.

Variable	HR (95% CI)	*P**
All tumors (n=788)		
Tumor grade		
Grade 2 vs. grade 1	2.52 (1.55–4.08)	<0.001
Grade 3 vs. grade 1	3.47 (2.20–5.47)	<0.001
LVSI (present vs. absent)	2.16 (1.32–3.54)	0.002
Age (continuous)	1.04 (1.01–1.06)	0.003
POLE mutation	0.43 (0.13–1.37)	0.15
EECs only (n=770)		
POLE mutation†	0.44 (0.14–1.42)	0.17

**P*-value calculated by two-sided Cox proportional hazards test. †*POLE* proofreading exonuclease domain mutations detected from sequencing exons 9 and 13 only (contain ~80% pathogenic variants)
EEC=endometrioid endometrial cancer, LVSI=lymphovascular space invasion.

Supplementary Table 6. Final multivariable Cox models for cancer-specific survival including *POLE* proofreading mutation using all PORTEC cases and limited to endometrioid ECs only.

Variable	HR (95% CI)	*P**
All tumors (n=788)		
Tumor grade		
Grade 2 vs. grade 1	2.69 (1.47–4.91)	<0.001
Grade 3 vs. grade 1	5.03 (2.97–8.51)	<0.001
LVSI (present vs. absent)	2.10 (1.16–3.78)	0.01
Age (continuous)	1.04 (1.01–1.08)	0.006
POLE mutation	0.19 (0.03–1.44)	0.11
EECs only (n=770)		
POLE mutation†	0.21 (0.03–1.50)	0.12

**P*-value calculated by two-sided Cox proportional hazards test. †*POLE* proofreading exonuclease domain mutations detected from sequencing exons 9 and 13 only (contain ~80% pathogenic variants)
EEC=endometrioid endometrial cancer, LVSI=lymphovascular space invasion.

Supplementary Table 7. *POLE* proofreading mutations detected in PORTEC-1/2 and additional series.

Nucleotide change	Amino acid change	PORTEC1&2 n=788 (%)	Leuven n=170 (%)	Zurich/Basel n=229 (%)	TCGA n=229 (%)
c.857C>G	p.Pro286Arg	31 (3.9)	5 (2.9)	5 (2.2)	8 (3.5)
c.890C>T	p.Ser297Phe	2 (0.3)	0	0	1 (0.4)
c.890C>A	p.Ser297Tyr	1 (0.1)			
c.895A>G*	p.Met299Va*	0	1 (0.6)	0	0
c.1231G>C/ c.1231G>T	p.Val411Leu	14 (1.8)	3 (1.8)	1 (0.4)	5 (2.2)
c.1270C>A	p.Leu424Ile	0	0	0	1 (0.4)
c.1270C>G	p.Leu424Val	0	0	0	1 (0.4)
c.1331T>A	p.Met444Lys	0	0	0	1 (0.4)
Other (non-exon 9/13)		NA	NA	NA	1 (0.4)
Total		48 (6.1)	9 (5.3)	6 (2.6)	18 (7.9)

*Variant not previously reported affecting residue absolutely conserved in Pol ε orthologues close to hotspot codon 297 and predicted to be deleterious by mutation assessor (score 2.35) and SIFT (score 0.00). "Other" indicates non-exon 9/13 variants associated with ultramutation in TCGA analysis.

Supplementary Table 8. Multivariable Cox model for recurrence-free survival of PORTEC grade 3 tumor subgroup including *POLE* proofreading mutation and standard prognostic variables.

Variable	HR (95% CI)	*P**
All grade 3 tumors (n=109)		
NEEC vs. EEC	1.96 (0.68–4.81)	0.20
LVSI (present vs. absent)	1.13 (0.25–2.87)	0.82
Myometrial invasion (>50% vs. <50%)	1.86 (0.89–3.95)	0.10
Age (continuous)	1.01 (0.96–1.05)	0.83
POLE mutation	0.11 (0.001–0.84)	0.03
Grade 3 EECs only (n=97)		
POLE mutation†	0.12 (0.001–0.87)	0.03

**P*-value calculated by two-sided Cox proportional hazards test with Firth correction. †*POLE* proofreading exonuclease domain mutations detected from sequencing exons 9 and 13 only (contain ~80% pathogenic variants).
NEEC=non-endometrioid endometrial cancer, EEC=endometrioid endometrial cancer, LVSI=lymphovascular space invasion.

Supplementary Table 9. Multivariable Cox model for cancer-specific survival of PORTEC grade 3 tumor subgroup including *POLE* proofreading mutation and standard prognostic variables.

Variable	HR (95% CI)	*P**
All grade 3 tumors (n=109)		
NEEC vs. EEC	1.84 (0.56–4.86)	0.28
LVSI (present vs. absent)	1.01 (0.27–2.82)	0.98
Myometrial invasion (>50% vs. <50%)	1.65 (0.75–3.66)	0.21
Age (continuous)	1.02 (0.97–1.08)	0.42
POLE mutation	0.14 (0.001–1.01)	0.05
Grade 3 EECs only (n=97)		
POLE mutation†	0.15 (0.001–1.10)	0.07

**P*-value calculated by two-sided Cox proportional hazards test with Firth correction. †*POLE* proofreading exonuclease domain mutations detected from sequencing exons 9 and 13 only (contain ~80% pathogenic variants). NEEC=non-endometrioid endometrial cancer, EEC=endometrioid endometrial cancer, LVSI=lymphovascular space invasion.

Supplementary Table 10. Demographic and clinicopathological characteristics of Leuven series according to *POLE* proofreading mutation.

		POLE wild-type n=161 (94.7%)	*POLE* mutant* n=9 (5.3%)	*P*†
Age, years				
	Median (range)	70 (36–93)	57 (45–81)	0.06
Tumor type				
	EEC	103 (64.0)	5 (55.6)	0.75
	NEEC	58 (36.0)	4 (44.4)	
FIGO stage				
	I	92 (57.1)	6 (66.7)	
	II	18 (11.2)	1 (11.1)	0.50
	III	30 (18.6)	0	
	IV	21 (13.0)	2 (22.2)	
Grade				
	1	41 (25.5)	2 (22.2)	
	2	32 (19.9)	1 (11.1)	0.73
	3	88 (54.7)	6 (66.7)	

**POLE* proofreading exonuclease domain mutations detected from sequencing exons 9 and 13 only contain ~90% pathogenic variants). †*P*-values represent comparison of *POLE* wild-type and *POLE* proofreading mutant groups calculated by unpaired t-test (age), Fisher's exact test (tumor type), or $\chi 2$ test (other). All statistical tests were two-sided. EEC=endometrioid endometrial cancer, NEEC=non-endometrioid endometrial cancer.

Supplementary Table 11. Demographic and clinicopathological characteristics of TCGA series according to *POLE* proofreading mutation.

		POLE wild-type n=211 (92.1%)	*POLE* mutant* n=18 (7.9%)	*P*†
Age, years				
	Median (range)	63 (34-90)	57 (33-87)	0.06
Tumor type				
	EEC	170 (80.6)	18 (100)	0.049
	NEEC	41 (19.4)	0	
FIGO stage				
	I	156 (73.9)	13 (72.2)	
	II	11 (5.2)	1 (5.6)	0.74
	III	34 (16.1)	4 (22.2)	
	IV	10 (4.7)	0	
Grade				
	1	65 (30.8)	6 (33.3)	
	2	69 (32.4)	4 (22.2)	0.61
	3	77 (36.5)	8 (44.5)	

**POLE* proofreading exonuclease domain mutations detected from whole exome sequencing. †*P*-values represent comparison of *POLE* wild-type and *POLE* proofreading mutant groups calculated by unpaired t-test (age), Fisher's exact test (tumor type), or χ2 test (other). All statistical tests were two-sided. EEC=endometrioid endometrial cancer, NEEC=non-endometrioid endometrial cancer.

Supplementary Table 12. Demographic and clinicopathological characteristics of Zurich/ Basel series according to *POLE* proofreading mutation.

		POLE wild-type n=223 (97.4%)	*POLE* mutant* n=6 (2.6%)	*P*†
Age, years				
	Median (range)	66 (33-88)	57 (52-89)	0.42
Tumor type				
	EEC	215 (96.4)	6 (100)	1.0
	NEEC	8 (3.6)	0	
FIGO stage				
	I	143 (64.1)	5 (83.3)	
	II	31 (13.9)	0	0.17
	III	42 (18.8)	0	
	IV	7 (3.1)	1 (16.7)	
Grade				
	1	147 (65.9)	2 (33.3)	
	2	48 (21.5)	0	0.004
	3	28 (12.6)	4 (66.7)	

**POLE* proofreading exonuclease domain mutations detected from sequencing exons 9 and 13 only (contain ~90% pathogenic variants). †*P*-values represent comparison of *POLE* wild-type and *POLE* proofreading mutant groups calculated by unpaired t-test (age), Fisher's exact test (tumor type), or χ2 test (other). EEC=endometrioid endometrial cancer, NEEC=non-endometrioid endometrial cancer. All statistical tests were two-sided.

Supplementary Table 13. Patient outcome according to *POLE* proofreading mutation in Leuven series.

		Univariable analysis		Multivariable analysis*	
	Events/total (%)	HR 95% CI)	*P**	HR (95% CI)	*P**
Recurrence					
POLE wild-type	51/161 (31.6)	0.14 (0.008-2.34)	0.17	0.18 (0.01-3.11)	0.24
POLE mutant	0/9 (0.0)				
Cancer specific survival					
POLE wild-type	30/161 (18.6)	0.24 (0.01–3.10)	0.20	0.66 (0.04-11.39)	0.78
POLE mutant	0/9 (0.0)				
Overall survival					
POLE wild-type	36/161 (22.4)	0.21 (0.01–3.60)	0.28	0.53 (0.03-8.96)	0.66
POLE mutant	0/9 (0.0)				

* Calculated using Cox proportional hazards two-sided test. Cox models use all informative cases irrespective of histology. Corresponding results from multivariable analyses following omission of *POLE*-wild-type NEECs from models are: RFS – HR=0.18, 95%CI 0.01-3.25, *P*=0.25; CSS – HR=1.07, 95%CI 0.52-21.95, *P*=0.97 (multivariable analysis of OS not done for EEC subset).

Supplementary Table 14. Patient outcome according to *POLE* proofreading mutation in the TCGA series.

		Univariable analysis		Multivariable analysis	
	Events/total (%)	HR (95% CI)	*P**	HR (95% CI)	*P**
Recurrence					
POLE wild-type	40/211 (19.0)	0.164 (0.0083-2.35)	0.20	0.12 (0.0070-2.11)	0.15
POLE mutant	0/18 (0.0)				
Cancer specific survival					
POLE wild-type	NR	NR	NR	NR	NR
POLE mutant	NR				
Overall survival					
POLE wild-type	21/211 (9.9)	0.29 (0.016-5.15)	0.43	0.34 (0.018-6.48)	0.48
POLE mutant	0/18 (0.0)				

* Calculated using Cox proportional hazards two-sided test. Cox models use all informative cases irrespective of histology. Corresponding results from multivariable analyses following omission of *POLE*-wild-type NEECs from models are: RFS – HR=0.18, 95%CI 0.01-3.08, *P*=0.24 (multivariable analysis of OS not done for EEC subset). NR – not reported.

Supplementary Table 15. Patient outcome according to *POLE* proofreading mutation in the Zurich/Basel series.

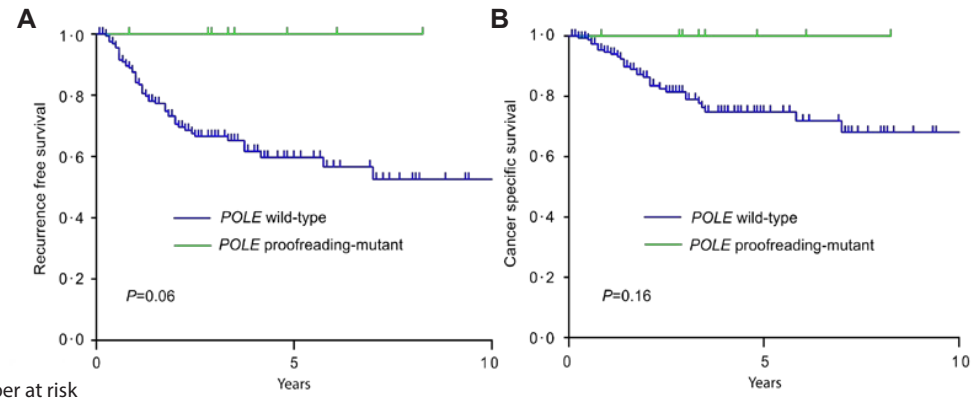
		Univariable analysis		Multivariable analysis	
	Events/total (%)	HR (95% CI)	*P**	HR (95% CI)	*P**
Recurrence					
POLE wild-type	NR	NA	NA	NA	NA
POLE mutant	NR				
Cancer specific survival					
POLE wild-type	24/223 (10.8)	0.73 (0.04-12.70)	0.82	0.21 (0.01-4.26)	0.31
POLE mutant	0/6 (0.0)				
Overall survival					
POLE wild-type	57/223 (25.6)	0.87 (0.17-4.48)	0.86	0.42 (0.08-2.27)	0.31
POLE mutant	0/16 (0.0)				

* Calculated using Cox proportional hazards two-sided test with Firth correction. Cox models use all informative cases irrespective of histology. Corresponding results from multivariable analyses following omission of *POLE*-wild-type NEECs from models are: CSS – HR=0.23, 95%CI 0.01-4.63, P=0.34. NR – not reported. NA – not available.

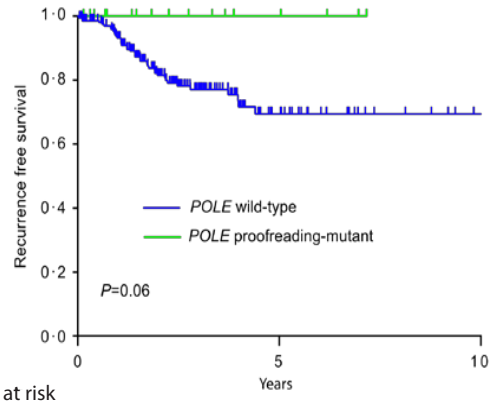
Supplementary Table 16. Comparison of *POLE* proofreading-mutant endometrioid endometrial cancers by grade in PORTEC studies and additional series.

		POLE proofreading mutant* EECs		
		Grade 1/2 n=48 (%)	Grade 3 n=29 (%)	*P*†
FIGO stage				
	I	44 (91.7)	22 (75.9)	0.14
	II	2 (4.1)	2 (6.9)	
	III	1 (2.1)	3 (10.3)	
	IV	1 (2.1)	2 (6.9)	
Depth of invasion				
	<50%	17 (35.4)	15 (51.7)	0.15
	>50%	28 (58.3)	12 (41.4)‡	
	Not known	3 (6.3)	2 (6.9)	
LVSI				
	No	42 (87.4)	18 (62.1)	0.06
	Yes	3 (6.3)	6 (20.7)	
	Not known	3 (6.3)	5 (17.2)	
Recurrence				
	No	45 (93.8)	29 (100)	–
	Yes	3 (6.3)	0	

**POLE* proofreading exonuclease domain mutations detected from sequencing exons 9 and 13 only in PORTEC, Leuven and Zurich/Basel series (contain ~90% pathogenic variants), and whole exome sequencing in TCGA series. †*P*-values represent comparison of informative grade 3 and grade 1/2 ECs by or χ^2 test (stage) and Fisher's exact test (invasion, LVSI). All statistical tests were two-sided. ‡ May underestimate the true frequency of deep myometrial invasion in high-grade *POLE*-mutant tumors due to exclusion of grade 3 ECs with >50% myometrial invasion from PORTEC studies. In *POLE*-mutant ECs from the unselected additional series deep myometrial invasion was more common in grade 3 than in grade 1/2 tumors (75% vs. 25%, *P*=0.04, Fisher's exact test). LVSI=lymphovascular space invasion.

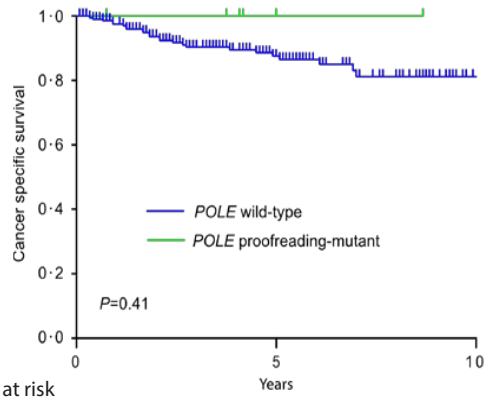


Supplementary Figure 1. Outcome according to *POLE* proofreading mutation in Leuven series. Probability of differential recurrence-free survival (A) and cancer-specific survival (B) according to *POLE* proofreading mutation status are shown. *P*-values are obtained by log-rank test. All statistical tests were two-sided.



Number at risk			
POLE wild-type	221	26	2
POLE-mutant	18	5	0

Supplementary Figure 2. Recurrence-free survival according to *POLE* proofreading mutation in TCGA series. Two-sided *P*-value obtained by log-rank test.



Number at risk			
POLE wild-type	223	81	9
POLE-mutant	6	2	0

Supplementary Figure 3. Cancer-specific survival according to *POLE* proofreading mutation in Zurich/Basel series. Two-sided *P*-value obtained by log-rank test.

Chapter 4

Improved risk assessment by integrating molecular alterations and clinicopathological factors in the PORTEC endometrial cancer trials cohort

Stelloo E, Nout RA, Osse EM, Jürgenliemk-Schulz IJ, Jobsen JJ, Lutgens LC, van der Steen-Banasik EM, Nijman HW, Putter H, Bosse T, Creutzberg CL, Smit VT

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Abstract

Purpose: Recommendations for adjuvant treatment for women with early-stage endometrial carcinoma (EC) are based on clinicopathological features. Comprehensive genomic characterization defined four subgroups: p53-mutant, microsatellite instability (MSI), *POLE*-mutant and no specific molecular profile (NSMP). We aimed to confirm the prognostic capacity of these subgroups in large randomized trial populations, investigate potential other prognostic classifiers, and integrate these into an integrated molecular risk assessment guiding adjuvant therapy.

Experimental design: Analysis of MSI, hotspot mutations in 14 genes including *POLE*, protein expression of p53, ARID1a, β-catenin, L1CAM, PTEN, ER, and PR was undertaken on 947 available early-stage endometrioid ECs from the PORTEC-1 and -2 trials, mostly high-intermediate risk (n=614). Prognostic value was determined using univariable and multivariable Cox proportional hazard models. Areas under the curve of different risk stratification models were compared.

Results: Molecular analyses were feasible in >96% of the patients and confirmed the four molecular subgroups: p53-mutant (9%), MSI (26%), *POLE*-mutant (6%), and NSMP (59%). Integration of prognostic molecular alterations with established clinicopathological factors resulted in a stronger model with improved risk prognostication. Approximately 15% of high-intermediate risk patients had unfavorable features (substantial LVSI, p53-mutant, and/or >10% L1CAM), 50% favorable features (*POLE*-mutant, NSMP being microsatellite stable and *CTNNB1*-wild type), and 35% intermediate features (MSI or *CTNNB1*-mutant).

Conclusions: Integrating clinicopathological and molecular factors improves the risk assessment of patients with early-stage EC. Assessment of this integrated risk profile is feasible in daily practice, and holds promise to reduce both over- and undertreatment.

Introduction

Endometrial cancer (EC) is the most common gynecological cancer in developed countries.[1] Over 50% of women with EC present with early-stage, low-risk disease, and are treated with surgery alone.[2] Adjuvant therapy recommendations are based on the individual patient's risk of disease recurrence using clinicopathological factors such as age, stage, histological subtype, tumor grade, and lymphovascular space invasion (LVSI).[3] EC patients are generally stratified in three risk groups; however, various definitions exist.[4-6] The PORTEC-1 and -2 (PostOperative Radiation Therapy for EC) clinical trials have contributed evidence that adjuvant radiotherapy can be safely omitted in patients with low-intermediate risk features, and that EC patients with high-intermediate risk features can effectively be treated with vaginal brachytherapy.[4,7] Despite this clinicopathological risk stratification considerable over- and undertreatment remains: seven patients with stage I high-intermediate risk EC need to receive vaginal brachytherapy to prevent one recurrence, while 8% of patients develop distant metastases that may have been prevented or delayed with adjuvant chemotherapy. We hypothesized that the clinicopathological risk assessment might be improved by integration of molecular biomarkers predictive of individual tumor behavior.

Many studies addressing the prognostic significance of molecular alterations in EC have focused on one or two biomarkers.[8,9] Integrated genomic characterization by The Cancer Genome Atlas (TCGA) defined four distinct EC subgroups with possible prognostic value.[10] Using methods broadly available in clinical practice these four subgroups can be easily determined by their surrogate markers: p53, microsatellite instability (MSI), and *POLE* resulting in a practically and clinically useful molecular classification tool.[11,12] In relatively small series of unselected ECs, the combination of both the clinicopathological and molecular classification improved the clinicopathological risk assessment.[12] At present it is unclear how other potential molecular prognosticators, such as mutations in *CTNNB1*, *PIK3CA* and L1CAM overexpression should be integrated in the suggested TCGA subgroups.

The aims of this study were to confirm and validate the prognostic significance of the proposed molecular classification tool in early-stage endometrioid ECs (EECs), mainly high-intermediate risk, from two large randomized trials (PORTEC-1 and -2) with mature long-term follow-up data and to investigate whether incorporation of other molecular alterations and established clinicopathological risk factors will result in an improved risk assessment.

Methods

Patients and study design

For both PORTEC-1 and -2 trials central pathology review was undertaken, during which formalin-fixed paraffin-embedded (FFPE) tumor material was collected. All tumor samples with confirmed endometrioid histology were included in the current analysis. The design and clinical results of both randomized trials have been published previously.[4,7] In brief, PORTEC-1 (1990-1997) included 714 patients with stage I EC, grade 1 or 2 with deep myometrial invasion, or grade 2 or 3 with superficial invasion. PORTEC-2 (2000-2006) included 427 EC patients with high-intermediate risk features: stage I, age >60 years, grade 1-2 with deep invasion or grade 3 with superficial invasion and stage IIA disease (except grade 3 with deep invasion). The PORTEC study protocols were approved by the Dutch Cancer Society and the medical ethics committees at participating centers. All patients provided informed consent. Data on patient and tumor characteristics, including results of pathology review and outcome, were obtained from the trial databases. The presence of substantial LVSI, diffuse or multifocal LVSI around the tumor, was evaluated and previously reported.[13] The REMARK criteria were followed, wherever possible, throughout this study.[14]

Procedures

For immunohistochemical analyses, all slides were evaluated by two investigators and a gyneco-pathologist, blinded for patient characteristics and outcome. Evaluations were done independently with discrepancies resolved at simultaneous viewing. For DNA analyses, tumor DNA was isolated as previously reported.[11]

p53 expression, MSI, and *POLE* exonuclease domain mutation status were assessed, as described previously, to identify the four molecular EC subgroups.[11] In short, immunohistochemical expression of p53 (clone DO-7, 1:2000; Neomarkers) was scored positive if >50% of the tumor cells showed a strong positive nuclear staining, or when discrete geographical patterns showed >50% tumor cell positivity. Tumors in which no p53 staining of the tumor was observed and cases with only DNA present (n=119) were sequenced for exon 5-8 *TP53* mutations.[15] The MSI status was determined using the Promega MSI analysis system (version 1.2). Tumors with instability in at least two markers were defined as being MSI whereas those showing no instability were classified as being stable (MSS). Tumors in which instability at one repeat was observed or MSI status could not be determined due to poor DNA quality (n=121) were stained manually for the mismatch repair proteins MLH1 (clone ES05, 1:100; DAKO), MSH2 (clone FE11, 1:200, DAKO), MSH6 (clone EPR3945, 1:800, Genetex), and PMS2 (clone EP51, 1:75, DAKO).[11] Both methodologies, MSI assay and mismatch repair protein expression, are highly sensitive methods for the identification of a defective DNA mismatch repair system.[16] Tumors were then considered MSI if tumor cells showed loss of nuclear staining of at least one of the mismatch repair proteins, and MSS if tumor cells showed nuclear positivity for

all mismatch repair proteins. *POLE* exonuclease domain hotspot mutations (named *POLE* mutations throughout this paper) were detected by Sanger sequencing of exon 9 and 13. KASPar competitive allele specific PCR (LGC Genomics) assays were used to screen for *POLE* variants at codons 286, 297 and 411 in tumors with poor DNA quality (n=98, primer sequences are available upon request). Part of these results were previously published.[17]

To assess mutations in other frequently altered genes in EC, we used the Sequenom MassARRAY system and the GynCarta multigene analysis 2.0 (Sequenom) to test for 159 hotspot mutations in *BRAF*, *CDKNA2*, *CTNNB1*, *FBXW7*, *FGFR2*, *FGFR3*, *FOXL2*, *HRAS*, *KRAS*, *NRAS*, *PIK3CA*, *PPP2R1A*, *PTEN* as described previously.[15] Further immunohistochemical analyses were performed for estrogen receptor (ER), progesterone receptor (PR; clone PGR636, 1:200; DAKO), PTEN (clone 6H2.1, 1:200; DAKO), β-catenin (clone 14, 1:1600; BD transduction), and ARID1a (clone PSG-3, 1:800; Santa Cruz) expression. Immunohistochemical procedures were as described previously except for ER expression analysis (clone EP1, DAKO, 1:100, Tris-EDTA pH 9.0, 3,3'-diaminobenzidine+).[11,15] ER and PR were scored positive when at least >10% of tumor cells showed nuclear expression. PTEN, β-catenin, and ARID1a staining were evaluated as described previously.[11,15] In short, PTEN staining was evaluated in three categories as negative, positive and heterogeneous. Activated Wnt-signaling was defined as nuclear staining of ß-catenin. ARID1a was scored as negative, weak positive or strong positive nuclear staining or as 'clonal loss'. Previously published results of immunohistochemical L1CAM expression (clone 14.10, 1:500; Covance Inc.) on the same patients in this study were integrated for analysis.[18] Tumors with >10% positive tumor cells were considered L1CAM positive.

Statistical analysis

Associations between clinicopathological features and molecular alterations were tested using Chi-square statistics or Fishers exact test in case of categorical and t test or analysis of variance (ANOVA) for continuous variables. Time-to-event analyses were calculated from the date of randomization to date of recurrence (vaginal and/or pelvic for locoregional recurrence, and distant metastases for distant recurrence) or to date of endometrial cancer death (disease specific survival) or to date of death (overall survival) or to date of any recurrence or death (recurrence-free survival); patients who were alive and without recurrence were censored at the date of last follow-up. Survival curves were calculated using the Kaplan–Meier method with log-rank test. Cox proportional hazards models were used to evaluate the prognostic value of each factor. Factors with *P*-values <0.10 were included in a multivariable Cox model with a stepwise method to include in the final model. In the last step, significant factors from the forward selection model (*P*-values <0.05) were included in a final Cox model together with established clinicopathological prognostic factors: age as continuous variable, grade (1-2 vs. 3), LVSI (substantial vs. none or mild), and adjuvant treatment (vaginal brachytherapy, external beam radiotherapy or no additional therapy). Discrimination between the risk

stratification models was quantified using the area under the receiver operating curve with 95% confidence intervals (CIs). All reported p-values were based on two–sided tests with *P*-values <0.05 considered statistically significant (IBM SPSS 20.0).

Results

In total, 947 (83% of randomized patients) EECs from PORTEC-1 and -2 were available (Figure 1). Analysis of classifying alterations (p53, MSI, *POLE*) was successful in 809/836 (97%) cases for which sufficient material was available. For 111 PORTEC-cases only FFPE slides were available for DNA isolation, which provided 52 (47%) additional successfully analyzed cases. Patient, tumor and treatment characteristics did not differ between included, excluded and failed cases (Supplementary Table S1). The median follow-up was 131 months (range 0.2-219.2 months).

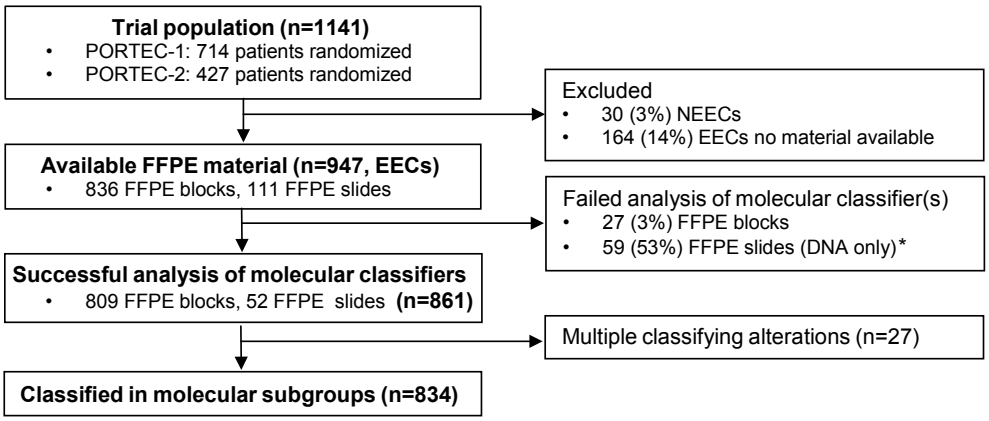

Figure 1. Flow chart of sample analyses. *The majority of cases with incomplete analysis were PORTEC-1 cases from which only FFPE tumor slides were available EEC= endometrioid endometrial cancer; NEEC=non-endometrioid endometrial cancer.

The four molecular subgroups displayed marked differences in clinicopathological characteristics, alterations in potential other classifiers, and clinical outcome (Table 1, Figure 2, Supplementary Table S2). In total, 834 EECs could be classified in one of the four subgroups: 74 (9%) p53-mutant, 219 (26%) MSI, 49 (6%) *POLE*-mutant, and 492 (59%) NSMP. Twenty-seven (3%) tumors were found to have more than one classifying alteration (p53, MSI or *POLE*). p53-mutant tumors were significantly associated with grade 3, loss of hormone receptors, >10% L1CAM expression, *PPP2R1a*, and *FBXW7* mutations. MSI tumors presented more frequently with substantial LVSI, and abnormal ARID1a expression. *POLE* mutations occurred more frequently in younger women, grade 3, and often co-occurred with *PTEN* mutations. In contrast, the NSMP tumors were more frequently grade 1, and *CTNNB1* mutant. The prognosis was unfavorable in the p53-mutant group, intermediate in the MSI and NSMP group, and the *POLE*-mutant group had a favorable prognosis with no local and only

two distant recurrences (Figure 2). In addition, women with a *POLE*- and p53-mutant tumor developed no recurrences (0/7), whereas some of the women with MSI tumors with *POLE* (2/6) or p53 mutation (2/13), or both (1/1) developed recurrences (Supplementary Table S3). Within the four subgroups, distant recurrence and endometrial cancer-related death rates were similar.

Table 1. Clinicopathological characteristics and alterations in potential other molecular classifiers according to the four molecular subgroups in early-stage endometrial cancer (n=834).

	Total n=834 (100%)	p53-mutant n=74 (8.9%)	MSI n=219 (26.3%)	*POLE*-mutant n=49 (5.9%)	NSMP n=492 (59.0%)	*P*-value
Age, years						
Mean (range)	68 (41-90)	69 (51-86)	69 (43-89)	62 (46-81)	68 (41-90)	0.000
< 60	138 (16.5)	7 (9.5)	35 (16.0)	19 (38.8)	77 (15.7)	
60-70	360 (43.2)	32 (43.2)	89 (40.6)	18 (36.7)	221 (44.9)	0.001
> 70	336 (40.3)	35 (47.3)	95 (42.4)	12 (24.5)	194 (39.4)	
Grade						
1-2	724 (86.8)	48 (64.9)	135 (83.6)	33 (73.4)	457 (92.9)	0.000
3	110 (13.2)	26 (35.1)	36 (16.4)	13 (26.6)	35 (7.1)	
Myometrial invasion						
<50%	251 (30.1)	35 (47.3)	71 (32.4)	25 (51.0)	120 (24.4)	0.000
>50%	583 (69.9)	39 (52.7)	148 (67.6)	24 (49.0)	372 (75.6)	
LVSI*						
Absent/Focal	784 (95.5)	70 (94.6)	194 (91.1)	47 (100)	473 (97.1)	0.002
Substantial	37 (4.5)	4 (5.4)	19 (8.9)	0	14 (2.9)	
Risk group						
Low	242 (29.0)	22 (29.7)	62 (28.3)	24 (49.0)	134 (27.2)	
High-intermediate	546 (65.5)	44 (59.5)	143 (65.3)	23 (46.9)	336 (68.3)	0.013
High	46 (5.5)	8 (10.8)	14 (6.4)	2 (4.1)	22 (4.5)	
Treatment						
NAT	241 (28.9)	17 (23.0)	63 (28.8)	16 (32.7)	145 (29.5)	
EBRT	409 (49.0)	38 (51.3)	113 (51.6)	25 (51.0)	233 (47.4)	0.688
VBT	184 (22.1)	19 (25.7)	43 (19.6)	8 (16.3)	114 (23.1)	
Mutations**						
CDKN2A	2 (0.2)	0	0	0	2 (0.4)	0.707
CTNNB1	157 (19.5)	5 (7.0)	19 (9.0)	8 (17.0)	125 (26.3)	0.000
FBXW7	40 (5.0)	8 (11.3)	13 (6.1)	1 (2.1)	18 (3.8)	0.032
FGFR2	80 (9.9)	2 (2.8)	20 (9.4)	0	58 (12.2)	0.007
KRAS	139 (17.3)	7 (9.9)	43 (20.3)	3 (6.4)	86 (18.1)	0.042
NRAS	25 (3.1)	1 (1.4)	8 (3.8)	0	16 (3.4)	0.456
PIK3CA	261 (32.4)	17 (23.9)	70 (33.0)	24 (51.1)	150 (31.6)	0.019
PPP2R1a	39 (4.8)	12 (16.9)	6 (2.8)	1 (2.1)	20 (4.2)	0.000
PTEN	349 (43.4)	15 (21.1)	106 (50.0)	34 (72.3)	194 (40.8)	0.000
Altered protein expression***						
>10% L1CAM	44 (5.6)	27 (39.7)	5 (2.4)	1 (2.6)	16 (3.4)	0.000
<10% ER	38 (5.0)	16 (24.2)	5 (2.5)	4 (10.5)	13 (2.8)	0.000
<10% PR	81 (10.6)	25 (39.1)	19 (9.4)	9 (23.7)	28 (6.1)	0.000
loss/clonal ARID1a	329 (45.4)	17 (27)	123 (63.7)	13 (35.1)	176 (40.8)	0.000
loss/hetero. PTEN	395 (51.5)	28 (43.1)	130 (64.4)	19 (48.7)	218 (47.3)	0.000
nuclear β-catenin	184 (23.6)	7 (10.6)	34 (16.3)	3 (7.7)	140 (30.1)	0.000

* Degree of LVSI unknown for 13 (1.6%) cases. ** Mutation analysis failed for 29 (3.5%) cases. ***Immunohistochemical analysis failed, or no available FFPE slides for 111 (13%) ARID1a, 56 (6.7%) β-catenin, 73 (9%) ER, 68 (8%) PTEN, 73 (9%) PR. LVSI=lymphovascular space invasion, NAT=no additional treatment, EBRT=external beam radiotherapy, VBT=vaginal brachytherapy, hetero.= heterogeneous.

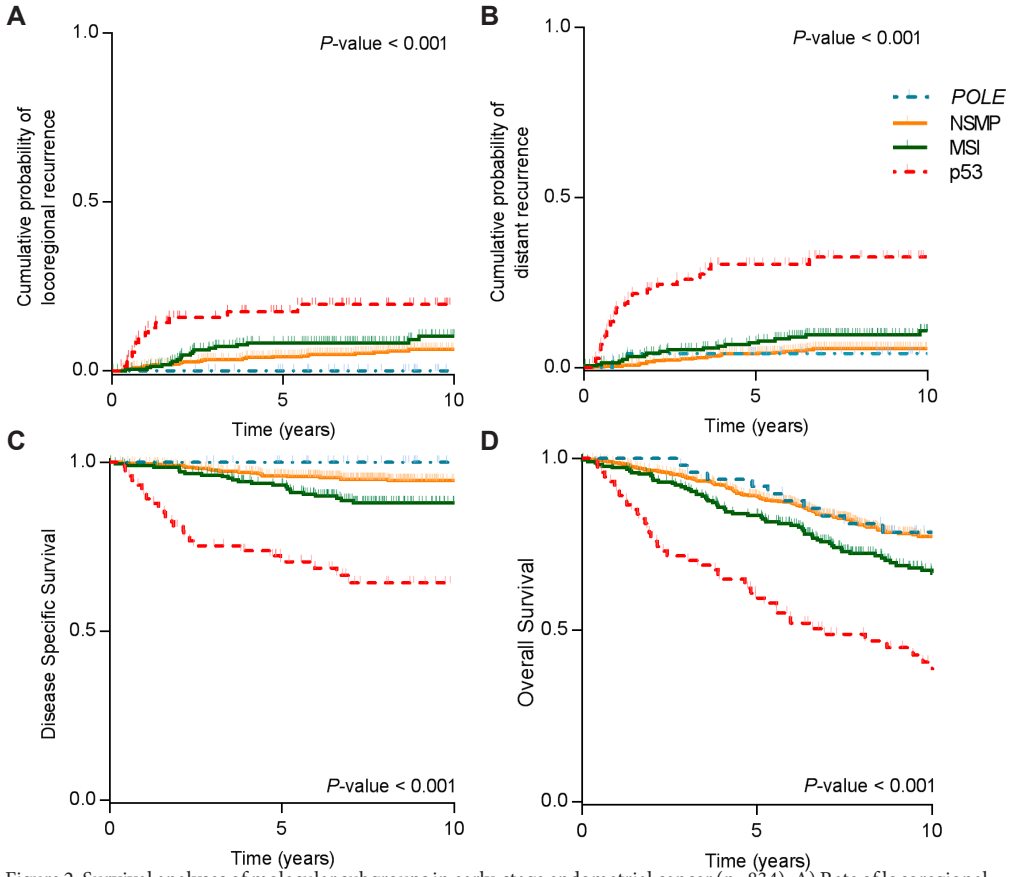

Figure 2. Survival analyses of molecular subgroups in early-stage endometrial cancer (n=834). A) Rate of locoregional recurrences, B) rate of distant recurrences, C) disease specific survival, and D) overall survival.

The prognostic value of the molecular subgroups and additional molecular alterations was evaluated in univariable analysis and multivariable analysis with the clinicopathological factors (age, grade, depth of myometrial invasion, LVSI) and treatment, both in the whole population (Supplementary Table S4) and in an analysis restricted to cases with high-intermediate risk features (Table 2-univariable analysis, Table 3-multivariable analysis). In both analyses, p53-mutant and substantial LVSI were the strongest prognostic factors for locoregional-, distant recurrence, and overall survival, while >10% L1CAM expression was prognostic for distant recurrence and overall survival. After excluding cases with favorable (*POLE*-mutant) and unfavorable factors (substantial LVSI, p53-mutant and >10% L1CAM), a final analysis found MSI prognostic for distant recurrence and overall survival, and *CTNNB1* exon 3 mutation status prognostic for distant recurrence (Table 3, Supplementary Table S4). Univariable prognostic factors, *FGFR2* mutation and loss of hormone receptor expression, lost its significance in multivariable analysis in the presence of other (un)favorable prognostic factors. Univariable analysis in 242 ECs with low-risk features showed a higher rate of locoregional and distant recurrences and lower overall survival in the eight patients with >10% L1CAM, and a trend for p53-mutant patients (Supplementary Table S5).

Table 2. Univariable analysis of clinicopathological characteristics, molecular subgroups, and potential other molecular classifiers in high-intermediate risk early-stage endometrial cancer (n=546).

		Locoregional Recurrence 42 events			Distant Recurrence 50 events			Overall Survival 182 events		
	Total n	HR	95% CI	*P*-value	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Age (cont.)	546	1.035	0.988-1.085	0.145	1.015	0.972-1.060	0.508	1.085	1.062-1.110	0.000
Grade										
1-2	492	1			1			1		
3	54	1.784	0.751-4.239	0.190	3.038	1.552-5.945	0.001	1.741	1.149-2.639	0.009
Myometrial invasion										
<50	62	1			1			1		
>50	484	0.539	0.239-1.216	0.137	0.304	0.161-0.574	0.000	0.586	0.392-0.877	0.009
LVSI										
Absent/mild	507	1			1			1		
Substantial	28	3.733	1.567-8.891	0.003	4.895	2.368-10.121	0.000	2.791	1.668-4.432	0.000
Given treatment										
NAT	113	1			1			1		
EBRT	276	0.311	0.150-0.642	0.002	0.873	0.425-1.796	0.713	0.833	0.595-1.167	0.289
VBT	157	0.546	0.257-1.157	0.114	1.108	0.511-2.401	0.796	0.745	0.480-1.157	0.190
Molecular subgroup										
NSMP	336	1			1			1		
p53	44	6.787	3.069-15.012	0.000	11.083	5.629-21.821	0.000	4.861	3.098-7.073	0.000
MSI	143	2.476	1.182-4.776	0.015	2.220	1.180-4.447	0.025	1.853	1.329-2.584	0.000
POLE	23	-	-	0.970	0.869	0.116-6.532	0.891	0.907	0.367-2.237	0.832
CTNNB1										
No mutation	433	1			1			1		
Mutation	101	0.575	0.225-1.467	0.247	0.934	0.453-1.929	0.854	0.669	0.438-1.023	0.063
FBXW7										
No mutation	512	1			1			1		
Mutation	22	0.666	0.091-4.848	0.688	0.530	0.073-3.847	0.531	1.569	0.827-2.977	0.168
FGFR2										
No mutation	468	1			1			1		
Mutation	66	0.746	0.256-2.095	0.578	0.296	0.072-1.219	0.092	0.556	0.316-0.979	0.042
KRAS										
No mutation	453	1			1			1		
Mutation	81	0.998	0.419-2.379	0.997	1.322	0.639-2.734	0.452	1.035	0.686-1.561	0.871
NRAS										
No mutation	519	1			1			1		
Mutation	15	-	-	0.430	-	-	0.398	0.635	0.231-1.690	0.354
PIK3CA										
No mutation	358	1			1			1		
Mutation	176	0.572	0.272-1.201	0.140	0.814	0.436-1.516	0.516	0.921	0.668-1.271	0.618
PPP2R1A										
No mutation	504	1			1			1		
Mutation	30	1.599	0.492-5.203	0.435	2.128	0.842-5.375	0.110	1.640	0.932-2.888	0.086
PTEN										
No mutation	305	1			1			1		
Mutation	229	0.908	0.484-1.702	0.763	0.517	0.277-0.965	0.038	0.797	0.588-1.080	0.144
L1CAM										
<10%	496	1			1			1		
>10%	30	3.283	1.283-8.404	0.013	7.718	3.993-14.917	0.000	3.763	2.379-5.953	0.000
ER										
>10%	499	1			1			1		
<10%	21	3.547	1.259-9.993	0.017	6.194	2.882-13.310	0.000	2.139	1.183-3.865	0.012
PR										
>10%	465	1			1			1		
<10%	51	2.828	1.297-6.165	0.009	5.684	3.042-10.622	0.000	2.096	1.379-3.188	0.001
ARID1a										
Positive	249	1			1			1		
Loss/clonal	228	0.792	0.423-1.483	0.467	0.827	0.455-1.503	0.533	0.878	0.643-1.200	0.415
PTEN										
Positive	232	1			1			1		
Loss/hetero.	283	0.979	0.529-1.812	0.946	0.988	0.553-1.765	0.967	1.043	0.769-1.414	0.788
β-catenin										
Membrane	399	1			1			1		
Nuclear	126	0.758	0.350-1.643	0.483	0.846	0.420-1.704	0.640	0.704	0.471-1.051	0.086

Cont.=continuous, hetero.=heterogeneous.

Table 3. Multivariable analysis on the prognostic role of the clinicopathological characteristics, molecular subgroups, and potential other molecular classifiers in high- intermediate risk (HIR) endometrial cancers (EC) (n=546) and in a subset of HIR EC without substantial LVSI, >10% L1CAM, p53 and *POLE* mutation (n=443).

All cases of HIR EC (n=546)

	Locoregional Recurrence 41 events			Distant Recurrence 46 events			Overall Survival 170 events		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Age (Cont.)	1.032	0.984-1.081	0.197	1.016	0.972-1.062	0.469	1.076	1.051-1.101	0.000
Grade									
1-2	1			1			1		
3	0.203	0.021-1.946	0.167	0.162	0.029-0.904	0.038	0.262	0.057-1.204	0.085
Myometrial invasion									
<50%	1			1			1		
>50%	0.201	0.024-1.678	0.138	0.126	0.026-0.624	0.011	0.254	0.058-1.101	0.067
LVSI									
Absent/mild	1			1			1		
Substantial	3.190	1.301-7.821	0.011	4.303	1.833-10.09	0.001	2.637	1.542-4.509	0.000
Treatment									
NAT	1			1			1		
EBRT	0.277	0.133-0.574	0.001	1.154	0.498-2.677	0.738	0.897	0.623-1.292	0.559
VBT	0.466	0.212-1.027	0.058	1.134	0.465-2.769	0.782	0.707	0.445-1.123	0.142
Molecular subgroup									
NSMP	1			1			1		
p53	7.340	3.168-17.00	0.000	5.766	2.400-13.85	0.000	3.777	2.364-6.037	0.000
MSI	2.319	1.105-4.866	0.026	2.154	1.022-4.540	0.044	1.879	1.307-2.700	0.001
POLE	-	-	0.973	0.883	0.113-6.890	0.906	1.105	0.394-3.101	0.850
L1CAM									
<10%				1			1		
>10%				4.303	1.833-10.09	0.001	2.462	1.453-4.170	0.001

HIR EC without substantial LVSI, >10% L1CAM, p53 and *POLE* mutation (n=443)

	Locoregional Recurrence 27 events			Distant Recurrence 23 events			Overall Survival 127 events		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Age (Cont.)	1.063	1.000-1.130	0.052	1.007	0.944-1.074	0.837	1.102	1.070-1.134	0.000
Grade									
1-2	1			1			1		
3	0.060	0.004-0.842	0.037	0.350	0.033-3.765	0.387	0.409	0.050-3.381	0.407
Myometrial invasion									
<50%	1			1			1		
>50%	0.076	0.009-0.668	0.020	0.099	0.011-0.859	0.036	0.277	0.037-2.070	0.211
Treatment									
NAT	1			1			1		
EBRT	0.249	0.106-0.585	0.001	0.862	0.329-2.262	0.764	0.806	0.541-1.201	0.289
VBT	0.181	0.054-0.605	0.005	0.511	0.139-1.877	0.312	0.559	0.312-1.003	0.051
Molecular subgroup									
NSMP	1			1			1		
MSI	1.816	0.815-4.048	0.145	2.520	1.049-6.051	0.039	1.672	1.146-2.438	0.008
CTNNB1									
No mutation				1					
Mutation				2.959	1.234-7.098	0.015			

Cont.=continuous.

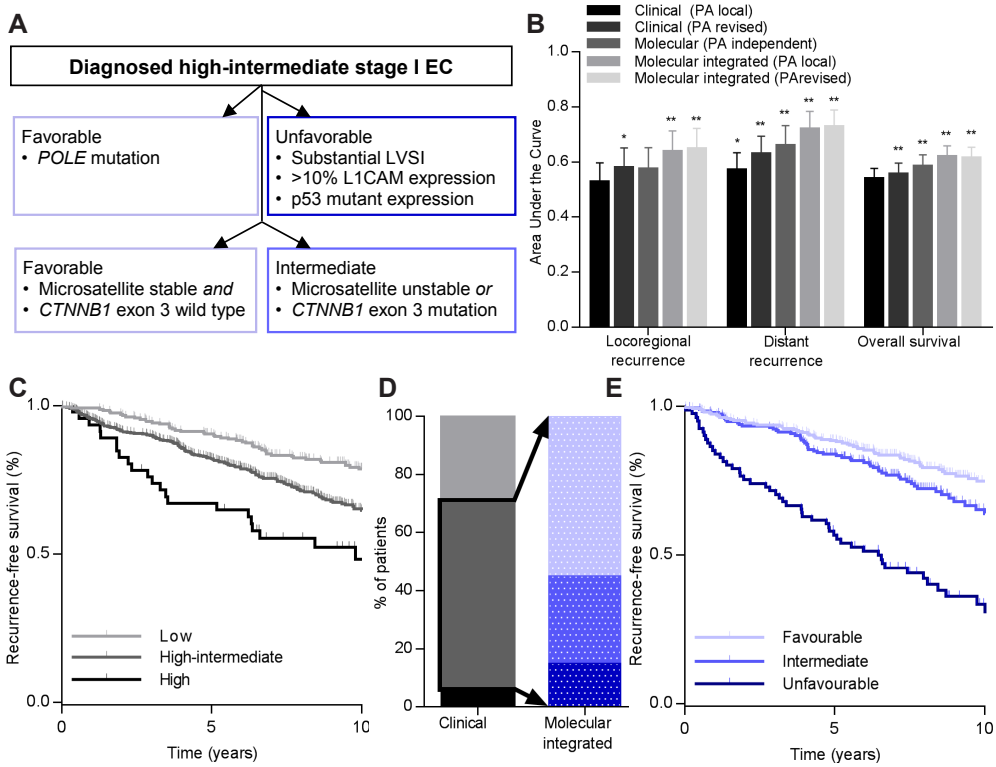

Figure 3. Molecular integrated risk assessment. A) Flow chart of the molecular integrated risk model. B) Area under the curve for the clinical- and molecular-, and molecular integrated risk assessment, with and without central pathology review (*P-value<0.05, **P-value<0.01). C) Recurrence-free survival of clinical risk assessment in early-stage endometrial cancer (n=834, P-value<0.001). D) Bar chart of the proportion of clinically low-, high-intermediate-, and high-risk patients based on central pathology review (left) and the proportion of clinically high-intermediate risk patients reclassified into favorable, intermediate and unfavorable molecular integrated risk groups. E) Recurrence-free survival of molecular integrated risk assessment in early-stage high-intermediate risk endometrial cancer (n=546, P-value<0.001).

Based on the outcomes of multivariable analysis a molecular integrated risk assessment was defined that combines clinicopathological and molecular risk factors (Figure 3A). Substantial LVSI, p53-mutant and >10% L1CAM tumors were designated unfavorable, while in the remaining cases both MSI and *CTNNB1* mutant were distinguished from the favorable group of *POLE*-mutant tumors and NSMP tumors being MSS and *CTNNB1* wild type (Figure 3A). Since PORTEC-1 included patients that are currently considered low risk, and central pathology review in both trials identified additional low- and high-risk cases, the area under the curve (AUC) was estimated for the molecular integrated risk assessment taking these different starting points into account (Figure 3B-C). Compared to the original pathology reports, central pathology review or molecular classification improved the AUC. However, AUCs of the integrated molecular risk assessment showed a substantial improvement, without additional improvement when using findings of central pathology review. Approximately 15% of the high-intermediate risk patients had unfavorable features, and 50% had favorable

features leaving 35% intermediate (Figure 3D-E). In tumors with unfavorable features, targetable alterations were found: 65% *PI3K/AKT* alterations, 9% *FBXW7* mutations, 7% *FGFR2* mutations, 28% L1CAM positivity 78% ER positivity, and 61% PR positivity.

Discussion

In 834 early-stage EECs from two randomized trials (PORTEC-1 and -2) with mature long-term follow-up, the prognostic impact of the four molecular subgroups, originally proposed by the TCGA, was confirmed.[10] Clinically applicable molecular analysis methods for surrogate markers were used and proved feasible in >96% of EEC patients. Integration of prognostic molecular alterations with established clinicopathological factors results in a stronger risk assessment. As a consequence, within the high- intermediate risk population, who are currently thought to be relatively homogenous with regard to clinical outcomes, approximately 15% patients with a marked unfavorable and 50% with a favorable prognosis could be identified.

L1CAM, p53, and LVSI were consistent independent prognostic factors for distant recurrence, overall and disease specific survival. p53-mutant tumors exhibit a high degree of genomic instability linked to tumor progression, and invasion by upregulation of p53-mutant target genes, and *TP53* mutation is well known for its prognostic impact in EC.[8,10] LVSI, especially when quantified as substantial, and L1CAM have similar strong negative prognostic value. LVSI strongly increases the risk of tumor spread via lymphatics and capillaries. L1CAM is known to enhance motility and migration of tumor cells. Both were recently published in this same population as single risk factors[13,18] and by Zeimet *et al.*[19], but were now confirmed to be independent prognostic factors in an integrated analysis. In contrast, patients with *POLE*-mutant or MSS and *CTNNB1* wild type tumors displayed a more favorable prognosis. The favorable outcome of *POLE*-mutant ECs with their striking mutation burden may be explained by an increased immunogenicity, and became evident in EC recently.[20,21] *CTNNB1* mutations result in activation of Wnt signalling contributing to tumor progression, abnormal expression of cell proliferation, and progression genes. Similarly to our results, a previous report showed that ECs carrying a *CTNNB1* mutation characterize a more aggressive subset within low-grade early-stage EEC.[10,22] The prognostic importance of MSI has been controversial, although the strongest association with poor clinical outcome has been observed in early-stage EC similar to our observation.[23] This report integrates a large number of single prognostic factors in the context of clinical trial material resulting in a comprehensive overview.

In this large cohort, only few (3%) tumors had multiple classifying alterations (e.g. *POLE* and MSI). Classification of this small subset would require further analyses, such as mutational load and copy-number status. Supek *et al.* reported that colorectal and stomach tumors

with both MSI and *POLE* mutation had an overall mutational load similar to MSI tumors, whereas two out of three MSI/*POLE* endometrial tumors had a much higher mutational load and different mutational signature.[24] Furthermore, Shinbrot *et al.* showed that the *TP53* gene is frequently affected by *POLE* mutation induced strand-specific mutations.[25] These data support that mutational load, mutation signature, and pattern may be useful for molecular classification of rare tumors that present with combinations of MSI, *POLE*, or *TP53* mutations. With the advent of next generation sequencing technologies, these can be easily analyzed.

Several molecular alterations, such as hormone receptor expression, *CTNNB1* and *FGFR2* mutations, have been previously reported as having prognostic potential in single biomarker studies.[8,9,13,19,22,26,27] Some univariable factors, *FGFR2* mutation and hormone receptor status, lost significance in multivariable analysis. This may be due to the fact that *FGFR2* mutations were equally frequent in MSI and NSMP ECs, and that hormone receptor loss was mainly found in p53-mutant and L1CAM positive ECs but was also frequently observed in *POLE*-mutant ECs.[10,26-28] MSI, p53 and L1CAM proved stronger independent prognosticators in this analysis. *CTNNB1* mutation status was sufficiently strong to emerge in multivariable analysis, stressing its independent prognostic significance. Using this combined approach, an improved risk assessment resulted in which *POLE*, L1CAM, MSI and *CTNNB1* are integrated with histopathological factors.

Previous studies have shown improved risk stratification obtained by central pathology review.[29,30] The reviewed pathology in our analyses had the advantage to exclude prototypical non-endometrioid cancers. With regard to grading, lack of prognostic relevance of grade 2 was shown, advocating the use of a two-tiered grading system, as was also proposed by others.[31-33] The increased AUC of the model based on central pathology review as compared to the original inclusion pathology confirms these findings. The molecular integrated risk model showed an even higher increase in AUC; however, central pathology review did not add any additional value to the molecular integrated risk model. The molecular integrated risk model has three major advantages. Firstly, it is based on more objective variables, such as mutational status of *POLE*. Secondly, the molecular integrated risk model identifies significantly more patients with favorable features that would otherwise be classified as high-intermediate risk with central pathology review alone. Finally, this approach has also the advantage to facilitate pre-screening for Lynch syndrome.

Despite the strength of a randomized trial population, mature long-term follow-up, large group of early-stage EEC, and straightforward molecular analysis, this study has some limitations. Our focused and practical approach provides analyses that can easily be implemented in prospective studies and clinical practice. Most common hotspot mutations were analyzed but this does not rule out the possibility that other clinical relevant alterations may have

been missed. Although, molecular alterations were highly concordant between curettage and hysterectomy specimen,[15,17,18] intratumor heterogeneity may interfere with prediction of the patient's prognosis and requires further study. LVSI and the classic-histopathology, included in the integrated risk model, cannot be evaluated on preoperative specimen, therefore, it is recommended not to rely on preoperative specimens. No automated immunohisto-chemical protocols were used, while it is likely that robust, standardized automated staining procedures are the preferred method in diagnostic pathology. Molecular alterations in our integrated risk model have been proven in single biomarker studies; however, this integrated risk model needs to be validated or prospectively analyzed. Since the majority of our patient cohort has received adjuvant radiotherapy, the decision to omit adjuvant radiotherapy especially in the favorable subgroup remains to be elucidated in a prospective study. There is also need to further investigate whether certain molecular defined subgroups of EC may be more sensitive to radiotherapy. Nevertheless, we believe our data is unique and informative for patient's outcome, and may guide molecular-based trials and therapies for EC.

The proposed molecular integrated risk model outperforms the current clinicopathologic approach; therefore, the question arises whether this integrated model can be used for new clinical studies and guide treatment decisions. Especially in high-intermediate EC, this risk model may substantially reduce overtreatment of favorable cases, and select unfavorable cases who might need more intensive treatment. The clinical utility for tailoring adjuvant therapy, the feasibility of determining the molecular integrated profile within tight time limits and the cost-effectiveness aspects of this approach (e.g. costs of molecular testing vs. saving costs of adjuvant radiotherapy) will be prospectively established in a planned prospective trial PORTEC-4. Within ~10% of low-risk patients, p53 and L1CAM seem prognostic indicators for high recurrence rate and impaired survival, which is in line with Talhouk *et al.*[12] However, the small number of events in this subgroup limits these findings. Factors that are associated with favorable outcome or predict chemotherapy response in high-risk EC remain to be elucidated in future studies.

In conclusion, integration of molecular risk factors with clinicopathological factors in early-stage EC leads to improved risk stratification with potential clinical utility. This molecular integrated risk prediction holds promise to reduce both over- and undertreatment and should form the basis for future prospective clinical studies.

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Supplementary files

Supplementary Table 1. Clinicopathological characteristics of the PORTEC-1 and -2 trial populations: comparison of cases included in the current analysis and those excluded for lack of material (n=164), non-endometrioid histology (n=30) or failed molecular analysis (n=86).

	PORTEC-1			PORTEC-2		
	Included n=477	Excluded n=237	*P*-value	Included n=384	Excluded n=43	*P*-value
Age, years						
Mean (range)	66 (41-90)	66 (43-88)	0.387	70 (52-89)	70 (46-85)	0.471
< 60	131 (27.5)	69 (29.1)		14 (3.6)	2 (4.7)	
60-70	179 (37.5)	92 (38.8)	0.731	190 (49.5)	18 (41.9)	0.585
> 70	167 (35.0)	76 (32.1)		180 (46.9)	23 (53.4)	
Grade						
1-2	400 (83.9)	201 (84.8)	0.743	345 (89.8)	29 (67.4)	0.000
3	77 (16.1)	36 (15.2)		39 (10.2)	13 (30.2)	0.212**
Myometrial invasion						
<50%	198 (41.5)	96 (40.5)	0.798	61 (15.9)	10 (23.3)	0.379
>50%	279 (58.5)	141 (59.5)		323 (84.1)	33 (76.7)	
LVSI*						
Absent/Focal	452 (95.6)	125 (95.4)	0.945	356 (94.9)	29 (96.7)	0.701
Substantial	21 (4.4)	6 (4.6)		19 (5.1)	1 (3.3)	
Risk group						
Low	216 (45.3)	106 (44.7)		36 (9.3)	2 (4.7)	0.000
High-intermediate	234 (49.1)	112 (47.3)	0.477	327 (85.2)	28 (65.1)	0.339**
High	27 (5.7)	19 (8.0)		21 (5.5)	13 (30.2)	
Treatment						
NAT	246 (51.6)	123 (51.9)		2 (0.5)	1 (2.3)	
EBRT	231 (48.4)	114 (48.1)	0.934	190 (49.5)	19 (44.2)	0.727
VBT	0	0		192 (50.0)	23 (53.5)	

LVSI=lymphovascular space invasion, NAT=no additional treatment, EBRT=external beam radiotherapy, VBT=vaginal brachytherapy. *Degree of LVSI unknown for 13 included cases, and 119 excluded cases. **Endometrioid EC only.

Supplementary Table 2. Hotspot mutation frequency according to the four molecular subgroups in early-stage endometrial cancer (n=834).►

	p53-mutant n=74	MSI n=219	*POLE*-mutant n=49	NSMP n=492	Total n=834
PTEN[1] (%)	15 (20)	105 (48)	34 (69)	195 (40)	349 (43)
p.R130G	5	24	3	75	107/790
p.R130fs*4	6	18	19	45	88/801
p.R233*	3	14	0	22	39/799
p.L318fs*2	2	11	0	17	30/796
p.R130*	2	7	0	11	20/790
p.T321fs*3	0	10	0	7	17/786
p.N323fs*2	0	10	0	7	17/791
p.K267fs*9	0	13	0	1	14/827
p.R173C	0	1	8	5	14/805
p.E7*	0	0	8	2	10/802
p.R130P	0	2	0	8	10/800
p.K267fs*31	0	5	0	4	9/798
p.R130L	0	2	0	6	8/800
p.R173H	0	2	3	3	8/801
p.K6fs*4	0	1	1	2	4/801
p.Q214*	0	1	0	3	4/798
p.R234W	0	2	0	2	4/787
p.248fs*5	0	2	0	2	4/801
p.R355*	0	1	0	3	4/803
p.V290fs*1	0	3	0	0	3/800
p.T321fs*23	0	1	0	1	2/797
p.N323fs*21	0	1	0	1	2/826
PIK3CA[1] (%)	17 (23)	69 (32)	24 (49)	151 (31)	261 (32)
p.R88Q	6	24	13	30	73/789
p.H1047R	4	13	0	34	51/800
p.E545K	3	5	0	27	35/800
p.E542K	0	5	2	15	22/809
p.M1043I	0	1	5	11	17/794
p.Y1021C	1	4	4	4	13/825
p.H1047Y	1	9	0	2	12/807
p.Q546K	1	5	0	5	11/804
p.Q546R	0	2	0	9	11/782
p.E545A	1	2	1	6	10/791
p.T1025A	0	2	4	3	9/785
p.H1047L	0	1	0	7	8/800
p.M1043V	0	1	0	4	5/805
p.E545G	0	1	0	3	4/791
p.Q546L	0	0	0	2	2/782
p.Q546P	0	0	0	2	2/782
p.E545D	0	0	0	1	1/793
p.Q546E	0	1	0	0	1/804
CTNNB1[1] (%)	5 (7)	18 (8)	8 (16)	126 (26)	157 (20)
p.S37T	2	1	1	34	38/804
p.S45F	0	0	1	11	12/801
p.S33F	0	1	0	10	11/796
p.T41I	0	2	1	8	11/796
p.D32N	0	2	1	6	9/801
p.S33Y	0	1	1	7	9/796
p.G34R	1	4	0	4	9/828
p.T41A	1	1	0	7	9/814
p.D32Y	0	0	0	8	8/801
p.G34E	0	3	2	3	8/793
p.S45P	0	1	0	6	7/805
p.S37C	1	0	0	5	6/804
p.S33C	0	0	0	5	5/796

Supplementary Table 2 continued.

		p53-mutant n=74	MSI n=219	*POLE*-mutant n=49	NSMP n=492	Total n=834
	p.D32G	0	0	0	4	4/826
	p.S33P	0	1	0	3	4/814
	p.T41I	0	2	1	8	11/796
	p.D32N	0	2	1	6	9/801
	p.S33Y	0	1	1	7	9/796
	p.G34R	1	4	0	4	9/828
	p.T41A	1	1	0	7	9/814
	p.D32Y	0	0	0	8	8/801
	p.G34E	0	3	2	3	8/793
	p.S45P	0	1	0	6	7/805
	p.S37C	1	0	0	5	6/804
	p.S33C	0	0	0	5	5/796
	p.D32G	0	0	0	4	4/826
	p.S33P	0	1	0	3	4/814
	p.G34V	0	0	0	3	3/793
	p.D32H	0	0	0	2	2/801
	p.D32V	0	0	0	2	2/826
	p.S33A	0	1	0	1	2/814
	p.S37P	0	0	0	2	2/804
	p.S45Y	1	0	1	0	2/801
	p.S37A	0	0	0	1	1/804
	p.S37Y	0	0	0	1	1/804
	p.S45C	0	0	0	1	1/801
KRAS[1] (%)		7 (9)	43 (20)	3 (6)	86 (17)	139 (17)
	p.G12D	2	14	1	28	45/795
	p.G12V	3	10	1	23	37/795
	p.G13D	1	13	1	12	27/801
	p.G12A	0	4	0	10	14/795
	p.G12C	1	1	0	8	10/795
	p.G12S	0	0	0	3	3/795
	p.G13S	0	0	0	2	2/775
	p.Q61H(G)	0	1	0	1	2/791
	p.G13C	0	0	0	1	1/775
	p.G13R	1	0	0	0	1/775
	p.Q61L	0	0	0	0	1/784
FGFR2[1] (%)		2 (3)	19 (9)	0 (0)	59 (12)	80 (10)
	p.S252W	1	12	0	34	47/798
	p.N549K	1	1	0	15	17/795
	p.K659E	0	2	0	7	9/803
	p.C382R	0	4	0	2	6/805
	p.Y375C	0	2	0	1	3/806
POLE (%)		0 (0)	0 (0)	49 (100)	0 (0)	49 (6)
	p.P286R	0	0	32	0	32/834
	p.V411L	0	0	14	0	14/834
	p.S297F	0	0	3	0	3/834
FBXW7[1] (%)		8 (11)	13 (6)	1 (2)	18 (4)	40 (5)
	p.R465H	2	6	0	9	17/825
	p.R505C	4	3	0	5	12/799
	p.R479Q	2	3	1	1	7/803
	p.R465C	1	1	0	3	5/813
	p.R479L	0	1	0	0	1/803

Supplementary Table 2 continued.

	p53-mutant n=74	MSI n=219	*POLE*-mutant n=49	NSMP n=492	Total n=834
PPP2R1A[1] (%)	12 (16)	6 (3)	1 (2)	20 (4)	39 (5)
p.R183W	1	1	0	14	16/783
p.S256F	3	0	0	3	6/778
p.P179L	4	0	0	1	5/807
p.R183Q	0	2	1	2	5/779
p.R258H	0	3	0	1	4/784
p.S256Y	3	0	0	0	3/778
p.P179R	1	0	0	0	1/807
NRAS (%)	1 (1)	8 (4)	0 (0)	16 (3)	25 (3)
p.Q61L	1	3	0	2	6/800
p.Q61R	0	0	0	5	5/800
p.G12D	0	2	0	2	4/828
p.G12S	0	1	0	3	4/806
p.Q61K	0	0	0	2	2/811
p.G12A	0	0	0	1	1/828
p.G12C	0	1	0	0	1/806
p.G12V	0	1	0	0	1/828
p.G13R	0	0	0	1	1/788
CDKN2A (%)	0 (0)	0 (0)	0 (0)	2 (<1)	2 (<1)
p.R80*	0	0	0	1	1/805
p.D108A	0	0	0	1	1/799
BRAF (%)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
FGFR3 (%)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
FOXL2 (%)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
HRAS (%)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

[1] Some tumors had multiple mutations in one gene. Frequencies presented as n (%), where n represents the number of samples showing the mutation. Analyzed hot spot mutations which were not detected are not shown.

Supplementary Table 3. Clinicopathological characteristics, additional mutations and protein expression alterations in tumors with multiple classifying alterations.

	p53 & MSI n=13	p53 & *POLE* n=7	MSI & *POLE* n=6	p53 & MSI & *POLE* n=1
Age, years				
Mean (range)	64 (52-73)	62 (49-76)	70 (61-79)	74 (-)
< 60	3 (23.1)	4 (57.1)	0	0
60-70	6 (46.1)	0	3 (50.0)	0
70	4 (30.8)	3 (42.9)	3 (50.0)	1
Grade				
1-2	10 (76.9)	5 (71.4)	5 (83.3)	1
3	3 (23.1)	2 (28.6)	1 (16.7)	0
Myometrial invasion				
<50%	6 (46.1)	2 (28.6)	0	0
>50%	7 (53.9)	5 (71.4)	6 (100)	1
LVSI				
Absent	12 (92.3)	7 (100)	4 (66.7)	1
Substantial	1 (7.7)	0	2 (33.3)	0
Risk group				
Low	6 (46.1)	4 (57.1)	0	0
High-intermediate	6 (46.1)	3 (42.9)	5 (83.3)	1
High	1 (7.7)	0	1 (16.7)	0
Treatment				
NAT	3 (23.1)	3 (42.9)	0	1
EBRT	5 (38.4)	4 (57.1)	3 (50.0)	0
VBT	5 (38.4)	0	3 (50.0)	0
Mutations				
CDKN2A	1 (7.7)	0	0	0
FBXW7	3 (23.1)	3 (42.9)	1 (16.7)	0
KRAS	1 (7.7)	0	0	0
PIK3CA	3 (23.1)	4 (57.1)	2 (33.3)	1
PPP2R1a	1 (7.7)	0	1 (16.7)	0
PTEN	5 (38.4)	6 (85.7)	4 (30.8)	1
Altered protein expression				
>10% L1CAM	1 (7.7)	2 (28.6)	1 (16.7)	0
<10% ER	2 (18.2)	2 (33.3)	1 (16.7)	0
<10% PR	3 (27.3)	2 (28.6)	1 (20.0)	0
loss/clonal ARID1a	4 (30.8)	1 (14.3)	3 (50.0)	0
loss/ heterogeneous PTEN	6 (46.1)	3 (42.9)	5 (83.3)	0
nuclear β-catenin	2 (15.4)	1 (14.3)	2 (33.3)	0
Survival				
Alive	10 (76.9)	7 (100)	4 (66.7)	0
Dead	3 (23.1)	0	2 (33.3)	1
Recurrence				
Locoregional	0	0	1 (16.7)	0
Distant	2 (15.4)	0	1 (16.7)	1

LVSI=lymphovascular space invasion, NAT=no additional treatment, EBRT=external beam radiotherapy, VBT=vaginal brachytherapy

Supplementary Table 4. Multivariable analysis on the prognostic role of the clinicopathological characteristics, molecular subgroups, and potential other classifiers in all cases of early-stage endometrial cancer (n=834) and in the subset of EC without substantial LVSI, >10% L1CAM, p53 and *POLE* mutation (n=620).

All cases (n=834)

	Locoregional Recurrence 60 events			Distant Recurrence 65 events			Overall Survival 252 events		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Age (Cont.)	1.040	1.007-1.075	0.018	1.010	0.978-1.044	0.536	1.079	1.061-1.097	0.000
Grade									
1-2	1			1			1		
3	1.852	0.981-3.496	0.057	2.543	1.402-4.613	0.002	1.456	1.030-2.058	0.033
Myometrial invasion									
<50%	1			1			1		
>50%	1.315	0.727-2.381	0.365	1.681	0.913-3.094	0.096	1.077	0.798-1.455	0.627
LVSI									
Absent/mild	1			1			1		
Substantial	3.224	1.431-7.267	0.005	3.150	1.508-6.581	0.002	2.027	1.235-3.328	0.005
Treatment									
NAT	1			1			1		
EBRT	0.217	0.117-0.402	0.000	1.437	0.749-2.757	0.276	1.003	0.752-1.339	0.982
VBT	0.404	0.204-0.799	0.009	1.552	0.743-3.242	0.242	0.840	0.569-1.241	0.382
Molecular subgroup									
NSMP	1			1			1		
p53	4.089	2.060-8.116	0.000	4.422	2.221-8.803	0.000	2.475	1.682-3.642	0.000
MSI	1.425	0.797-2.645	0.224	1.622	0.876-3.004	0.124	1.444	1.071-1.948	0.016
POLE	-	-	0.964	1.060	0.245-4.592	0.938	1.247	0.625-2.488	0.531
L1CAM									
<10%				1			1		
>10%				3.028	1.540-5.953	0.001	2.098	1.366-3.221	0.001

Cases without substantial LVSI, >10% L1CAM, p53 and *POLE* mutation (n=620)

	Locoregional Recurrence 36 events			Distant Recurrence 30 events			Overall Survival 175 events		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Age (Cont.)	1.057	1.012-1.104	0.013	0.999	0.952-1.048	0.966	1.094	1.071-1.117	0.000
Grade									
1-2	1			1			1		
3	2.134	0.892-5.106	0.089	6.583	2.751-15.75	0.000	1.609	0.999-2.590	0.051
Myometrial invasion									
<50%	1			1			1		
>50%	1.199	0.549-2.622	0.649	1.539	0.639-3.709	0.336	0.914	0.635-1.315	0.628
Treatment									
NAT	1			1			1		
EBRT	0.256	0.118-0.555	0.001	1.456	0.610-3.476	0.397	1.050	0.747-1.476	0.777
VBT	0.218	0.071-0.663	0.007	1.065	0.333-3.402	0.916	0.807	0.486-1.339	0.407
Molecular subgroup									
NSMP	1			1			1		
MSI	1.181	0.579-2.409	0.647	2.181	0.997-.4770	0.051	1.431	1.036-1.976	0.030
CTNNB1									
No mutation				1					
Mutation				2.834	1.284-6.257	0.010			

Cont.=continuous, LVSI=lymphovascular space invasion, NAT=no additional treatment, EBRT=external beam radiotherapy, VBT=vaginal brachytherapy

Supplementary Table 5. Univariable analysis of clinicopathological characteristics, molecular subgroups, and potential other classifiers in low-risk early-stage endometrial cancer (n=242).

		Locoregional Recurrence 12 events			Distant Recurrence 10 events			Overall Survival 67 events		
	n	HR	95% CI	*P*-value	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Age (cont.)	242	1.064	1.001-1.131	0.045	1.005	0.935-1.079	0.900	1.087	1.057-1.117	0.000
Grade										
1-2	227	1			1			1		
3	15	1.511	0.195-11.706	0.693	1.775	0.225-14.015	0.586	1.356	0.544-3.379	0.513
Myometrial invasion										
<50	189	1			1			1		
>50	53	0.305	0.039-2.366	0.256	0.880	0.187-4.143	0.871	0.763	0.416-1.399	0.382
LVSI										
Absent/mild	234	1			1			1		
Substantial	6	-	-	0.705	-	-	0.729	-	-	0.428
Given treatment										
NAT	112	1			1			1		
EBRT	111	0.098	0.012-0.762	0.026	2.022	0.506-8.084	0.319	1.067	0.651-1.751	0.796
VBT	19	0.681	0.086-5.400	0.716	2.017	0.210-19.404	0.544	1.722	0.592-5.011	0.318
Molecular subgroup										
NSMP	134	1			1			1		
p53	22	0.849	0.106-6.787	0.877	3.939	0.941-16.487	0.061	1.989	0.977-4.048	0.058
MSI	62	0.819	0.217-3.089	0.769	1.154	0.135-9.877	0.896	1.231	0.694-2.182	0.478
POLE	24	-	-	0.983	0.439	0.051-3.760	0.453	0.716	0.279-1.836	0.487
CTNNB1										
No mutation	176	1			1			1		
Mutation	53	1.664	0.501-5.527	0.406	2.579	0.693-9.606	0.158	0.909	0.502-1.646	0.753
FBXW7										
No mutation	217	1			1			1		
Mutation	12	1.563	0.201-12.131	0.669	-	-	0.634	0.443	0.108-1.818	0.259
FGFR2										
No mutation	217	1			1			1		
Mutation	12	-	-	0.589	2.442	0.305-19.539	0.400	1.236	0.445-3.434	0.685
KRAS										
No mutation	183	1			1			1		
Mutation	46	2.009	0.605-6.672	0.255	3.216	0.863-11.978	0.082	0.746	0.379-1.466	0.395
NRAS										
No mutation	221	1			1			1		
Mutation	8	-	-	0.671	-	-	0.718	1.998	0.725-5.506	0.181
PIK3CA										
No mutation	157	1			1			1		
Mutation	72	0.440	0.096-2.009	0.289	0.271	0.034-2.164	0.218	1.007	0.592-1.713	0.979
PPP2R1A										
No mutation	223	1			1			1		
Mutation	6	-	-	0.713	4.841	0.605-38.721	0.137	0.528	0.073-3.813	0.527
PTEN										
No mutation	129	1			1			1		
Mutation	100	0.628	0.189-2.087	0.448	0.643	0.161-2.572	0.533	0.751	0.452-1.247	0.268

Supplementary Table 5 continued.

		Locoregional Recurrence 12 events			Distant Recurrence 10 events			Overall Survival 67 events		
	n	HR	95% CI	*P*-value	HR	95% CI	*P*-value	HR	95% CI	*P*-value
L1CAM										
<10%	208	1			1			1		
>10%	8	10.72	2.211-52.000	0.003	10.49	2.107-52.263	0.004	4.167	1.765-9.835	0.001
ER										
>10%	188	1			1			1		
<10%	11	2.280	0.285-18.254	0.437	6.752	1.360-33.532	0.020	1.270	0.456-3.540	0.648
PR										
>10%	184	1			1			1		
<10%	20	1.259	0.157-10.071	0.828	3.365	0.679-16.681	0.137	1.897	0.929-3.874	0.079
ARID1a										
Positive	122	1			1			1		
Loss/clonal	82	0.417	0.087-2.008	0.276	1.508	0.377-6.029	0.562	0.980	0.579-1.659	0.940
PTEN										
Positive	122	1			1			1		
Loss/hetero.	86	1.818	0.488-6.774	0.373	0.460	0.093-2.281	0.342	1.023	0.604-1.735	0.931
β-catenin										
Membrane	155	1			1			1		
Nuclear	55	1.380	0.345-5.526	0.649	0.905	0.183-4.485	0.903	0.892	0.489-1.628	0.710

Cont.=continuous, LVSI=lymphovascular space invasion, NAT=no additional treatment, EBRT= external beam radiotherapy, VBT=vaginal brachytherapy, hetero.=heterogeneous.

Chapter 5

Refining prognosis and identifying targetable pathways within high-risk endometrial cancer; a *Trans*PORTEC initiative

Stelloo E, Bosse T, Nout RA, MacKay HJ, Church DN, Nijman HW, Leary A, Edmondson RJ, Powell ME, Crosbie EJ, Kitchener HC, Mileshkin L, Pollock PM, Smit VT, Creutzberg CL

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Abstract

This study aimed to investigate whether molecular analysis can be used to refine risk assessment, direct adjuvant therapy, and identify actionable alterations in high-risk endometrial cancer. *Trans*PORTEC, an international consortium related to the PORTEC3 trial, was established for translational research in high-risk endometrial cancer. In this explorative study, routine molecular analyses were used to detect prognostic subgroups: p53 immunohistochemistry, microsatellite instability and *POLE* proofreading mutation. Furthermore, DNA was analyzed for hotspot mutations in 13 additional genes (*BRAF*, *CDKNA2*, *CTNNB1*, *FBXW7*, *FGFR2*, *FGFR3*, *FOXL2*, *HRAS*, *KRAS*, *NRAS*, *PIK3CA*, *PPP2R1A*, and *PTEN*) and protein expression of ER, PR, PTEN, and ARID1a was analyzed. Rates of distant metastasis, recurrence-free, and overall survival were calculated using the Kaplan–Meier method and log-rank test. In total, samples of 116 high-risk endometrial cancer patients were included: 86 endometrioid; 12 serous; and 18 clear cell. For endometrioid, serous, and clear cell cancers, 5-year recurrence-free survival rates were 68%, 27%, and 50% (P=0.014) and distant metastasis rates 23%, 64%, and 50% (*P*=0.001), respectively. Four prognostic subgroups were identified: (1) a group of p53-mutant tumors; (2) microsatellite unstable tumors; (3) POLE proofreading-mutant tumors; and (4) a group with no specific molecular profile (NSMP). In group 3 (*POLE*-mutant; n=14) and group 2 (microsatellite unstable; n=19) patients, no distant metastasis occurred, compared with 50% distant metastasis rate in group 1 (p53-mutant; n=36) and 39% in group 4 (NSMP; *P*<0.001). Five-year recurrence-free survival was 93% and 95% for group 3 (*POLE*-mutant) and group 2 (microsatellite unstable) vs 42% (group 1, p53-mutant) and 52% (group 4, NSMP; *P*<0.001). Targetable *FBXW7* and *FGFR2* mutations (6%), alterations in the PI3K-AKT pathway (60%) and hormone receptor positivity (45%) were frequently found. In conclusion, molecular analysis of high-risk endometrial cancer identifies four distinct prognostic subgroups, with potential therapeutic implications. High frequencies of targetable alterations were identified and may serve as targets for individualized treatment.

Introduction

Risk classification of endometrial carcinomas is based upon a combination of clinical and histopathological factors and is used in guiding adjuvant therapy. High-risk factors are (combinations of) advanced age, high-grade, non-endometrioid histology, extensive lymphovascular space invasion, and more advanced disease stage. 15–20% of patients with endometrial cancer have high-risk disease and an aggressive clinical course. There has been widespread uncertainty among clinicians over the best treatment approach for this subgroup of patients, including extent of surgery, type and extent of radiotherapy, and chemotherapy. Two randomized trials PORTEC3 and GOG249 have recently completed an accrual and two trials are going on (GOG258 and the ENGOT-EN2-DGCC/EORTC55102 trial) to evaluate the role of adjuvant chemotherapy with or without pelvic radiation for patients with high-risk endometrial cancer. Significant inter-observer variability exists, even amongst expert gyneco-pathologists, when subtyping and grading endometrial.[1-4] In most cases, tumor cell type and grade are diagnosed based on hematoxylin and eosin slides according to the WHO histopathological criteria.[5] The use of immunomarkers for distinguishing subtype has increased during the past years. Identifying specific molecular alterations which determine tumor behavior and metastatic potential is needed to improve risk classification, inform treatment decisions, and identify targetable pathways in high-risk endometrial cancers. *Trans*PORTEC, an international consortium related to the PORTEC3 trial, was set up to conduct such a translational research in high-risk endometrial cancer (www.msbi.nl/transportec).

Over the past decade, multiple groups have identified common molecular alterations of several important genes in endometrial cancer.[6-11] These alterations have been fitted in the dualistic model of endometrial carcinogenesis discriminating endometrial cancers in the more indolent endometrioid cancers (type 1) and the more aggressive non-endometrioid cancers (type 2). Recently, comprehensive molecular profiling of 373 endometrial cancers suggested that the traditional dualistic model can be improved upon classification of endometrial cancers into four molecular subgroups with a potential prognostic significance: DNA polymerase epsilon (*POLE*) ultramutated, microsatellite unstable hypermutated, copy-number low and copy-number high (serous-like and mostly *TP53* mutant).[12] At present such an extensive analysis is impractical and too expensive for a routine clinical utilization. However, testing for the surrogate markers of these subgroups (e.g., p53 immunohistochemistry, microsatellite instability, and *POLE* proofreading mutation) may be cost effective and would be easy to apply in the current clinical practice. In this study, we aimed to investigate whether such molecular analyses can be used to detect prognostic subgroups in a series of high-risk endometrial cancers. Importantly, this pilot study includes clear cell tumors – a

histological subtype not characterized by The Cancer Genome Atlas. In addition, we aimed to determine the frequency of molecularly targetable alterations in high-risk endometrial cancers,[6,13-16] which is of interest given the poor outcome of high-risk endometrial cancers with current management.

Materials and methods

Patient and Tissue Selection

Tumor tissues were selected from partner institutions within the *Trans*PORTEC consortium, with the aim of obtaining a large series of high-risk endometrial cancers using inclusion criteria of the PORTEC3 study.[17] In total, formalin fixed paraffin-embedded tumor samples from 116 patients that fulfilled these criteria were collected from five participating institutions: Leiden University Medical Center, The Netherlands (n=14); University Medical Center Groningen, The Netherlands (n=46); University College London, United Kingdom (n=8); St Marys Hospital Manchester, United Kingdom (n=34); and Gustave Roussy Paris, France (n=14). Paraffin-embedded tissue blocks containing representative tumor were selected. Hematoxylin-eosin-stained slides were viewed by experienced gynecopathologists (TB and VS) to select an area of tumor tissue containing at least 70% tumor cells.

Tissue Microarray Construction

Tissue microarrays were constructed from all samples with sufficient tumor volume (n=114) using a tissue microarray Master.[18] Tissue microarrays contained 1-mm tumor and tumor/stroma cores of each sample, in triplicate, and were randomly distributed. Colon, normal endometrial, kidney, liver, ovary, placenta, skin, testis, tonsil, and fallopian tube samples were included in the tissue microarrays for orientation purposes and as internal positive controls. Immunohistochemistry was performed on whole slide and tissue microarray for the first 59 cases to validate the utility of tissue microarray for protein expression analysis in endometrial cancer. Comparison of the results showed a concordance of >80% for PTEN, ARID1a, p53, ER, and MLH1 (data not shown).

Immunohistochemical Analysis

Immunohistochemistry on tissue microarrays and whole slides (4 µm) was performed as described previously.[19,20] Details of the procedures and primary antibodies are described in Supplementary Table 1. As negative controls, slides were incubated in phosphate-buffered saline without primary specific antibodies. Two observers scored the tissue microarrays and whole slides independently. The observers were blinded for patient characteristics and outcome, and discrepancies were resolved at a multihead microscope. p53 was scored positive if 450% of the tumor cells showed a strong

positive nuclear staining, or when discrete geographical patterns showed >50% tumor cell positivity.[21] 'Indefinite' cases in which no staining of the tumor was observed were sequenced for *TP53* mutations. PTEN staining was evaluated in three categories as negative, positive, and heterogenous.[22] ARID1a was scored as negative, weak positive, or strong positive nuclear staining or as 'clonal loss'.[23] In the final analyses, 'clonal loss' was reclassified as 'loss of expression' as this pattern has been indicated to correspond with *ARID1a* mutations.[24] The ER, PR, and MLH1 scores for all three tissue microarray tumor cores were determined. ER and PR were scored positive when at least one tumor core showed any nuclear expression. MLH1 nuclear staining was scored positive or negative, if all cores were concordant. 'Clonal loss' of MLH1 was scored if one of the cores was discordant. Cases were scored 'failed' when two of the three cores could not be evaluated, because of the absence of the core, tumor, or internal control for staining.

DNA Isolation

Prior to DNA isolation, tumor DNA was enriched in the FFPE blocks by taking three 0.6-mm tissue cores from the tumor focus by using a tissue microarrayer (Beecher Instruments), to reach a tumor percentage >70%. Normal DNA was isolated from cores in the adjacent normal myometrium. In five cases, 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).[25]

Mutation Analysis

All samples were analyzed by using the Sequenom MassARRAY system and the GynCarta Assay version 2.0 (Sequenom) to test for the presence or absence of 159 hotspot mutations in 13 genes (*BRAF*, *CDKNA2*, *CTNNB1*, *FBXW7*, *FGFR2*, *FGFR3*, *FOXL2*, *HRAS*, *KRAS*, *NRAS*, *PIK3CA*, *PPP2R1A*, and *PTEN*) as described previously.[26] This gene panel only covers 40% of *PTEN* mutations found in endometrial cancer and therefore loss of PTEN protein expression was analyzed by immunohistochemistry. Mutations in *POLE* exons 9 and 13, which together contain >90% of the pathogenic *POLE* exonuclease domain mutations, were detected by using Sanger sequencing.[11] Sanger sequencing for exon 5-8 of *TP53* was performed on those samples that showed an 'indefinite' p53 immunohistochemical staining pattern as described previously.[9]

Microsatellite Instability

The microsatellite status of each tumor was determined using the Promega microsatellite instability analysis system (version 1.2, Promega), as described previously.[9] Tumors with instability in two or more of these markers were defined as being high-frequency microsatellite unstable, whereas those with instability at one repeat or showing no instability were classified as being stable. Microsatellite unstable tumors in which no

loss of MLH1 protein expression was observed were stained for MSH2, MSH6, and PMS2 to confirm loss of one of the mismatch repair proteins (Supplementary Table 1).

Methylation Specific PCR

Tumors with loss of MLH1 protein expression were selected for further testing for methylation status of the 5' regulatory region of *MLH1*, using methylation-specific PCR, with primers that have been previously described.[27] Contamination of the carcinoma tissue by stromal or inflammatory cells was unavoidable and tumors with a partially methylated phenotype were scored as methylated.

Statistics

Rates of distant metastasis, recurrence-free, and overall survival were calculated with Kaplan–Meier method and log-rank test starting at the date of diagnosis. For analysis of overall survival, all deaths irrespective of cause were considered an event; for recurrence-free survival all recurrences (local, regional, and distant) were considered as an event. IBM SPSS software version 21.0 was used for all statistical analysis.

Table 1 Patient characteristics.

		n=116 (%)
Age		
	Mean, range	66 (21-85)
	<60	38 (32.8)
	60-70	31 (26.7)
	>70	47 (40.5)
FIGO stage 2009		
	I	42 (36.2)
	II	21 (18.1)
	III	41 (35.3)
	IV	11 (9.5)
	Unknown	1 (0.9)
Tumor type		
	Endometrioid	86 (74.1)
	Serous	12 (10.3)
	Clear cell	18 (15.5)
Grade		
	1	13 (11.2)
	2	5 (4.3)
	3	98 (84.5)
Depth of invasion		
	<50%	23 (19.8)
	>50%	87 (75.0)
	Unknown	6 (5.2)
lymphovascular space invasion		
	Absent	40 (34.5)
	Present	55 (47.4)
	Unknown	23 (18.1)
Any adjuvant therapy		
	Yes	82 (70.7)
	No	10 (8.6)
	Unknown	24 (20.7)
Adjuvant radiotherapy*		
	EBRT	55 (47.4)
	VBT	1 (0.9)
	EBRT + VBT	21 (18.1)
	None	15 (12.9)
	Unknown	24 (20.7)
Adjuvant chemotherapy		
	Yes	16 (13.8)
	No	76 (65.5)
	Unknown	24 (20.7)

EBRT=external beam radiotherapy,
VBT=vaginal brachytherapy.

Results

Clinicopathologic Characteristics

Clinicopathological characteristics are shown in Table 1. In total, 18 (16%) clear cell, 12 (10%) serous, and 86 (74%) endometrioid (33 FIGO stage I grade 3, 18 stage II, 28 stage III, and 6 stage IV) endometrial cancers were included. Median follow-up was months (range 0.3–165.5 months). For endometrioid, serous and clear cell cancers 5-year recurrence-free survival rates were 68%, 27%, and 50% (*P*=0.014), distant metastasis rates 23%, 64%, and 50% (*P*=0.001) and overall survival 67%, 18%, and 39% (*P*=0.002). The corresponding Kaplan–Meier curves are shown in Figure 1. In univariable analysis, age (*P*=0.031) was a prognostic factor for decreased recurrence-free survival, in contrast to grade (*P*=0.988) and deep myometrial invasion (*P*=0.150). However, it should be taken into account that the patients were selected on those high-risk factors. None of the clinicopathological factors (age, grade, and myometrial invasion) were prognostic for distant metastasis.

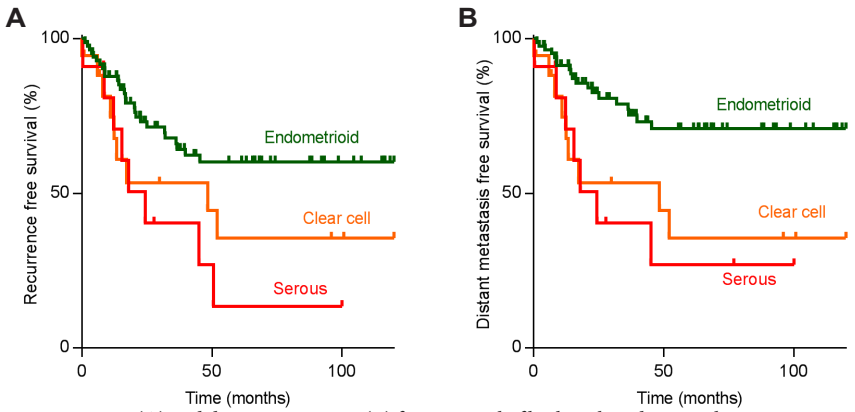

Figure 1. Recurrence (A) and distant metastasis (B) free survival of high-risk endometrial cancer patients stratified by tumor type. For endometrioid, serous and clear cell cancers 5-year recurrence-free survival rates were 68%, 27%, and 50% (*P*=0.014), distant metastasis rates 23, 64, and 50% (*P*=0.001) and overall survival 67%, 18%, and 39% (*P*=0.002).

Molecular Subgroups Within High-Risk Endometrial Cancers

The distribution and frequency of alterations differed substantially across the endometrial cancer subtypes, with the highest number of alterations in the endometrioid tumors (Supplementary Table 2-4). The co-occurrence of alterations was observed in a higher frequency in endometrioid (n=50, 58%) and serous (n=7, 58%) tumors compared with clear cell (n=6, 33%) subtypes (*P*=0.062). Combining the detected alterations resulted in the identification of four molecular subgroups; (1) a group of p53-mutant tumors (n=39, 34%); (2) microsatellite unstable tumors (n=19, 16%); (3) *POLE* proofreading-mutant tumors (n=14, 12%) and (4) a group with no specific molecular profile (NSMP; n=44, 38%) (Figure 2). A subset of tumors with endometrioid (n=20, 23%) and clear cell morphology (n=8, 44%)

had a p53 mutant-like expression similar to all serous cancers. This group 1 (p53-mutant) tumors had relatively few alterations in *PPP2R1a*, *FBXW7*, and PI3K-AKT pathway. A p53 mutant-like expression was inversely correlated with a microsatellite instability. Only one endometrioid cancer showed a p53 mutant-like expression and the microsatellite instability with loss of MLH1 protein expression due to promoter hypermethylation. In group 3 (*POLE*-mutant), two endometrioid tumors showed a microsatellite instability lacking *MLH1* promoter hypermethylation. Furthermore, the group 3 (*POLE*-mutant) tumors were highly associated with *PIK3CA* hotspot mutations and showed an inverse relationship with p53 mutant-like expression. Group 2 (microsatellite unstable) tumors consisted of 17 endometrioid and 2 clear cell cancers. All microsatellite unstable tumors showed loss of protein expression of one or two mismatch repair proteins (MLH1, MSH2, MSH6, and PMS2). DNA promoter hypermethylation of *MLH1* was observed in 9 of the 12 microsatellite unstable tumors with the loss of MLH1 protein expression. In the other cases, microsatellite instability could not be attributed to *MLH1* promoter methylation and microsatellite instability must therefore be the result of alternative mechanisms (e.g., Lynch syndrome; Supplementary Table 4). In group 4 (NSMP), we did not detect microsatellite instability, p53 mutant-like expression or *POLE* proofreading mutations. These remaining tumors can be characterized mostly by endometrioid and clear cell morphology, high frequency of PI3K-AKT alterations, high levels of ER/PR expression and *CTNNB1* mutations. Additionally, twelve tumors within this subgroup (27%) had none of the alterations tested.

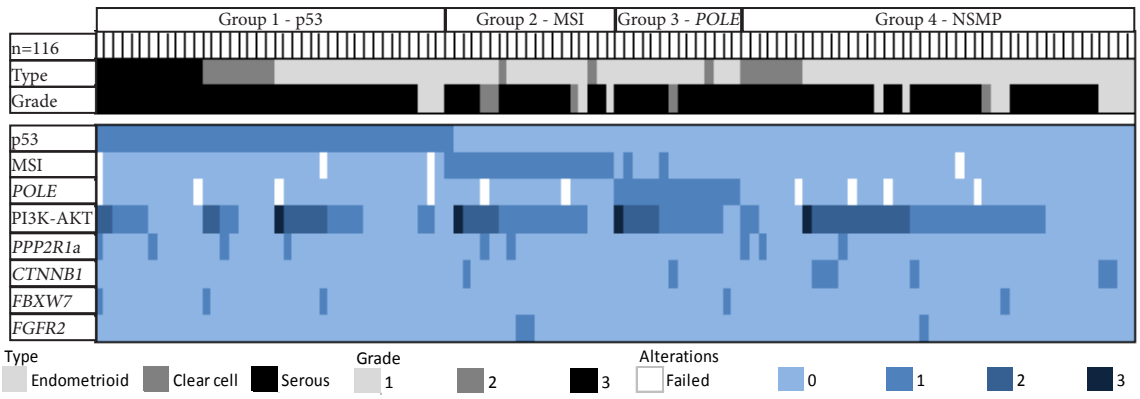

Figure 2. Molecular landscape of high-risk endometrial cancer. Routine analysis focusing on frequent hotspot mutations and known molecular drivers of endometrial cancer results in the identification of four molecular subgroups within high-risk endometrial cancer: (1) p53-mutant, (2) microsatellite unstable, (3) *POLE* proofreading-mutant, and (4) with no specific molecular profile (NSMP). PI3K-AKT pathway alterations are hotspot mutations in *PIK3CA*, *KRAS*, *NRAS*, loss of PTEN, or loss of ARID1a expression.

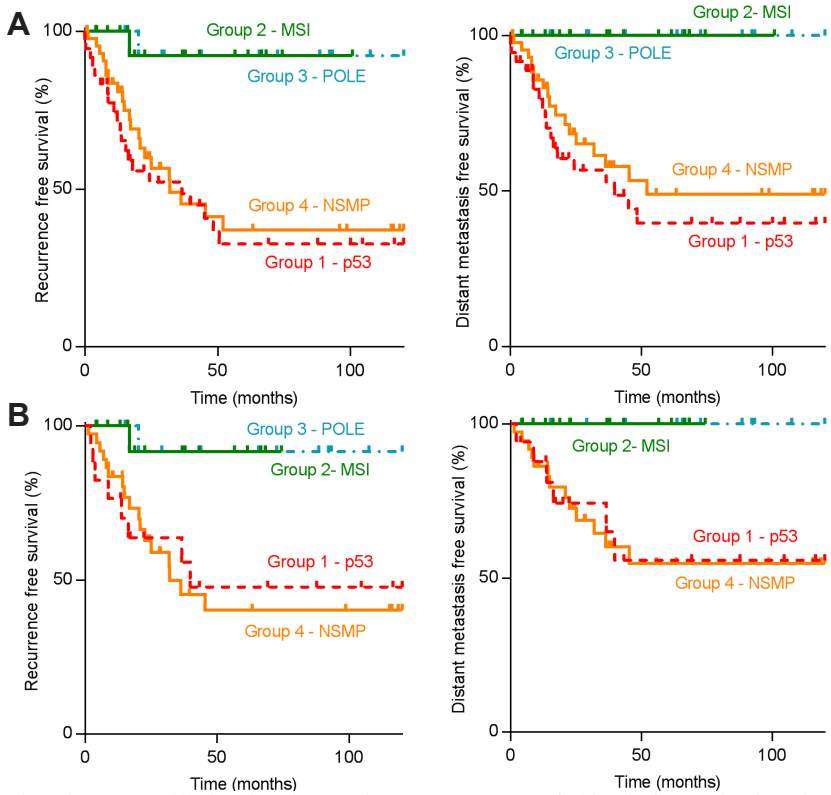

Figure 3. Clinical outcome of high-risk endometrial cancer patients stratified by the four molecular subgroups; (1) p53-mutant, (2) microsatellite unstable (MSI), (3) *POLE* proofreading-mutant, and subgroup (4) with no specific molecular profile (NSMP). Recurrence-free and distant metastasis-free survival of all high-risk patients (a) and endometrioid high-risk patients (b) stratified by the four molecular subgroups. Both in group 3 (*POLE*-mutant; n=14) and group 2 (microsatellite unstable; n=19) endometrial cancer patients no distant metastasis occurred, compared with group 1 (p53-mutant; n=36, 5-year distant metastasis 50%) and group 4 patients (NSMP; n=44, 39%; *P*<0.001). Five-year recurrence-free survival was 93% and 95% for group 3 (*POLE*-mutant) and group 2 (microsatellite unstable) vs. 42% (group 1-p53-mutant) and 52% (group 4-NSMP; *P*<0.001). Even after exclusion of the non-endometrioid cancer patients, group 3 (*POLE*-mutant) and group 2 (microsatellite unstable) endometrioid cancer patients still shows improved recurrence and distant metastasis free survival (*P*=0.004 and *P*=0.004).

Correlation of Molecular Subgroups with Clinical Outcome

Next, we analyzed the prognostic significance of the identified molecular subgroups and found that the four molecular subgroups were associated with different clinical outcomes. Both in group 3 (*POLE*-mutant; n=14) and group 2 (microsatellite unstable; n=19) endometrial cancer patients, no distant metastasis occurred, compared with 5-year rates of distant metastasis of 50% and 39%, respectively, among group 1 (p53-mutant) and group 4 (NSMP; P<0.001). Five-year recurrence-free survival was 93% and 95%, respectively, for group 3 (*POLE*-mutant) and group 2 (microsatellite unstable) cases, vs. 42% and 52% for group 1 (p53-mutant) and group 4 (NSMP; *P*<0.001; Figure 3a). Overall survival at 5 years was 93% in group 3 (*POLE*-mutant) patients, 63% in group 2 (microsatellite unstable; died from other causes), 40% in group 1 (p53-mutant) and 61% in group 4 (NSMP). Furthermore, *POLE*-mutant status was associated with a younger age, in contrast to p53-mutant status,

which was associated with older age. After exclusion of the non-endometrioid tumors, our analysis was still able to discriminate endometrioid patients with good vs. poor prognosis. Group 3 (*POLE*-mutant) and group 2 (microsatellite unstable) cancer patients were both associated with a better recurrence and distant metastasis-free survival, as compared with group 1 (p53-mutant) and group 4 (NSMP) endometrioid cancer patients (Figure 3b). Interestingly, group 1 (p53-mutant) endometrioid tumors and group 4 (NSMP) of high-risk endometrial tumors showed no differences in the clinical course.

Targetable Pathways within Molecular Subgroups

The frequencies of targetable alterations for each identified molecular subgroup is shown in Table 2. Group 1 (p53-mutant) and group 4 (NSMP) patients seem to have a poor outcome under the current treatment. Most cases within these two subgroups had alterations in the PI3K-AKT pathway (60%) or were hormone receptor-positive (45%) and potentially targetable with PI3K-AKT-mTOR inhibitors or hormonal therapies. In 6% of the cases, somatic mutations in *FBXW7* and *FGFR2* were identified, which could potentially be targetable with HDAC inhibitors or FGFR inhibitors (e.g. BGI398, AZD4547).

Table 2 Frequency of targetable alterations within the four subgroups; (1) p53-mutant, (2) microsatellite unstable, (3) *POLE* proofreading-mutant and (4) with no specific molecular profile (NSMP).

Targetable alteration (%)	Group 1 p53 n=39	Group 2 MSI n=19	Group 3 *POLE* n=14	Group 4 NSMP n=44	Potential drugs
PI3K-AKT pathway					
PIK3CA	7 (18)	6 (32)	11 (71)	13 (30)	PI3K, AKT, mTOR inhibitors
PTEN*	16 (41)	8 (42)	6 (43)	14 (32)	
ARID1a*	5 (13)	7 (37)	1 (7)	11 (26)	
KRAS	3 (8)	2 (11)	0	4 (9)	
NRAS	1 (3)	0	1 (7)	0	
Hormone receptor positivity					
ER	23 (59)	14 (74)	9 (64)	33 (74)	Hormonal therapy
PR	16 (41)	11 (58)	7 (50)	25 (57)	
Other targetable genes					
FBXW7	3 (8)	1 (5)	1 (7)	1 (2)	HDAC inhibitors
FGFR2	0	2 (11)	0	1 (2)	FGFR inhibitors

* Alterations based on immunohistochemistry.

Discussion

This research shows that molecular subclassification of high-risk endometrial cancer can be effectively used to identify distinct subsets with prognostic significance. We found highly significant and clinically relevant differences in relapse and survival rates between the molecular subgroups, which can be used to determine adjuvant therapy in clinical practice. The technology required for this molecular classification is suitable for daily clinicopathological practice. This practical approach resulted in the confirmation of the four molecular subgroups proposed by The Cancer Genome Atlas, and additionally identified potentially targetable pathways for high-risk endometrial cancers. These results can be translated into

clinical practice by tailoring adjuvant therapy and/or directing targeted treatment in future studies. The same molecular analyses have been shown to work successfully on pre-operative endometrial biopsy or curettages with high concordance with the hysterectomy specimen.[19] The findings also illustrate that endometrial cancers currently classified as 'high-risk' are in fact a heterogeneous group of tumors with diverse molecular alterations and variable clinical outcome. Our data confirm that even in a selected cohort of high-risk patients serous and clear cell histology portends poor prognosis when compared with endometrioid tumors.[28] However, even after exclusion of the non-endometrioid tumors, molecular analysis can discriminate patients with a good and poor prognosis.

Importantly, our analyses further support previous studies that showed an association of *POLE* proofreading mutations with younger age and favorable prognosis.[11,29,30] It is noteworthy that in this cohort, the frequency of *POLE* proofreading mutation was 12%, consistent with its association with high tumor grade[10,11,29] and higher than the 6% frequency detected in unselected low- and intermediate-risk endometrial cancers.[10,11,29,30] In addition, we found that microsatellite instability was associated with a reduced risk of recurrence and distant metastases. The impact of microsatellite instability on the prognosis of women with endometrial cancer is controversial. Some studies reported that microsatellite instability is associated with a favorable prognosis,[31,32] whereas in other studies a significant worse prognosis was found.[33,34] Possible explanations for these discrepancies include cohort differences, lack of statistical power, and diversity of methodology. Further studies will be required to clarify the effects of *POLE* mutations and microsatellite instability on the biological behavior of endometrial tumor cells and the associated mechanisms. Collectively our data suggest that *POLE* proofreading mutations and microsatellite instability may be useful as biomarkers to identify patients, mostly with grade 3 and clinically high-risk disease who, in fact, have a good prognosis and may not require intensive postoperative radiotherapy or even chemotherapy. In addition, our data provide a rationale to develop treatment strategies that take into account these genetic alterations.

Within the molecular subgroups we identified potentially targetable pathways alterations. This is of particular interest for high-risk endometrial cancer patients with a poorer outcome under the current treatment regimens, such as the group 1 (p53-mutant) and group 4 (NSMP) cancer patients without p53 mutant-like expression, *POLE* mutation and/or microsatellite instability. Most of the cases in group 4 (NSMP) had alterations in the PI3K-AKT pathway or were hormone receptor-positive and potentially targetable with PI3K-AKT-mTOR inhibitors, anti-hormone therapies, or combination therapies with dual inhibitors (Table 2). A selected group of cancers may be targetable with HDAC inhibitors or FGFR inhibitors based on the mutation frequencies of *FBXW7* and *FGFR2*. Previously, higher frequencies of these targetable alterations were reported in the comparable p53-mutant/serous-like and microsatellite stable copy-number low subgroup.[12] The use of exome sequencing may explain in part the higher

rate of detected mutations. However, the identified variants may also include non-pathogenic variants. Further studies are required to determine whether new therapies targeting these alterations improve the survival.

In the current analysis, we included a relatively large set of clear cell cancers (n=18). In our focused molecular analysis these cases were partly included not only within group 1 (p53-mutant/serous-like) but also in the other three subgroups of endometrioid cancers. Overlapping molecular features in clear cell cancers have been noted previously, including loss of ARID1a and p53 mutant-like expression.[35-37] This questions whether clear cell cancer are molecularly distinct or whether these cancers are morphological variants of serous and endometrioid cancers harboring the same spectrum of molecular alterations. The data presented here are not conclusive yet and a broader unbiased molecular analysis will be required to answer this question.

This report on a subgroup of high-risk endometrial cancers was set up as an exploratory study and therefore results should be interpreted as such. The retrospective nature, limited sample size, differences in adjuvant treatment regimens and follow-up data are obvious limitations. Furthermore, with our focused approach analyzing known drivers and hotspot mutations we do not provide an unbiased molecular profile, but tested previously identified molecular clusters. This focused approach can be viewed as a strength of our study, in that it enhances the clinical applicability. However, as a consequence, we did not identify novel molecular drivers in a group of tumors without any of the alterations tested.

In conclusion, we showed that relatively straightforward molecular analysis can be used to refine the risk assessment of endometrial cancer patients that are currently classified as high-risk based on clinicopathological factors. Our results indicate that group 3 (*POLE*-mutant) and group 2 (microsatellite unstable) high-risk patients have a favorable prognosis and therefore the current risk assessment of these patients may be overestimated, possibly resulting in overtreatment. Group 1 tumors that have a p53-mutant like expression and group 4 tumors with no specific molecular profile are truly high-risk cancers. For truly high-risk patients, studies should be directed toward identifying targetable pathways. Our data provide a rationale to investigate not only the use of PI3K-AKT pathway inhibitors in this selected patient group but also hormonal treatment in those tumors with retained receptor expression remains an option. The molecular approach used in this work will be extended and tested in the International *Trans*PORTEC Consortium Studies of the large randomized cohort of endometrial cancers of patients who participated in the PORTEC3 trial, with the advantages of clear, randomized treatment groups and complete follow-up data. This would result in a novel approach in which routine molecular analyses are incorporated in the workup of endometrial cancer to refine the risk assessment based upon clinical and histopathological factors and identify targetable alterations, resulting in the reduction of over- and undertreatment.

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Supplementary files

Supplementary Table 1. Procedures and details of the primary antibodies used for immunohistochemistry.

Protein	Origin	Clone	Dilution	Specie	Antigen retrieval	DAB/ DAB+	whole slide /TMA	Manually/ Automated*
ARID1a	SantaCruz	PSG-3	1:800	mouse	Tris-EDTA	DAB+	whole slide	manually
β-catenin	BD Transduction	14	1:1600	mouse	Citrate	DAB	whole slide	manually
ER	DAKO	1D5	1:100	mouse	Citrate	DAB	TMA	manually
MLH1	DAKO	ES05	1:100	mouse	Tris-EDTA	DAB+	TMA	manually
MSH2	DAKO	FE11	1:400	mouse	Tris-EDTA	DAB+	whole slide	automated
MSH6	GeneTex	EPR3945	1:100	rabbit	Tris-EDTA	DAB+	whole slide	automated
p53	NeoMarkers	DO-7	1:2000	mouse	Tris-EDTA	DAB	whole slide	manually
PMS2	DAKO	EP51	1:10	rabbit	Tris-EDTA	DAB+	whole slide	automated
PR	DAKO	PGR636	1:200	mouse	Citrate	DAB	TMA	manually
PTEN	DAKO	6H2.1	1:200	mouse	Tris-EDTA	DAB+	whole slide	manually

*Procedures as described previously.[18,19]

Supplementary Table 2. Hotspot mutation frequency in high-risk endometrial cancers.

	Endometrioid n=86	Serous n=12	Clear cell n=18	Total n=116
PIK3CA (%)[1]	30 (35)	1 (8)	4 (22)	35 (30)
p.R88Q	10	0	1	11/112
p.H1047R	4	0	1	5/113
p.E545K	3	0	1	4/115
p.E542K	3	1	0	4/113
p.T1025A	4	0	0	4/111
p.Q546K	1	0	1	2/114
p.Q546E	2	0	0	2/114
p.M1043V	1	0	0	1/114
p.Q546P	1	0	0	1/114
p.H1047Y	1	0	0	1/113
p.E545G	1	0	0	1/112
p.M1043I(T)	1	0	0	1/111
PTEN(%)[1]	26 (30)	0 (0)	0 (0)	26 (22)
p.R130fs*4	8	0	0	8/114
p.R130G	6	0	0	6/111
p.R233*	4	0	0	4/113
p.E7*	2	0	0	2/114
p.L318fs*2	2	0	0	2/112
p.K267fs*9	2	0	0	2/112
p.R173C	2	0	0	2/111
p.V290fs*1	1	0	0	1/115
p.N323fs*2	1	0	0	1/114
p.T321fs*23	1	0	0	1/114
p.R130L	1	0	0	1/112
p.R130*	1	0	0	1/111
POLE (%)	13 (15)	0 (0)	1 (6)	14 (12)
p.P286R	8	0	0	8/115
p.V411L	5	0	1	6/115
KRAS (%)	8 (9)	1 (8)	0 (0)	10 (8)
p.G13S	2	0	0	2/114
p.G12D	2	0	0	2/114
p.G12V	1	1	0	2/114
p.G12A	1	0	0	1/114
p.Q61H(A)	1	0	0	1/114
p.G13D	1	0	0	1/112
PPP2R1A (%)	4 (5)	2 (2)	3 (17)	9 (8)
p.R183W	1	0	2	3/113
p.P179L	0	1	1	2/114
p.R258H	1	0	0	1/114
p.R183Q	1	0	0	1/114
p.S256Y	1	0	0	1/113
p.S256F	0	1	0	1/113
CTNNB1 (%)	8 (9)	0 (0)	0 (0)	8 (7)
p.S45Y	1	0	0	1/114
p.S45F	1	0	0	1/114
p.D32G	1	0	0	1/114
p.G34R	1	0	0	1/114
p.G34E	1	0	0	1/114
p.S33C	1	0	0	1/113
p.S33Y	1	0	0	1/113
p.D32N	1	0	0	1/112

Supplementary Table 2 continued.

	Endometrioid n=86	Serous n=12	Clear cell n=18	Total n=116
FBXW7 (%)	3 (3)	1 (8)	2 (11)	6 (5)
p.R465C	2	1	2	5/114
p.R465H	1	0	0	1/112
FGFR2 (%)	3 (3)	0 (0)	0 (0)	3 (3)
p.C382R	2	0	0	2/114
p.S252W	1	0	0	1/113
NRAS (%)	2 (2)	0 (0)	0 (0)	2 (2)
p.Q61R	1	0	0	1/114
p.G12D	1	0	0	1/114
CDKN2A(%)	1 (1)	0 (0)	0 (0)	1 (1)
p.R80*	1	0	0	1/113
BRAF (%)	0 (0)	0 (0)	0 (0)	0 (0)
FGFR3 (%)	0 (0)	0 (0)	0 (0)	0 (0)
FOXL2 (%)	0 (0)	0 (0)	0 (0)	0 (0)
HRAS (%)	0 (0)	0 (0)	0 (0)	0 (0)

[1] One endometrioid endometrial tumor had 2 *PIK3CA* mutations and five had 2 *PTEN* mutations in the same tumor. n represents the number of samples showing the mutation. Analyzed hot spot mutations which were not detected are not shown.

Supplementary Table 3. Alterations in protein expression in high-risk endometrial cancers.

	Endometrioid n=86	Serous n=12	Clear cell n=18	Total n=116
p53				
Wildtype	66 (77)	0 (0)	10 (56)	76 (66)
Mutant-like	20 (23)	12 (100)	8 (44)	40 (34)
ARID1a				
Negative	21 (25)	0 (0)	3 (17)	24 (21)
Positive	64 (74)	12 (100)	15 (83)	91 (78)
Failed	1 (1)	0 (0)	0 (0)	1 (1)
PTEN				
Negative	29 (34)	2 (17)	1 (6)	32 (28)
Heterogeneous	7 (8)	3 (25)	2 (11)	12 (10)
Positive	50 (57)	7 (58)	15 (83)	72 (62)
MLH1				
Negative	13 (15)	0 (0)	1 (6)	14 (12)
Clonal	1 (1)	0 (0)	0 (0)	1 (1)
Positive	72 (84)	12 (100)	17 (94)	101 (87)
ER				
Negative	24 (28)	3 (25)	10 (55)	37 (32)
Positive	61 (71)	9 (75)	7 (39)	77 (67)
Failed	1 (1)	0 (0)	1 (6)	2 (1)
PR				
Negative	35 (41)	9 (75)	13 (72)	57 (49)
Positive	50 (59)	3 (25)	5 (28)	58 (51)

Supplementary Table 4. Microsatellite instability status of high-risk endometrial cancer patients.

Microsatellite status (%)	Endometrioid n=86	Serous n=12	Clear cell n=18	Total n=116
Microsatellite stable	64 (74)	11 (92)	15 (83)	90 (78)
Sporadic microsatellite instability	10 (12)	0 (0)	0 (0)	10 (9)
Microsatellite instability (no *MLH1* methylation)	9 (10)	0 (0)	3 (17)	12 (10)
Failed	3 (3)	1 (8)	0 (0)	4 (3)

Chapter 6

Practical guidance for mismatch repair-deficiency testing in endometrial cancer

Stelloo E, Jansen AM, Osse EM, Nout RA, Creutzberg CL, Ruano D, Church DN, Morreau H, Smit VT, van Wezel T, Bosse T

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Abstract

Background: Mismatch repair (MMR)-deficiency analysis is increasingly recommended for all endometrial cancers, as it identifies Lynch syndrome-patients, and is emerging as a prognostic classifier to guide adjuvant treatment. The aim of this study was to define the optimal approach for MMR-deficiency testing and to clarify discrepancies between microsatellite instability (MSI) analysis and immunohistochemical (IHC) analysis of MMR protein expression.

Patients and Methods: 696 endometrial cancers were analyzed for MSI (pentaplex panel) and MMR protein expression (IHC). Agreement between methodologies was calculated using Cohen's Kappa. *MLH1* promoter hypermethylation, dinucleotide microsatellite markers and somatic MMR and *POLE* exonuclease domain (EDM) gene variants (using next-generation/ Sanger sequencing) were analyzed in discordant cases.

Results: MSI was found in 180 patients. Complete loss of expression of one or more MMR proteins was observed in 196 cases. A PMS2- and MSH6-antibody panel detected all cases with loss of MMR protein expression. The results of MSI and MMR protein expression were concordant in 655/696 cases (kappa=0.854, $P<0.001$). Ambiguous cases (n=41, 6%) included: subclonal loss of MMR protein expression (n=18), microsatellite stable or MSI-low cases with loss of MMR protein expression (n=20), and MSI-low or MSI-high cases with retained MMR protein expression (n=3). Most of these cases could be explained by *MLH1* promoter hypermethylation. Five of seven cases with solitary loss of PMS2 or MSH6 protein expression carried somatic gene variants. Two MSI-high cases with retained MMR protein expression carried a *POLE*-EDM variant.

Conclusion: MSI and IHC analysis are highly concordant in endometrial cancer. This holds true for cases with subclonal loss of MMR protein expression. Discordant MMR-proficient/ MSI-high cases (<1%), may be explained by *POLE*-EDM variants.

Introduction

A defect in DNA mismatch repair (MMR) leads to the accumulation of mismatches, insertions and deletions in repeated sequences - a phenomenon named microsatellite instability (MSI). Approximately 20-30% of sporadic endometrial cancers (ECs) display MSI as a consequence of somatic promoter hypermethylation and silencing of *MLH1*.[1] Defective MMR due to pathogenic germline variants in MMR genes causes Lynch syndrome (LS), a tumor predisposition syndrome that accounts for 2% of ECs.[2]

Determination of MMR-deficiency in EC may be important for several reasons. First, recent studies have suggested that tumor molecular features, including MMR-deficiency, may improve prognostication and help guide adjuvant therapy for EC patients.[3,4] Second, accurate assessment of MMR-deficiency is essential to identify patients with EC caused by LS. However in contrast to colorectal cancer, where consensus guidelines for MMR-deficiency testing have been published,[5] there is no general agreement on screening EC patients for LS.[6,7] Finally, recent studies have shown that MMR-deficiency in colorectal and urothelial cancer is predictive of response to immunotherapy,[8,9] suggesting that MMR-deficient ECs may also benefit from these therapeutics.

MMR-deficiency can be detected by either MSI analysis and/or immunohistochemical (IHC) staining, typically for four MMR proteins. The National Cancer Institute microsatellite panel was optimized and correlated with IHC analysis (~95%) to detect MMR-deficiency in colorectal cancer.[10,11] IHC alone has become standard practice in multiple institutions. Experience in this setting is that while some tumors show uniform and widespread loss of MMR protein expression, cases with subclonal loss of MMR protein expression are also observed.[11,12] Such cases present with two populations of tumor cells; one with retained expression, and another with abrupt and complete regional loss of MMR protein expression.[12] Small studies have shown high agreement between MSI and loss of MMR protein expression in EC,[13-15] while others have described subclonal loss of MMR protein expression.[16-20] However, studies identifying the frequency of such staining patterns in large patient series are sparse.

In this study, we sought to establish the optimum method for MMR-deficiency testing by comparison of MSI with IHC analysis in a large series of ECs. We also investigated the frequency of subclonal loss of MMR protein expression and the number of potential LS cases. Cases showing disagreement between methodologies and those with subclonal loss of MMR protein expression were further characterized.

Methods

Study population

The population comprised, 854 ECs from the PORTEC-1 and -2 clinical trials based on availability of formalin-fixed paraffin-embedded slides and sufficient tumor material for DNA isolation.[21,22] Further details are summarized in Supplementary Table S1 and Supplementary methods.

MSI assay

DNA was isolated as previously described.[23] In cases with subclonal loss of MMR protein expression, tissue sections were used to microdissect the differentially expressed tumor areas. Tumor MSI status was determined as previously reported (Supplementary methods).[4] Tumors initially classified as MSS or MSI-L with concomitant loss of MMR protein expression underwent evaluation of three dinucleotide repeat markers,[24] and reclassified as MSI-H if instability was detected at two dinucleotide markers.

IHC analysis

IHC staining for MLH1, PMS2, MSH2, and MSH6 was performed on all tumors in which MSI status was successfully determined (Supplementary methods). The slides were evaluated in three categories as retained, loss and subclonal loss of protein expression with stromal- and/or lymphocytic cells as internal controls.[16] The cases with subclonal loss of protein expression were re-evaluated to determine the percentage of tumor cells with loss of MMR expression.

Methylation-specific PCR for MLH1

Tumors with loss of MLH1 protein expression underwent testing for hypermethylation status of the *MLH1* 5' regulatory region by methylation-specific PCR, as previously described.[25]

Somatic variant screening

Subject to DNA availability and quality, tumors in which the results of MSI analysis and MMR protein expression were discordant underwent targeted next generation sequencing (NGS) of MMR and *POLE* genes using the Ion Proton™ System (ThermoFisher, MA, USA) as previously described (Supplementary methods).[26] Three additional cases were similarly analyzed using the Ion AmpliSeq Comprehensive Cancer Panel (ThermoFisher) at The Welcome Trust Center for Human Genetics. Frameshift variants in the polycytosine tract in exon 5 of *MSH6* were analyzed using Sanger sequencing.[27]

Results

Combined analysis of MMR protein expression and MSI was possible in 696 (81%) ECs (Supplementary Table S1). The frequencies of MSS, MSI-H and MSI-L were 74%, 24% and 2%, respectively. Among the 516 tumors assessed as MSS, 496 (96%) showed retained expression of all four MMR proteins (Table 1). The remaining twenty MSS cases showed loss of MMR protein expression as follows: combined MLH1 and PMS2 loss (n=14), combined MSH2 and MSH6 loss (n=3), solitary MSH6 loss (n=3, Figure 1A-D). Of the 11 cases assessed as MSI-L, six displayed combined loss of MLH1 and PMS2 expression (complete in four cases, subclonal in two cases), two cases showed solitary loss of PMS2, a further two cases had solitary MSH6 loss and one case retained expression of all four MMR proteins.

Table 1. Details on the MSI status and MMR protein expression in early-stage EC (n=696).

	MMR protein expression					
	MLH1	PMS2	MSH6	MSH2	protein expression	Count
MSI status						
MSS	1	1	1	1	Retained	496
MSS	2	2	1	1	Subclonal loss	6
MSS	1	1	2	2	Subclonal loss	2
MSS	0	0	1	1	Loss	8
MSS	1	1	0	1	Loss	3
MSS	1	1	0	0	Loss	1
MSI-L	1	1	1	1	Retained	1
MSI-L	2	2	1	1	Subclonal loss	2
MSI-L	0	0	1	1	Loss	4
MSI-L	1	0	1	1	Loss	2
MSI-L	1	1	0	1	Loss	2
MSI-H	1	1	1	1	Retained	2
MSI-H	2	2	1	1	Subclonal loss	8
MSI-H	0	0	2	1	Loss/Subclonal loss	6
MSI-H	0	0	1	1	Loss	130
MSI-H	1	1	0	0	Loss	10
MSI-H	1	0	1	1	Loss	8
MSI-H	1	1	0	1	Loss	5

Mismatch repair protein expression was scored as following: 0 – Complete loss; 1 – Retained; 2 -Subclonal loss. MMR=mismatch repair, MSS=microsatellite stable, MSI-L/H=microsatellite unstable with low- or high-frequency.

The majority of MSI-H cases (130 of 169, 77%) showed complete loss of MLH1 and PMS2 expression (Table 1). Sporadic MSI due to *MLH1* hypermethylation was observed in 97% of these 130 MSI-H cases. Eight MSI-H cases showed areas of subclonal loss of MLH1 and PMS2 (Figure 1E-H), and six cases displayed subclonal loss of MSH6 in addition to complete loss of MLH1 and PMS2 protein expression (Figure 1I-L). In ten cases (6%), combined loss of MSH2 and MSH6 protein expression was observed. The remaining MSI-H tumors showed solitary loss of PMS2 (n=8) or MSH6 (n=5) protein expression, or retained expression of all MMR proteins (n=2).

Table 2. Details on ECs with subclonal loss of MMR protein expression.

Case	Subclonal loss of protein expression	% tumor with loss of expression	MSI status[a]	MSI status[b]		No. of affected dinucleotides[c]	*MLH1* hyper-methylation[d]	gene variant
				Area with retained expression	Area with focal loss of expression			
1	MLH1, PMS2	90%	MSI-H	-	MSI-H	-	yes	-
2	MLH1, PMS2	90%	MSI-H	-	MSI-H	-	yes	-
3	MLH1, PMS2	75%	MSI-H	MSS	MSI-H	-	yes	-
4	MLH1, PMS2	75%	MSI-H	MSS	MSI-H	-	yes	-
5	MLH1, PMS2	75%	MSI-H	MSS	MSI-H	-	yes	-
6	MLH1, PMS2	75%	MSI-H	MSS	MSI-H	-	yes	-
7	MLH1, PMS2	75%	MSI-H	MSS	MSI-H	-	yes	-
8	MLH1, PMS2	25-50%	MSI-H	MSS	MSI-H	-	yes	-
9	MLH1, PMS2	75%	MSI-L	MSS	MSI-H	-	yes	-
10	MLH1, PMS2	25-50%	MSI-L	MSS	MSI-H	-	yes	-
11	MLH1, PMS2	>75%	MSS	MSS	MSI-H	-	yes	-
12	MLH1, PMS2	25-50%	MSS	MSS	MSI-H	-	yes	-
13	MLH1, PMS2	25-50%	MSS	MSS	MSS	0	yes	-
14	MLH1, PMS2	25-50%	MSS	MSS	MSS	Failed	yes	-
15	MLH1, PMS2	10%	MSS	MSS	MSS	0	Failed	None
16	MLH1, PMS2	10%	MSS	MSS	MSI-H	-	Failed	None
17	MSH2, MSH6	25-50%	MSS	MSS	MSS	-	-	-
18	MSH2, MSH6	10%	MSS	-	-	-	-	-

[a] first round of MSI analysis, [b] second round of MSI analysis in differently expressed areas (3 cases had too small areas) [c] instability in dinucleotide markers was only analyzed in discordant cases after the second round of MSI analysis [d] *MLH1* promoter hypermethylation was only assessed in cases with loss of/subclonal loss of MLH1 protein expression. Grey colored lines are unexplained/failed cases. MSS=microsatellite stable, MSI-L/H=microsatellite unstable with low- or high-frequency.

Table 3. Details on ECs with discordant MSI status and MMR protein expression.

Case	Protein expression	MSI status	No. of affected dinucleotides[a]	*MLH1* hyper-methylation[b]	Somatic variants[c]	Variant allele frequency	Classification of pathogenicity[d]
19	retained	MSI-H	-	-	*POLE* p.(V411L), *PMS2* p.(R107W), p.(R563*)	0.36, 0.37, 0.42	-, class 3, class 5
20	retained	MSI-H	-	no	*POLE* p.(A428T)	0.46	-
21	retained	MSI-L	0	-	None	-	-
22	MLH1-PMS2 loss	MSI-L	0	yes	-	-	-
23	MLH1-PMS2 loss	MSI-L	0	yes	-	-	-
24	MLH1-PMS2 loss	MSI-L	2	yes	-	-	-
25	MLH1-PMS2 loss	MSI-L	Failed	yes	-	-	-
26	MLH1-PMS2 loss	MSS	Failed	no	*POLE* p.(P286R)	0.69	-
27	MLH1-PMS2 loss	MSS	0	Failed	None	-	-
28	MLH1-PMS2 loss	MSS	0	yes	-	-	-
29	MLH1-PMS2 loss	MSS	Failed	yes	-	-	-
30	MLH1-PMS2 loss	MSS	0	yes	-	-	-
31	MLH1-PMS2 loss	MSS	0	yes	-	-	-
32	MLH1-PMS2 loss	MSS	0	yes	-	-	-
33	MLH1-PMS2 loss	MSS	0	yes	-	-	-
34	PMS2 loss	MSI-L	3	-	*PMS2* p.(M1?),p.(V302Tfs*4)	0.40, 0.27	class 3, class5
35	PMS2 loss	MSI-L	Failed	-	-	-	-
36	MSH6 loss	MSI-L	0	-	*MSH6* p.(R495*), p.(N975Kfs*10)	0.35, 0.41	class 5, class 5
37	MSH6 loss	MSI-L	0	-	*MSH6* p.(R922*)	0.47	class 5
38	MSH6 loss	MSS	0	-	*MSH6* p.(S445Nfs*7)	0.44	class 5
39	MSH6 loss	MSS	0	-	*MSH6* p.(Q415H)	0.43	class 3
40	MSH6 loss	MSS	Failed	-	-	-	-
41	MSH2-MSH6 loss	MSS	Failed	-	-	-	-

[a]instability in dinucleotide markers was only analyzed in discordant MSI-L and MSS cases [b]*MLH1* promoter hypermethylation was only assessed in cases with loss/focal loss of MLH1 protein expression [c]Mismatch repair gene variants were analyzed in those cases without *MLH1* promoter hypermethylation [d]classification of pathogenicity was based upon InSiGHT guidelines. Class 3 variants are predicted to be pathogenic by at least 2 out of 3 protein prediction programs used: (Align GVGD, SIFT and MutationTaster). Grey colored lines are unexplained/failed cases. MSS=microsatellite stable, MSI-L/H=microsatellite unstable with low- or high-frequency.

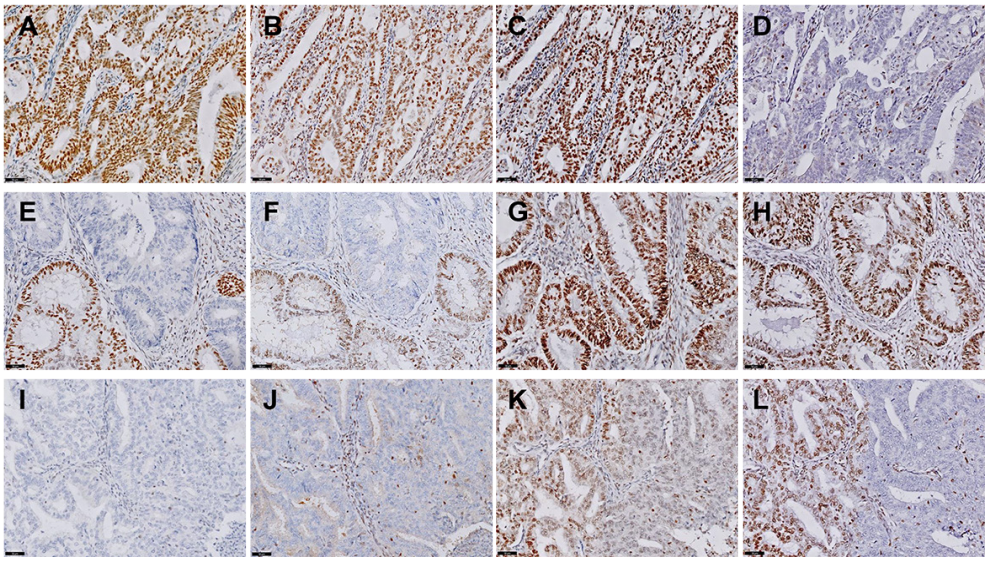

Figure 1. Representative images of MMR protein expression in EC. MMR protein expression of a MSS case with subclonal loss of MLH1 and PMS2 protein expression (A-D, case 13), a MSI-H case with complete loss of MLH1 and PMS2 and subclonal loss of MSH6 protein expression (E-H), a MSS case with loss of MSH6 protein expression (I-L, case 38). A-E-I) MLH1 protein expression, B-F-J) PMS2 protein expression, C-G-K) MSH2 protein expression and D-H-L) MSH6 protein expression. Scale bar represents 50 μM.

Overall, concordance between MSI and IHC analysis was observed in 655 of 696 cases (94%, kappa=0.854; 95%CI 0.811-0.897, P<0.001). A PMS2- and MSH6-antibody panel was as effective as the four-antibody panel in detecting MMR protein abnormalities. Twenty-seven concordant cases without *MLH1* promoter hypermethylation were identified as potential LS, but the underlying defect was not further tested. Discordant cases (n=41, 6%) included: subclonal loss of MMR expression (n=18), MSS or MSI-low cases with loss of MMR expression (n=20), and MSI-low or MSI-high cases with retained MMR protein expression (n=3). Details on the sample analysis of discordant cases are shown in Supplementary Figure S1.

All cases with subclonal loss of MMR protein expression (n=18) were evaluated in more detail by analyzing MSI in mono- and dinucleotide markers, *MLH1* promoter hypermethylation and somatic MMR- and *POLE*-exonuclease domain (EDM) variants in microdissected tumor areas (Table 2). Among 16 tumors with subclonal MLH1 and PMS2 loss, 14 had areas of differential expression that were sufficiently large to permit microdissection. Among these, MSI testing of microdissected areas was concordant with IHC analysis in 11 cases; tumor areas with retained MMR expression were MSS, whereas areas with loss of MMR expression showed MSI-H. A further three tumors showed microsatellite stability of markers in microdissected areas regardless of MMR protein expression (case 13-15, Table 2). All fourteen cases were found to have somatic promoter hypermethylation of *MLH1*. One case with subclonal loss of MSH2 and MSH6 protein expression showed microsatellite stability in the differently expressed areas

(case 17, Table 2). Unfortunately, both cases with subclonal loss of MSH2 and MSH6 protein expression had limited DNA available, and could not be analyzed in more detail.

Analysis of microdissected material from the six MSI-H cases with subclonal loss of MSH6 in addition tocomplete MLH1 and PMS2 protein loss demonstrated frameshift variants in the polycytosine tract of *MSH6* in areas with MSH6 loss and stable polycytosine tracts in areas with retained MSH6. Five of these cases displayed *MLH1* promoter hypermethylation.

We proceeded to perform detailed analysis of the 23 cases with discordant MSI status and MMR protein expression by examination of dinucleotide markers, *MLH1* promoter hypermethylation and/or NGS of the MMR- and *POLE* genes (Table 3). The two MSI-H cases (case 19-20) with retained MMR protein expression had a *POLE*-EDM variant, p.(V411L), and p.(A428T). The *POLE*-EDM p.(V411L) mutant case also harbored a truncating (p.(R563*)) and missense p.(R107W) variant in *PMS2*. The solitary MSI-L case with retained MMR protein expression (case 21) showed stability in dinucleotide markers and no somatic MMR or *POLE*-EDM gene variants.

One of 12 cases classified as MSS or MSI-L despite combined loss of MLH1 and PMS2 protein expression, showed mobility shifts in the dinucleotide markers (case 24, Table 3). Analysis of the *MLH1* promoter was successful in 11 of these cases, and revealed promoter hypermethylation in ten cases, while the single case lacking *MLH1* promoter hypermethylation was found to harbor a pathogenic *POLE*-EDM variant, p.(P286R).

Of the two MSI-L tumors with solitary PMS2 loss (case 34-35, Table 3), only one had sufficient DNA quality for further analysis. This confirmed MSI in the dinucleotide markers and revealed two likely pathogenic somatic *PMS2* variants, a start loss (p.(Met1?)) and a frameshift variant (p.(Val302Thrfs*4)).

Four of five cases classified as MSS/MSI-L with solitary MSH6 loss were informative for further analysis. All four showed microsatellite stable dinucleotide markers. Three tumors carried two (n=1) or one (n=2) pathogenic *MSH6* variants, while one tumor carried one somatic VUS predicted to affect function by two out of three protein prediction software used (Table 3). Case 41 with loss of MSH2 and MSH6 protein expression and a MSS phenotype had limited DNA, and was therefore excluded for further analysis.

Discussion

Accurate identification of MMR-deficiency in EC may be important to identify patients with a higher risk of recurrence,[3,4,28] and those whose tumors may be a consequence of LS. Similarly

to two small studies, we demonstrated high agreement (94%) between MSI and IHC analysis in 696 ECs.[13,14] Most discordant cases involved loss of MMR protein expression and a MSS/MSI-L phenotype and could be explained by *MLH1* promoter hypermethylation or MMR variants. In addition, subclonal loss of MMR protein expression generally corresponded to *MLH1* promoter hypermethylation and subclonal MSI within microdissected area of the tumor.

Importantly, the present study demonstrated that <3% of cases displayed subclonal loss of MMR protein expression. The fact that MSI and *MLH1* promoter hypermethylation were commonly found in areas with subclonal loss of MLH1 and PMS2 protein expression indicates sporadic intratumor heterogeneity.[16,18,19] However, *MLH1* germline epimutations cannot be totally excluded.[2] Subclonal loss of MSH6 expression, either in conjunction with or without MSH2 protein expression was also previously observed in EC but the underlying molecular mechanisms remain unclear.[12,20] In accordance with our findings, subclonal loss of MSH6 in cases with complete loss of MLH1 and PMS2 protein expression has been related to secondary MSI events in *MSH6*.[20,29] Although numbers are limited, subclonal loss of MMR protein expression is not associated with LS.

Our data suggest that cases with subclonal loss of MMR protein expression are best classified as MMR-deficient, even though the areas with retained expression are MSS. With regard to MMR-deficiency as a prognostic or predictive marker, it remains to be determined whether subclonal loss of MMR protein expression has the same biological behavior as tumors with MMR-proficiency. In view of the limited numbers of cases with subclonal loss of MMR protein expression (~3%), (inter)national collaborations are essential to obtain sufficient cases for such an analysis. Pending these future studies, we suggest for uniformity to classify tumors with 10% subclonal loss of MMR protein expression, as being MMR-deficient.

The interpretation of MSI-L cases remains controversial in EC and it is uncertain whether such cases are best considered as MSS or MSI. Similar numbers of DNA slippage events were observed in MSS and MSI-L ECs.[30] To date, no extensive research on the clinical implications of MSI-L in ECs has been performed, and the number of MSI-L cases in our study (n=11) was too low to permit such an analysis as well. However, most of these showed loss of MMR protein expression and would generally be regarded as abnormal by strategies that rely on IHC alone. Noteworthy, several studies have also shown MSI-L and MSS in association with loss of MMR expression and/or pathogenic germline MMR variants.[13,14,31]

Our study shows high agreement between IHC and MSI analysis, but not 100%. Of note, other assessments of DNA defects by IHC analysis e.g. *HER2* gene amplification only reaches 69-98% agreement.[32] Assessment of MMR protein expression is preferred over MSI analysis for the following reasons: lower costs, widely available, and determination of affected MMR gene.

Our findings confirm the utility of testing MMR-deficiency using a PMS2- and MSH6-IHC approach,[33] which can be followed by MLH1- and MSH2-IHC in case loss of PMS2 or MSH6 was observed. To overcome suboptimal fixation, drawback of IHC analysis, pathologists can rely on IHC analysis in pre-operative EC specimen.[23] IHC with standard well accepted techniques would appear adequate to identify EC patients with LS and to serve as a biomarker for trials of EC patients harboring MMR-deficiency.

It is debatable whether not screening for germline MMR variants is a limitation of this study. Of note, 5% of all cases in this study can be classified as potential LS (no *MLH1* promoter hypermethylation). Somatic screening of the discordant cases did show somatic variants but not in all cases. However, we cannot exclude the possibility of missed large genomic rearrangements within the tested genes, which is a limitation of NGS. Further analysis would improve understanding the molecular basis of the discordant cases, however, this study did not aim to determine the sensitivity and specificity of the two methodologies to identify LS. MSI and IHC analysis are highly concordant therefore germline testing is not needed to conclude which approach is best suitable for identifying patients with LS.

In conclusion, MSI and IHC analysis are highly concordant, also in cases with subclonal loss of MMR expression, therefore, an IHC approach is sufficient for determining MMR-deficiency in EC. Pathologists should be aware of the MMR protein expression patterns, including subclonal loss, to ensure correct classification in daily diagnostic pathology.

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Supplementary files

Supplementary Methods

Patients and study design

In total, 854 early-stage ECs from the randomized PORTEC-1 and -2 clinical trials based on availability of formalin-fixed paraffin-embedded slides and sufficient tumor material for DNA isolation were included in this study. Full details and results of both studies have been published previously.[21,22] PORTEC-1 (1990-1997) included 714 patients with stage I endometrial cancer, grade 1 or 2 with deep myometrial invasion, or grade 2 or 3 with superficial invasion. The PORTEC-2 study included 427 endometrial cancer patients between 2002 and 2006 with high-intermediate risk features: stage I, age >60 years, grade 1-2 with deep invasion or grade 3 with superficial invasion and stage IIA disease (except grade 3 with deep invasion). The PORTEC study protocols were approved by the Dutch Cancer Society and the medical ethics committees at participating centers. All patients provided informed consent for collection of their data and somatic analysis of tumor alterations. During the clinical trial period, universal LS screening was not performed. Patient and tumor characteristics, including results of pathology review, were obtained from the trial databases.

MSI assay

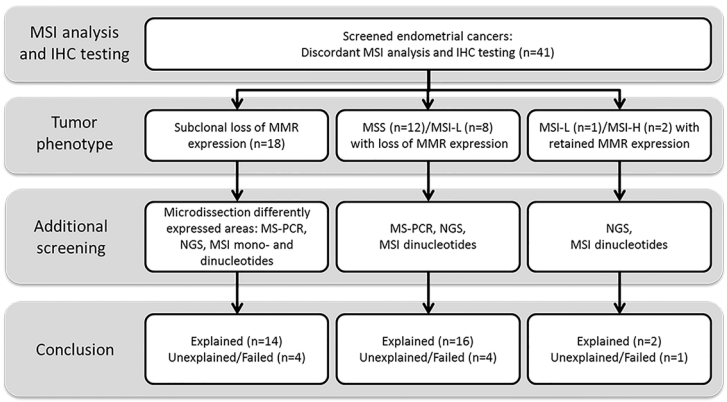
Tumor MSI status was determined as previously reported (Promega MSI analysis system (version 1.2)).[4] Tumors with instability in at least two of the five mononucleotide repeat markers were defined as being microsatellite instability-high (MSI-H), whereas those showing no instability were classified as being microsatellite stable (MSS). Tumors in which instability was detected at a single repeat were retested together with DNA extracted from unaffected myometrium to exclude biallelicity of the marker, and defined as microsatellite instability-low (MSI-L) if somatic instability was confirmed.

IHC analysis

IHC staining for MLH1, PMS2, MSH2, and MSH6 was performed on all tumors in which MSI status was successfully determined. Antigen retrieval was achieved by microwave oven procedure in 10 mmol/L Tris-EDTA buffer, pH 9.0. Sections were incubated overnight with primary antibodies against MLH1 (clone ES05, 1:100; DAKO), MSH2 (clone FE11, 1:200, DAKO), MSH6 (clone EPR3945, 1:800, Genetex) at room temperature, and PMS2 (clone EP51, 1:75, DAKO) at 4°C. Sections were incubated at room temperature with Envision FLEX+ Linker (DAKO) for 15 minutes followed by 30 minutes incubation with secondary antibody (Poly-HRP-GAM/R/R; DPV0110HRP; ImmunoLogic). 3,3'-diaminobenzidine+ was used as a chromogen. The slides were counterstained with hematoxylin, dehydrated and mounted. Slides were evaluated by two observers blinded for patient and tumor characteristics, and discrepancies were discussed and reviewed at a multihead microscope until consensus was reached.

Somatic variant screening

A custom panel was designed with the AmpliSeq™ Designer tool containing *MLH1*, *MSH2*, *MSH6*, *PMS2*, the *POLE*- and *POLD1* exonuclease domains (EDM) and exons 2, 6, 7, 12 and 13 of *MUTYH*.[26] The panel consisted of 201 amplicons (21378 bp), covering 99.3%, 99.3%, 100 and 76.8% of the coding regions of *MLH1*, *MSH2*, *MSH6* and *PMS2*, respectively. Variants were annotated to the following Genbank reference sequences: NM_000249.3 (*MLH1*), NM_000251.2 (*MSH2*), NM_000179.2 (*MSH6*), NM_000535.5 (*PMS2*), NM_006231.2 (*POLE*), NM_001256849.1 (*POLD1*) and NM_001128425.1 (*MUTYH*). Raw data analysis, alignments, variant calling and data analysis was performed as previously described.[26]



Supplementary Figure 1. Flowchart of sample analyses of cases with discordant MSI status and MMR protein expression. Explained cases are further described in the white colored lines and unexplained/failed cases in the grey colored lines in Table 2 and Table 3.

Supplementary Table S1. Clinicopathological characteristics according to the MSI status in early-stage EC (n=696).

	Total n=696 (100%)	MSS n=516 (74.1%)	MSI-L n=11 (1.6%)	MSI-H n=169 (24.3%)	*P*-value
Age, years					
Mean (range)	69 (41-88)	68 (41-88)	67 (61-79)	69 (43-88)	0.305
< 60	101 (14.5)	76 (14.7)	0	25 (14.8)	
60-70	300 (43.1)	224 (43.4)	9 (81.8)	67 (39.6)	0.096
> 70	295 (42.4)	216 (41.9)	2 (18.2)	77 (45.6)	
Tumor type					
EEC	679 (97.6)	500 (96.9)	11 (100)	168 (99.4)	0.162
NEEC	17 (2.4)	16 (3.1)	0	1 (0.6)*	
Grade					
1-2	587 (84.4)	442 (85.7)	7 (63.6)	138 (81.7)	0.075
3	109 (15.6)	74 (14.3)	4 (36.4)	31 (18.3)	
Myometrial invasion					
<50%	200 (28.7)	143 (27.7)	4 (36.4)	53 (31.4)	0.564
>50%	496 (71.3)	373 (72.3)	7 (63.6)	116 (68.6)	
Lymph vascular space invasion**					
Absent/Mild	643 (95.8)	493 (98.2)	9 (90.0)	141 (88.7)	0.000
Substantial	28 (4.2)	9 (1.8)	1 (10.0)	18 (11.3)	
Risk group					
Low	179 (25.7)	135 (26.2)	2 (18.2)	42 (24.8)	
High-intermediate	465 (66.8)	343 (66.5)	8 (72.7)	114 (67.4)	0.997
High	52 (7.5)	38 (7.3)	1 (0.9)	13 (0.8)	
Treatment					
NAT	181 (26.0)	133 (25.8)	2 (18.2)	46 (27.2)	
EBRT	336 (48.3)	248 (48.1)	5 (45.4)	83 (49.1)	0.876
VBT	179 (25.7)	135 (26.1)	4 (36.4)	40 (23.7)	

* The MSI-H NEEC with serous histology showed complete loss of MSH2 and MSH6 expression and harbored a pathogenic *TP53* mutation . **25 unknown. MSS=microsatellite stable, MSI-L/H= microsatellite unstable with low- or high-frequency, EEC=endometrioid endometrial cancer, NEEC= non-endometrioid, NAT=no additional treatment, EBRT=external beam radiotherapy, VBT=vaginal brachytherapy.

Chapter 7

Microsatellite instability derived *JAK1* frameshift mutations are associated with tumor immune evasion in endometrioid endometrial cancer

Stelloo E*, Versluis MA*, Nijman HW, de Bruyn M, Plat A, Osse EM, van Dijk RH, Nout RA, Creutzberg CL, de Bock GH, Smit VT, Bosse T, Hollema H

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Abstract

JAK1 frameshift mutations may promote cancer cell immune evasion by impeding upregulation of the antigen presentation pathway in microsatellite unstable endometrial cancers (ECs). This study investigated the *JAK1* mutation frequency, its functional implication in immune evasion and its prognostic significance in microsatellite unstable EC. Microsatellite instability and three microsatellite repeats within *JAK1* were analyzed in 181 ECs. Sixty-two (34%) ECs showed microsatellite instability, of which 22 (35%) had a *JAK1* mutation. LMP7, TAP1 and HLA class I protein expression and the presence of CD8-positive T-cells were analyzed in the microsatellite unstable ECs. *JAK1* mutant microsatellite unstable ECs showed impaired upregulation of LMP7 (P=0.074) and HLA class I (P<0.001), validated using RNAseq data of the TCGA. TAP1 expression and presence of CD8-positive T-cells were not related to *JAK1* mutations. In 198 additional microsatellite unstable ECs, the *JAK1* mutation frequency was confirmed but no prognostic significance was found. For, *JAK1* wildtype (n=135, 72%) and mutant (n=52, 28%) ECs, 10-year recurrence free rates were 84% and 77% (P=0.301). These observations show that *JAK1* mutations are highly frequent in microsatellite unstable EC, not associated with survival, but are associated with impaired upregulation of LMP7 and HLA class I and may therefore facilitate immune escape.

Introduction

About 30% of endometrial cancers, predominantly of endometrioid histology, can be molecularly characterized by microsatellite instability (MSI). MSI is a hypermutable phenotype caused by the loss of DNA mismatch repair (MMR) activity mostly due to sporadic *MLH1* promoter hypermethylation.[1] Tumors that exhibit this phenotype have numerous insertions and deletions also in coding microsatellites causing frameshift mutations and loss of protein function. The coding microsatellite-containing genes frequently affected by MSI are believed to be involved in progression of MSI tumors.[2] Some target genes, such as *BAX*, are altered in diverse MSI tumor types (e.g. colorectal- and ovarian cancer), whereas others, such as *JAK1*, have a very restricted occurrence in MSI endometrial cancers.[3-6] MSI endometrial cancers show a remarkably high number of *JAK1* frameshift mutations that may have clinical implications.[4,5]

JAK1 plays a role in the JAK/STAT pathway, which is activated by cytokines such as IFNγ, that influence several cellular processes such as cell growth and immune response.[7-9] Ren *et al.* have shown that *JAK1* mutant gynecological cancer cell lines were defective in interferon gamma (IFNγ) induced STAT1 tyrosine phosphorylation and thereby impede upregulation of antigen processing machinery components such as LMP2 and TAP1.[10] Impaired antigen processing and presentation due to hindered expression of LMP and/or TAP proteins are associated with lack of HLA class I upregulation and resistance to cytotoxic T-cell mediated lysis.[11,12] HLA class I expression has been reported as a prognostic marker in endometrial cancer patients[13-15] and upregulation of HLA class I was frequently impaired in MSI endometrial cancers.[14,16] The high rate of *JAK1* mutations in MSI endometrial cancer is suggestive of an adaptation favoring tumor survival by blocking the JAK/STAT pathway activity, and impeding an adequate immune response.

MSI tumors exhibit a high number of somatic mutations that could facilitate an immune response by presentation of neo-antigen-epitopes in the context of HLA class I molecules. Programmed death 1 expressed on cytotoxic T-cells is a checkpoint involved in immune suppression. Checkpoint inhibitors, as potential mechanism for T-cell activation, recently showed promising results in treatment of mismatch repair deficient tumors independent of tumor origin.[17] However, *JAK1* mutations and other mechanisms involved in impeding antigen presentation and expression of antigen processing machinery components in MSI endometrial cancers may interfere with new treatment regiments for MSI tumors such as the programmed death 1 inhibitor pembroluzimab.[17,18]

In this study, MSI and *JAK1* mutation status were analyzed in a study cohort of 181 tissue samples of endometrial cancer patients with the aim to evaluate that the *JAK1* locus is frequently affected by MSI, and to determine its functional implication in immune evasion

by analyzing expression of antigen presenting machinery components and the presence of cytotoxic T-cells specifically in MSI endometrial cancers. Finally, the effect of *JAK1* mutation status on survival was evaluated in a large independent cohort of 198 MSI endometrial cancer patients with mature long-term follow-up from the PORTEC-1 and -2 clinical trials.[19,20]

Methods

Patients

DNA analysis and immunohistochemical staining was performed on a study cohort of 181 endometrial cancers with endometrioid histology, treated at the University Medical Center Groningen between 1985-2004 or at the University Medical Center Leiden between 2000-2013. Classification and grading was done according to the World Health Organization criteria and staging was according to FIGO guidelines (2009). No follow-up data was available for this study cohort.

To validate our findings regarding the *JAK1* mutation frequency in MSI endometrial cancer, an independent cohort of 198 MSI early-stage endometrial cancers derived from the randomized PORTEC-1 and -2 clinical trials was used.[19,20] To estimate the impact of *JAK1* mutation on survival the same cohort of 198 MSI endometrial cancers was used.[21]

MSI and JAK1 mutation status

DNA was isolated as previously described.[22] The MSI status of each tumor was determined using the Promega MSI analysis system (version 1.2, Promega). Tumors with instability in at least two markers were defined as being high-frequency MSI whereas those showing no instability or instability in one marker were classified as being stable (MSS). *JAK1* frameshift mutations (k142fs, p430fs, k860fs) were detected by Sanger sequencing. The following primers were used: exon 5-F: 5'-GTCACATCTGGGTCCCCTTTGCCAC-3', exon 5-R: 5'-CACAAACTCCAGCTTCTCCTGGGC-3', exon9-F:5'-GTCGAGGAGGCCTTGTCCTTTGTGTC-3', exon 9-R: 5'-ACACGGGCTCTCTGCACACC-3', exon 19-F: 5'-GTATCGACTGCCTTTCACTCTG-3', exon 19-R: 5'-CTTACCTCTCCCAAGTCACGG-3'.

Immunohistochemistry

Formalin-fixed paraffin embedded 4-µm tissue sections of MSI endometrial cancers (n=58) with sufficient tumor tissue were immunohistochemically stained for expression of LMP7, TAP1, HLA class I (HCA2 and HC10) and CD8 (marker of cytotoxic T-cells). Sections were deparaffinized in xylene, rehydrated in graded concentration of ethanol and microwave antigen retrieval was performed in 10 mM citrate pH 6.0 (LMP7, TAP1), 10 mM Tris/1 mM EDTA pH 9.0 (LMP7, TAP1, HCA2, HC10 and CD8) before staining. Endogenous peroxidase was blocked by incubation in a 0.3% hydrogen peroxide solution. LMP7 and TAP1 were

stained using anti-LMP7 mouse monoclonal 1B3 (Novus Biologicals) and anti-TAP1 rabbit polyclonal H300 (Santa Cruz) as primary antibodies by incubation overnight at 4°C (dilution 1:100 and 1:50 respectively). HLA class I was stained using HCA2 and HC10 as previously described.[16] Antigen-antibody reactions were visualized using 3.3'-diaminobenzidine (DAB) and slides were counterstained with hematoxylin.

Evaluation of immunohistochemistry

Two observers blinded to clinicopathological features, MSI and *JAK1* mutation status independently evaluated the stained slides. Expression of LMP7 and TAP1 was scored using a semiquantative scale as described previously.[13,23] This score is based on the percentage of cells stained and the intensity of staining. The percentage of cells was scored on a 6 point scale with 0 for 0%, 1 for 1-5%, 2 for 5-25%, 3 for 25-50%, 4 for 50-75% and 5 for 75-100%. The intensity was scored on a 4 point scale with 0 indicating absence of staining and 3 indicating strong staining. The expression of LMP7, and TAP1 was categorized in impaired (score 0-2), normal (score 3-6) and upregulated (score 7-8) expression. For analysis of HLA class I expression, the percentage of tumor cells with membranous HCA2 and HC10 staining was quantified as previously described.[16] The expression of HLA class I was defined as follows: impaired HLA class I expression: less than 5% of tumor cells expressing both HCA2 and HC10, normal HLA class I expression; less than 5% of tumor cells expressing either of the markers, and upregulated HLA class I expression: 5% or more expressing both markers.

The number of CD8-positive T-cells was calculated using the average number of stained cells in 8 fields at 40x magnification. The average was calculated for four locations: intraepithelial at the tumor center, intraepithelial at the invasive margin, intrastromal in the tumor center and intrastromal at the invasive margin. For statistical analysis values for CD8 were dichotomized using the median as a cut off.

The Cancer Genome Atlas (TCGA) RNAseq data

Details of the TCGA RNAseq analysis have been previously reported.[1] Level 3 RSEM normalized RNA data profiled using the Illumina HiSeq RNAseq v2 were retrieved at the TCGA data portal. MSI events, differences in length of microsatellites, in 30 MSI EC patients were reported by Kim *et al.*[4] In total, 25 MSI endometrial cancers with both RSEM normalized and DNA slippage event data were informative for analysis.

Statistical analysis

JAK1 mutation status was compared between cases with and without microsatellite instability using Chi-square tests. Similarly, Chi-square tests were used to detect differences in expression of LMP7, TAP1, HLA class I and number of CD8-positive T-cells below or above the median for cases with and without *JAK1* mutation. The non-parametric Mann-Whitney test was used for all comparisons of continuous data and Spearman's rho to analyze correlation between

variables. RNAseq data was visualized by unsupervised clustering using RStudio.

To evaluate the impact of *JAK1* mutation status on survival in MSI endometrial cancer patients that participated in the randomized PORTEC-1 and -2 clinical trials, time-to-event analyses were calculated from the date of randomization to date of recurrence (vaginal, pelvic and/or distant recurrence) or to date of death (overall survival); patients who were alive and without recurrence were censored at the date of last follow-up. Survival curves were calculated using the Kaplan–Meier method with log-rank test. Analyses were performed using SPSS (v20, IBM statistics, Chicago, IL, USA).

Results

Of the 181 endometrial cancers from the study cohort, MSI was detected in 62 (34%) cases, in nine cases MSI status remained unclear due to technical failure. Twenty-two (35%) MSI endometrial cancers had a *JAK1* frameshift mutation, mainly at position K860, whereas only 3 of 110 (3%) MSS endometrial cancers had a *JAK1* mutation ($P<0.001$, Supplementary Table 1). Two of these three *JAK1* mutant MSS cases showed focal loss of MLH1 protein expression in part of the tumor as a result of *MLH1* promoter hypermethylation. There were no significant differences in age, FIGO stage, differentiation grade or tumor type between *JAK1* wildtype and mutant MSI endometrial cancers (Table 1). However, *JAK1* mutations were associated with deeper myometrial invasion ($P=0.030$; odds ratio 3.500, 95%CI 1.102-11.116).

Table 1. Clinicopathological characteristics of 58 MSI endometrial cancers of the study cohort and 187 MSI endometrial cancers of the PORTEC cohort according to *JAK1* mutation status.

	JAK1 wildtype n=36 (%)	*JAK1* mutation n=22 (%)	*P*-value
Age			
<60 years	18 (50.0)	11 (50.0)	1.000
>60 years	18 (50.0)	11 (50.0)	
Tumor type			
Endometrioid	35 (97.2)	22 (100)	0.430
Serous	1 (2.8)	0 (0.0)	
FIGO (2009)*			
I	22 (61.1)	11 (52.4)	
II	3 (8.3)	4 (19.0)	0.650
III	10 (27.8)	5 (23.8)	
IV	1 (2.8)	1 (4.8)	
Grade			
1	13 (36.1)	7 (31.8)	
2	14 (38.9)	10 (45.5)	0.885
3	9 (25.0)	5 (22.7)	
Myometrial invasion*			
<50%	21 (55.3)	6 (28.6)	0.030
>50%	15 (41.7)	15 (71.4)	

* 1 missing value

The functional implication of JAK1 in tumor immune evasion was analyzed by expression analysis of TAP1, LMP7, HLA class I and presence of CD8-positive T-cells in the MSI endometrial cancers (Table 2). Distribution of TAP1 expression was similar for both *JAK1* wildtype and mutant (P=0.151). Upregulation of LMP7 was impaired in *JAK1* mutant tumors, although not statistically significant (P=0.074). Upregulation of HLA class I was significantly impaired in *JAK1* mutant tumors (P<0.001). The expression of HLA class I was related to LMP7 expression in contrast to TAP1 (P=0.001 vs. P=0.381). Presence of CD8-positive T-cells was not related to *JAK1* mutation (Figure 1). In order to validate these findings in an independent cohort, RNAseq data was used from 25 TCGA MSI endometrial cancers with analyzed DNA slippage events. The 13 *JAK1* mutant endometrial cancers showed significantly lower expression of TAP1 (2.1-fold, P<0.001), LMP7 (3.0-fold, P<0.001), and HLA class I (2.5-fold, P<0.001) in comparison to *JAK1* wildtype endometrial cancers (Figure 2). Consistent with the results in our study cohort, *JAK1* mutation status did not correlate with CD8 expression (P=0.112).

Table 2. Expression of antigen processing machinery components TAP1, LMP7 and HLA class I in *JAK1* wildtype and mutant MSI endometrial cancers.

	JAK1 wildtype n=36 (%)	*JAK1* mutation n=22 (%)	*P*-value
TAP1*			0.151
Impaired/Normal	24 (68.6)	18 (85.7)	
Upregulated	11 (31.4)	3 (14.3)	
LMP7*			0.074
Impaired/Normal	8 (22.9)	10 (45.5)	
Upregulated	27 (77.1)	12 (54.5)	
HLA class I			<0.001
Impaired	7 (19.4)	12 (54.5)	
Normal	3 (8.3)	6 (27.3)	
Upregulated	26 (72.2)	4 (18.2)	

* 1 missing value

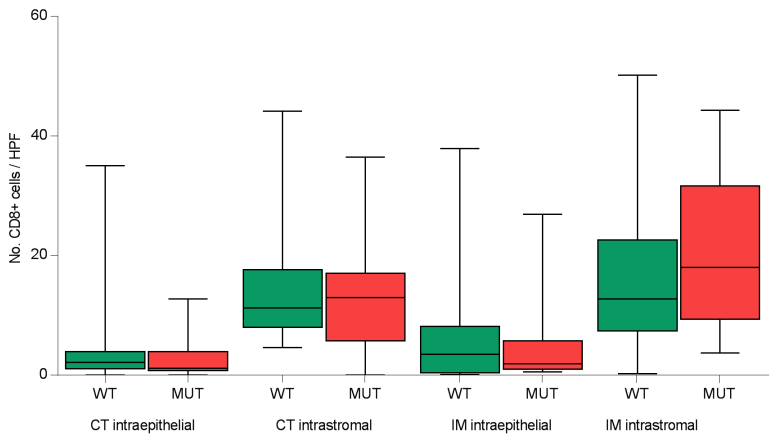

Figure 1. Quantification of CD8-positive T-cells in intraepithelial and intrastromal components in the center of the tumor (CT) and invasive margin (IM) in *JAK1* wildtype (WT) and mutant (MUT) MSI endometrial cancers.

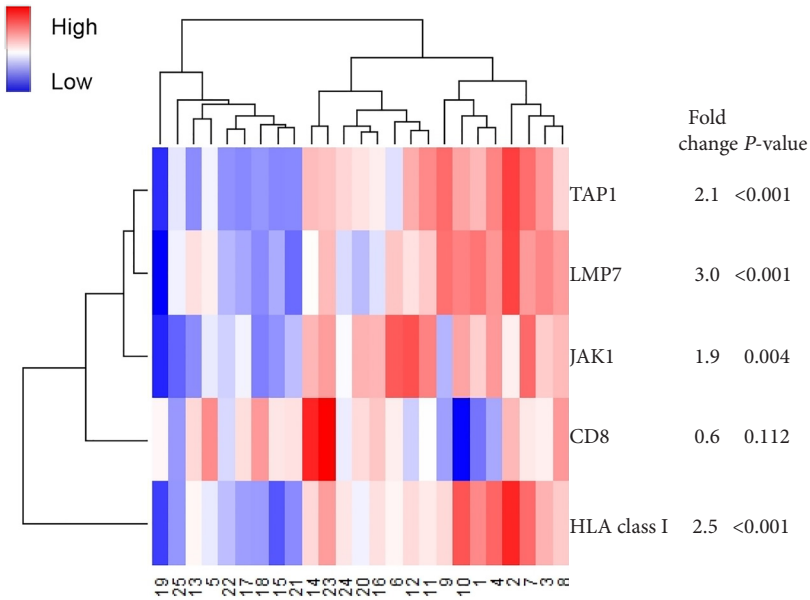

Figure 2. Heatmap of RNA expression of genes encoding for antigen machinery components. Case numbers 1-12 represent *JAK1* wildtype and 13-25 *JAK1* mutant MSI endometrial cancers. A broader analysis independent of MSI status was previously shown by Kim *et al.*

Two patient cohorts derived from the PORTEC-1 and -2 randomized trials with MSI early-stage endometrial cancers (n=198) with long-term mature follow-up data were used to investigate a possible prognostic effect of *JAK1* frameshift mutations. In this independent cohort, fifty-two (28%) of MSI endometrial cancers had a *JAK1* mutation (Supplementary table 1). No significant differences were found between *JAK1* mutation status and clinicopathological characteristics, and the association of *JAK1* mutation and deep myometrial invasion could not be confirmed (Table 1). This discrepancy could be explained by the fact that this cohort consisted of significant more tumors with deep myometrial invasion compared to the study cohort (Supplementary Table 2). For *JAK1* wildtype and mutant endometrial cancers, 10-year recurrence free rates were 84% versus 77%, respectively (P=0.301) and 10-year overall survival was 64.4% and 63.5% (P=0.716) (Figure 3). Neither did subanalysis (e.g. in grade 3 cancers, or when analyzing only pelvic and distant recurrences) show a significant difference in outcome between *JAK1* wildtype and mutant endometrial cancer patients. Survival analysis within the TCGA microsatellite unstable endometrial cancers with known *JAK1* mutation status (n=30) showed neither a survival benefit for *JAK1* wildtype tumors (data not shown).

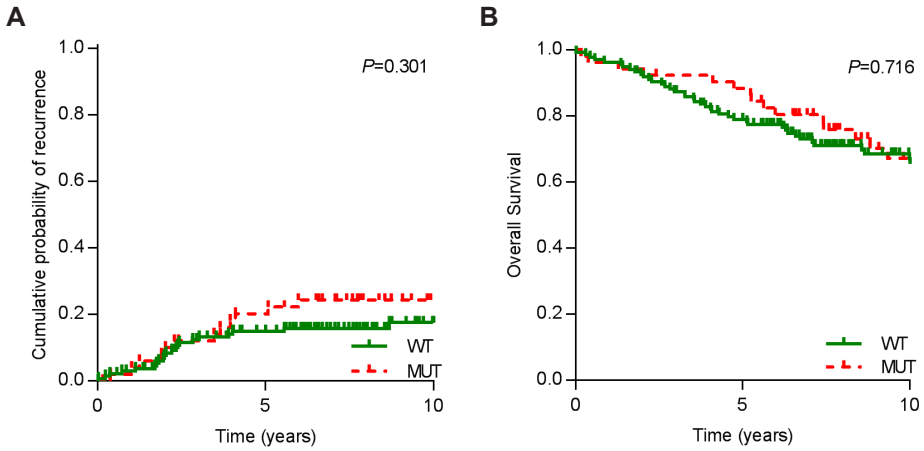

Figure 3. Clinical outcome of 198 MSI endometrial cancer stratified by *JAK1* mutations status. A) Overall recurrence rate and B) Overall survival. WT= wildtype; MUT=mutant.

Discussion

This research shows that frameshift mutations in *JAK1* frequently and almost exclusively occur in MSI endometrial cancers. *JAK1* mutations were associated with impaired upregulation of antigen presenting machinery proteins LMP7 and HLA class I. The association of *JAK1* mutations with limited expression of the antigen presentation pathway was validated using RNAseq data of the TCGA MSI endometrial cancers. Impaired upregulation of HLA class I expression interferes with tumor lysis by cytotoxic T-cells, and therefore *JAK1* mutations may facilitate an immune escape. However, no effect was observed of *JAK1* mutation status on recurrence rate and overall survival in a large independent cohort of 198 MSI endometrial cancers. These findings suggest a functional role for JAK1, although with no prognostic value which suggests that *JAK1* mutations are pivotal to cancer initiation and/or maintenance, in an intriguing tissue-specific manner.

The overall *JAK1* mutation frequency of 28% in our large series of MSI endometrial cancers is in line with findings from two recent smaller studies.[4,5] Both studies also showed that *JAK1* frameshift mutations in MSI tumors are tissue specific and significantly less important in colorectal cancers as compared with endometrial cancers. Similarly, Ren *et al.* identified *JAK1* frameshift mutations mainly in gynecological cancers, primarily in endometrial and cervical cancer, when sequencing more than 3,000 tumors from various human tissues.[10] Altogether, the occurrence of *JAK1* mutation specifically in endometrial cancer is suggestive of a positive selection for this mutation in endometrial cancer.

To date, there is very little evidence for the functionality of *JAK1* frameshift mutations in endometrial cancer. An *in vitro* study with one endometrial cancer and two ovarian cancer cell lines harboring *JAK1* frameshift mutations demonstrated that *JAK1* mutations impede STAT1 posphorylation and upregulation of antigen presenting machinery components LMP2 and TAP1.[10] Kim *et al.* have shown that *JAK1* mutations were associated with hampered JAK/STAT signaling and lymphocyte activation.[4] These findings suggest that *JAK1* mutations have a negative effect on tumor immune surveillance. Our study now confirmed impaired upregulation of LMP7 and HLA class I with no effect on TAP1 expression and the number of CD8-positive T-cells in *JAK1* mutant endometrial cancer tissue samples. The findings on protein expression were validated using RNAseq data of TCGA MSI endometrial cancers, except for TAP1. TAP1 gene expression may not be equal to its protein expression because of the small number of cases with RNAseq data (n=25) or due to the (post-)translational process into proteins. The lack of correlation between *JAK1* mutations and CD8-positive T-cells might be explained by the fact that recruitment and migration of T-cells do not rely on recognition of peptides presented by HLA class I molecules. In addition, previous studies have also shown a high correlation between LMP7 expression and HLA class I expression, but not with LMP2 or TAP1/2.[24,25] This further strengthens the argument that *JAK1* mutations favor immune escape via the JAK/STAT signaling, although, this study did not evaluate the activation status of the JAK/STAT signaling by phospho-STAT1 expression.

The association of *JAK1* mutations and lack of HLA class I upregulation in MSI endometrial cancers with no effect on clinical course was an unanticipated finding of our study. Bijen *et al.* and Yakabe *et al.* reported impaired upregulation of HLA class I as a prognostic marker for survival in endometrial cancer patients.[13,15] Of note, a large proportion of HLA class I negative endometrial cancers are MSI[14], therefore, separate analysis of MSI and MSS tumors would be of interest to determine the prognostic impact of HLA class I. This study indirectly showed no effect of HLA class I expression on survival via the *JAK1* mutation status in a large cohort of MSI, early-stage endometrial cancers. HLA class I expression was not analyzed on the precious PORTEC tissue samples as this will only validate previous findings of Ren *et al.*, the study and TCGA cohort and the fact that *JAK1* mutations have no effect on survival will remain. However, the relatively good prognosis of this cohort of early-stage cancers needs to be taken into account. The previous studies were performed in heterogeneous groups of endometrial cancers consisting of different histologic subtypes and different FIGO stages.[13-15] Therefore, the prognostic role remains to be determined in higher risk endometrial cancers. These results, in contrast to the finding that *JAK1* mutations favor immune evasion, may suggest a lack of negative selection of *JAK1* mutations specifically in endometrial cancer.

Considering the process of immune surveillance in cancer[11,12], it is likely that *JAK1* mutant tumors are still recognizable to cells of the innate immune system (natural killer cells) or that *JAK1* wildtype tumors have encountered other mechanisms to evade immune-mediated

killing. However, low numbers of natural killer cells that lack an association with HLA class I expression were observed in endometrial cancer (unpublished data by Versluis *et al.*). On the contrary, IFNγ production might be responsible for a CD4-positive T-cell-mediated antitumor immunity.[26] Furthermore, *JAK1* wildtype tumors may not demonstrate a survival benefit because of other strategies to dampen immune response such as upregulation of anti-apoptotic molecules, expression of immune-inhibitory ligands or secretion of immunosuppressive cytokines. Nevertheless, our findings imply that *JAK1* mutations may exert in part its oncogenic effects by immune escape, but we cannot exclude other contributions of JAK1 in the JAK/STAT signaling.[7]

Better understanding of the antigen-specific immune responses and tumor microenvironment may guide immunotherapy. Recently, immune checkpoint blockade were reported as promising therapies for tumors with a high mutational load, including mismatch repair deficient endometrial cancers, as a result of an increased neo-antigen specific T-cell response.[17] *JAK1* mutations in MSI endometrial cancers may interfere with the T-cell response due to impaired HLA class I or PD-L1 expression.[17,18] In melanoma, one patient without a clinical response to PD-1 blockade and increased T-cell response showed a loss of function *JAK1* mutation that unables PD-L1 upregulation. All other fifteen melanoma patients did not show genetic alterations in the interferon receptor signaling pathway.[18] These limited data suggest that *JAK1* mutations may be used as negative selective predictive biomarker for immune blockade therapy. However, additional studies are required on the immune microenvironment of *JAK1* mutant endometrial cancers (e.g. PD-L1 expression).

We have identified a high frequency of *JAK1* mutations in MSI endometrial cancers in two relatively large series of MSI endometrial cancers. In addition, *JAK1* mutations may have a negative effect on tumor immune surveillance due to lack of HLA class I upregulation on the cell surface. It must be noted that it remains unclear why *JAK1* mutations are limited to gynecological cancers and mainly to MSI endometrial cancer. However, *B2M* frameshift mutations, also leading to immune escape via loss of HLA class 1 expression, frequently occur in MSI colorectal cancer and are a rare phenomenon in MSI endometrial cancer.[14,27] In addition, Xiong *et al.* showed that JAK1 inhibition is associated with cell cycle arrest and apoptosis in colorectal cancer.[28] No effect on cell viability upon IFNγ was found in *JAK1* mutant gynecological cell lines.[10] In conclusion, we confirmed the remarkably high frequency of *JAK1* mutations and associations with impaired upregulation of antigen presenting machinery components in MSI endometrial cancers, which suggest a functional role for JAK1 in an intriguing tissue-specific manner.

Acknowledgements

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Supplementary files

Supplementary Table 1. *JAK1* mutation frequency in 181 endometrial cancers and 198 MSI endometrial cancers.

n=181*	MSI n=62 (%)	MSS n=110 (%)	*P*-value
JAK1 mutation status			<0.001
Wildtype	40 (64.5)	107 (97.3)	
K142fs	1 (1.6)	1 (0.9)	
P430fs	8 (12.9)	0	
K860fs	11 (17.8)	2 (1.8)	
P430fs & k860fs	2 (3.2)	0	

n=198**	MSI n=187 (%)
JAK1 mutation status	
Wildtype	135 (72.2)
K142fs	1 (0.5)
P430fs	7 (3.7)
K860fs	40 (21.4)
K142fs & K860fs	1 (0.5)
P430fs & k860fs	3 (1.6)

* Failed analysis for 9 cases ** Failed analysis for 11 cases.

Chapter 8

Discussion and future perspectives

Discussion and future perspectives

In this thesis, results on molecular alterations in endometrial cancer and their prognostic significance are presented with the ultimate goal to refine indications for adjuvant therapy. In addition, an improved risk assessment to guide adjuvant therapy in early-stage endometrial cancer is presented in which the prognostic molecular markers DNA polymerase epsilon (*POLE*) mutations, mismatch repair (MMR)-deficiency, *CTNNB1* (gene coding for β-catenin) mutations, L1 cell adhesion molecule (L1CAM) expression and tumor protein 53 (*TP53*) abnormalities are integrated with known clinicopathological risk factors. These promising markers, the current issues for their clinical implementation, and future perspectives in adjuvant treatment of endometrial cancer are discussed in this chapter.

POLE exonuclease domain mutations

In endometrial cancer, somatic mutations in *POLE* exonuclease domain were first identified in 2013.[1,2] *POLE* mutations were also identified in other cancer types, including melanoma, lung and colorectal cancer,[3] but the highest frequency (7%) was observed in endometrial cancer.[1,2] In addition, germline *POLE* mutations have been shown to predispose to colorectal and endometrial cancer, and other malignancies.[4] A *POLE* mutation affects the DNA binding and/or exonuclease activity of DNA polymerase epsilon, leading to misincorporation of bases during DNA replication and an excess of substitution mutations.[5,6] It is suggested that *POLE* mutations occur early during endometrial tumorigenesis, because of the minor overlap with other molecular markers of the genomic classification and its occurrence in endometrial precancerous lesions.[2,7] Following the observation that *POLE*-mutant endometrial cancers have a favorable outcome in The Cancer Genome Atlas study,[2] we and others have confirmed the prognostic impact of *POLE* mutations in higher quality clinical and/or independent cohorts.[8-14] These studies have also shown that *POLE* mutations are more common (10-20%) and even predict a favorable prognosis in high-grade and high-risk endometrial cancers.[8,9,12] Few *POLE*-mutant endometrial cancer patients have been reported who experienced relapse after primary surgery.

Studies to unravel the mechanism(s) by which these mutations lead to the excellent prognosis of these patients are limited. Several theories have been suggested. The first theory has been suggested because of the observation that the majority of endometrial cancer patients studied received adjuvant radiotherapy. An increased radiosensitivity of *POLE*-mutant tumors may explain their favorable outcome, however, limited available evidence points in the opposite direction. In two studies, locoregional recurrences were absent in patients with *POLE*-mutant endometrial cancers who received adjuvant radiotherapy as well as in those who received no additional treatment.[9,13] Of note, the number of *POLE*-mutant cancers and number of events

in the wildtype subgroup were small. Therefore, additional studies, sufficiently powered, are required to provide evidence that *POLE*-mutant endometrial cancers do not require (more aggressively) adjuvant therapy. In addition, the association of *POLE* mutations with radiosensitivity may also be investigated using a preclinical model, *POLE*-mutant cell line. A recent preclinical study exclusively investigated the sensitivity to chemotherapeutic agents.[15] No significant differences in sensitivity to 5-fluorouracil and oxaliplatin were found between *POLE*-wildtype and -mutant colorectal cancer cell lines.

The favorable prognosis of *POLE*-mutant cancers may also be explained by the high mutational burden that causes an enrichment of neo-antigens, which in turn stimulates an anti-tumor T-cell response.[16,17] This mechanism has been previously described to explain the good prognosis of mismatch repair-deficient colorectal cancers[18] and recently also confirmed for *POLE*-mutant colorectal cancers.[15] Tumor immune evasion by upregulation of immunosuppressive immune checkpoint molecules, including PD-1 and PD-L1, may explain the clinically detectable tumor mass and in some cases the lymphatic spread.[16,17] Upregulation of these immune checkpoints may suggest that the highly immunogenic *POLE*-mutant cancers are also sensitive to immune checkpoint inhibitors, similar to mismatch repair-deficient cancers.[19,20] Although recurrences rarely occur in *POLE*-mutant endometrial cancers, to date, two *POLE*-mutant endometrial cancer patients presenting with metastatic disease were treated with anti–PD-1 checkpoint inhibitors, and showed remarkable responses for over 7 and 14 months, respectively.[21,22] These findings suggest that it may be worthwhile to set up clinical trials of immune checkpoint inhibitors for advanced or recurrent *POLE*-mutant cancers. Basket trials based on the premise that *POLE* mutations predict tumor response to immune checkpoint inhibitors independent of tumor histologic origin hold promise to further advance this field.

Another explanation for the favorable prognosis of *POLE*-mutant cancers might be that the high mutational load exceeds an 'error threshold' leading to oncogenic stress and eventually apoptosis. Studies of mutant *POLE* in yeast have shown that the mutation rate can exceed the optimum for cell viability.[23] DNA mismatch repair proteins activated upon base mismatches are needed for DNA repair and to ensure cell viability. This is supported by the finding that *POLE*-mutant endometrial cancers are in general microsatellite stable.[2,10-12,24] Targeting the DNA repair pathway in advanced or recurrent *POLE*-mutant cancers may be considered to increase the mutational load to the 'error threshold' resulting in lethal mutagenesis and reduced viability. The emerging data on radiosensitivity, immune response and mutational burden may also demonstrate a combination of mechanisms responsible for the favorable prognosis of *POLE*-mutant cancers. For example, the effect of radiotherapy on promoting an immune response[25] may be further enhanced in *POLE*-mutant cancers.

MMR-deficiency

The majority of MMR-deficient endometrial cancers are due to hypermethylation of the *MLH1* gene promoter. This DNA promoter hypermethylation is strongly interrelated with histone lysine methylation mediating *MLH1* gene silencing.[26] MMR-deficient endometrial cancers are also associated with a genome-wide increase in DNA promoter CpG island hypermethylation.[2] Similarly, the CpG Island Methylator Phenotype (CIMP) has been reported in colorectal cancers.[27] The CIMP profile in colorectal cancer is strongly associated with *MLH1* promoter hypermethylation and *BRAF* mutations. However, there is no conclusive functional evidence supporting the observation that a *BRAF* mutation induces *MLH1* promoter hypermethylation or is a causal event for CIMP.[28,29] Interestingly, *BRAF* mutations are not involved in endometrial tumorigenesis.[30,31] To date, the cause and relevance of genome-wide DNA promoter hypermethylation in endometrial cancer, the link between specific molecular alterations, and similarities in the regulation of methylation across cancer types remains to be elucidated. In addition, reversal of DNA methylation and histone modifications could potentially be therapeutic, reactivating tumor suppressor genes, enhancing the anti-proliferative effect of chemotherapy or upregulating immune signaling, but this requires further investigation.[32-34] Promising preclinical data demonstrated the potential of DNA methyltransferase inhibitors and histone deacetylase inhibitors for tumor growth inhibition and apoptosis in endometrial cancer cell lines.[35,36] However, a more comprehensive analysis on the sensitivity of endometrial cancer cell lines of different histologies and molecular subgroups to epigenetic agents is needed. Then, the efficacy of such agents needs to be evaluated in animal model systems. This can potentially be performed in a panel of patient-derived tumor xenograft models which have been established and molecularly characterized.[37]

MMR-deficiency leads to base pair mismatches, insertions and deletions in microsatellite coding and non-coding regions – a phenomenon named microsatellite instability (MSI). Most of these mutations within coding microsatellites remain without consequences (passenger mutations), while other mutations are relevant for tumorigenesis. Genes with mutations in microsatellite regions are classified as 'true' MSI target genes based on mutation frequency and functional studies. It has been shown that the MSI target genes differ between MMR-deficient endometrial and colorectal cancers.[38-40] In fact, in colorectal cancer, mutations in the growth factor receptor *TGFBR2* and transcription factor *TCF-4* have been frequently found, but these have been rarely found in endometrial cancers. We and others have shown that *JAK1* inactivating mutations frequently and almost exclusively occur in MMR-deficient endometrial cancers.[40-44] MMR-deficient endometrial cancers are also highly immunogenic due to the neo-antigens as a result of microsatellite instability. However, *JAK1* mutations are associated with decreased expression of antigen presenting machinery proteins and may interfere with the T-cell response. Therefore, *JAK1* mutations may be used as negative predictive biomarkers for immunotherapy. Immunotherapy agents have been suggested for

MMR-deficient endometrial cancers, as recent studies have shown that MMR-deficiency in colorectal and urothelial cancer is predictive of response to immune checkpoint blockade.[19,20] Mutation analysis of *JAK1* in biopsies from patients with metastatic MMR-deficient cancers before and after treatment with immune checkpoint inhibitors will prove whether *JAK1* mutations are indicative for resistance to immunotherapy. Such a study in melanoma has described *JAK1* mutations as a mechanism of acquired resistance to immunotherapy.[45] Further investigation into the MSI target genes driving the tumorigenic process may help to understand the differences between MMR-deficient cancers and may guide adjuvant therapy.

Genes involved in double strand break repair by homologous recombination such as *BRCA1/2*, *ATR*, *RAD50* and *MRE11*, have also been found with mutations in their microsatellite regions as a result of the MMR-deficiency in endometrial cancer.[40,46-51] Although the gene mutation frequency varies between studies, e.g. 0-15% for *BRCA1* and 15-30% for *MRE11*, it has been reported that most MSI events occur in the homologous recombination pathway.[51] Homozygous mutations in these genes are known to cause double strand break repair defects, while MSI-derived heterozygous mutations show reduced protein expression of affected genes and reduced double strand break repair.[50-52] This suggests that DNA repair defects in addition to MMR-deficiency may be lethal, while a limited level of genetic instability may confer a growth advantage for tumor cells in carcinogenesis. Functional studies have shown that MMR-deficient cells with impaired DNA repair by homologous recombination may be sensitive to agents inducing double strand breaks, such as PARP inhibitors.[50,51] Therefore, advanced or metastatic MMR-deficient endometrial cancer patients may potentially benefit from PARP inhibitors, which are already approved for BRCA-deficient recurrent epithelial ovarian and breast cancers.[53]

A small proportion of MMR-deficient endometrial cancers are due to germline mutations in the MMR genes and are Lynch syndrome-associated cancers. The cumulative lifetime risk of endometrial cancer for women with Lynch syndrome is 50-60%.[54-56] These women are also at risk of colorectal cancer and a spectrum of other malignancies.[54-56] Although women with a diagnosis of endometrial cancer have a ~3-5% risk of Lynch syndrome, there is currently no general agreement on screening endometrial cancer patients for Lynch syndrome. In contrast, for colorectal cancer, consensus guidelines for MMR-deficiency testing have been published.[57] We and others have shown that an immunohistochemical approach is sufficient for determining MMR-deficiency in endometrial cancer.[58-61] In addition, one study suggested that the *POLE* mutation status may have implications for Lynch syndrome screening.[10] *POLE* mutations and MMR-deficiency rarely co-occur (~1%, Table 1),[1,2,10-12,17,24,62-66] however, to date none of these cancers were Lynch-associated.[10,66] Little is known about how Lynch syndrome-associated endometrial cancer differs from sporadic MMR-deficient and -proficient cancers with respect to prognosis. Two studies showed no significant difference in survival rate between Lynch syndrome-associated and sporadic MMR-deficient endometrial cancers.[67,68]

Table 1. Frequency of the co-occurrence of *POLE* mutations and MMR-deficiency (double classifiers) reported in the literature.

	no. of patients	*POLE* sequencing analysis	MMR-deficiency analysis	No. of MMR-deficient & *POLE*-mutant tumors	*MLH1* promoter methylation status	Affected MMR protein expression
Church et al[1]	173	exon 9-14	MSI assay	0	-	-
TCGA[2]	248	all exons	MSI assay	2 (0.8)	2/2 unmethylated	Unknown (2)
Meng et al[8]**	53	exon 9-14	IHC	1 (1.9)	-	MSH6 (1)
Stelloo et al[12]	116	exon 9, 13	MSI assay	2 (1.7)	2/2 unmethylated	MLH1-PMS2 (1), PMS2 (1)
Talhouk et al[11]	143	exon 9-14	IHC	1 (0.7)	-	MSH6 (1)
Billingsley et al[10]	544	exon 9-14	MSI assay	12 (2.2)	8/12 unmethylated	None (1), MSH6 (1), MSH2-MSH6 (1), unknown (5)
Howitt et al[17]	63	exon 9, 13	IHC	0	-	-
Hussein et al[62]	99	exon 9-14	IHC	1 (1.0)	Unknown	Unknown
Hoang et al[63]	14	exon 9-14	IHC	0	-	-
Stelloo et al[24]	861	exon 9, 13	MSI assay	7 (0.8)	3/7 unmethylated	None (1), PMS2 (3)
Wong et al[64]	47	exon 2-49***	MSI assay	2 (4.3)	Unknown	Unknown
Bakhsh et al[65]	483	exon 9-14	IHC	3 (0.6)	Unknown	MLH1-PMS2 (1), MSH2-MSH6 (1), MSH6 (1)
Haraldsdottir et al[66]	14	COLOseq	IHC/MSI assay	3 (21.4)	3/3 unmethylated	MLH1-PMS2 (1), MSH2-MSH6 (2)*
Total	2685	-	-	34 (1.3)	-	-

* all three cases somatic variants. ** MSI status only known for the 53 grade 3 EECs, ***also pathogenic variants in polymerase domain.

Table 2. Frequency of the co-occurrence of *POLE* mutations and p53 abnormalities (double classifiers) reported in the literature.

	no. of patients	*POLE* sequencing analysis	p53 IHC or sequencing analysis	no. of *POLE*- & p53-mutant tumors (%)
TCGA[2]	248	all exons	all exons	6 (2.4)
Stelloo et al[12]	116	exon 9, 13	IHC, indefinite exon 5-8	1 (0.9)
Hussein et al[62]	99	exon 9-14	IHC	1 (1.0)
Talhouk et al[11]	143	exon 9-14	IHC	1 (0.7)
Stelloo et al[24]	861	exon 9, 13	IHC, indefinite exon 5-8	8 (0.9)
Bakhsh et al[65]	483	exon 9-14	IHC	5 (1.0)
Total	1950	-	-	22 (1.1)

In contrast, improved survival has been reported in Lynch syndrome-associated compared to sporadic colorectal cancer matched by stage.[69,70] The survival benefit might be ascribed to the stronger immunogenic response in Lynch-associated colorectal cancer.[71] This implies that genes affected by the MMR-deficiency cause differences in neo-antigen responses, tumor and clinical behavior between MMR-deficient cancers.

We and others have shown an association between MMR-deficiency and lymphovascular space invasion (the presence of tumor cells in either blood vessels, lymphatic vessels, or both).[12,24,72-78] On the other hand, five studies found no association.[11,79-82] These conflicting findings may in part be explained by the lack of uniform scoring of lymphovascular space invasion. The mechanism underlying the correlation between MMR-deficiency and lymphovascular space invasion is unclear. Little is known about how tumor cells locate, migrate and intravasate into blood and lymphatic vessels. Mechanisms that have been described include tumor-induced or stromal microenvironment-induced (lymph-) angiogenesis, or endothelium releasing factors to recruit tumor cells.[83] Microsatellite instability affected target genes may be involved in the tumor-induced (lymph-) angiogenesis. A 3D-model in which endometrial tumor cells are co-cultured or flowed over endothelial cells may give more insight in the biological mechanism of lymphovascular space invasion. Lymphovascular space invasion is indicative of aggressive behavior,[84-86] therefore, development of targeted therapies preventing or inhibiting lymphovascular space invasion is critical to improve patient outcomes.

CTNNB1

CTNNB1 encodes for β-catenin which has two major roles in the cell. β-catenin forms a complex with the transmembrane protein E-cadherin at adherent junctions to play a role in cell-cell adhesion. In addition, free cytoplasmic β-catenin is immediately phosphorylated by the destruction complex containing AXIN1/2, APC, CSNK1A1 and GSK3β and subsequently degraded by the proteasome. Upon Wnt signaling, β-catenin stabilizes by the inactivation of the destruction complex and translocates to the nucleus to function as a transcriptional factor regulating expression of genes involved in proliferation and survival (e.g. *CCND1* and *MYC*).

Missense mutations in exon 3 of *CTNNB1* were frequently identified within the copy-number low group by The Cancer Genome Atlas, thereby contributing to refinement of the current molecular classification.[2] The copy-number low group also presented with low number of mutations, and *CTNNB1* mutations are detected as an early event in endometrial hyperplasia,[87] therefore, it is currently believed that the tumorigenesis is driven by CTNNB1 mutations. In accordance with previous studies, we showed that CTNNB1 mutations (~40%) characterized an aggressive subset within low-grade early-stage endometrial cancers.[24,88,89] The clinical significance of *CTNNB1* mutations, occurring less frequent (~10%)[2,12] in endometrial

cancer patients with high-risk features, remains to be elucidated. The mutations hinder the phosphorylation of serine and threonine residues by GSK3β, which results in nuclear accumulation of β-catenin and constitutively active Wnt signaling.[87,90,91] This is supported by the observation of increased expression of downstream targets of Wnt signaling, which were also associated with significantly poorer overall survival.[88] Thus far, there is no evidence that *CTNNB1* missense mutations, not identified in the β-catenin domain that interacts with E-cadherin, alter the interaction between β-catenin and E-cadherin. Accordingly, it has been suggested that nuclear β-catenin upon *CTNNB1* mutations drives tumorigenesis by its role in Wnt signaling.

The strong correlation of *CTNNB1* exon 3 mutations with β-catenin nuclear expression suggests the possibility to screen for alterations using an immunohistochemical approach.[87,90,91] However, we also observed that the β-catenin nuclear localization is not restricted to *CTNNB1*-mutant endometrial cancers. Alterations in the destruction complex, such as *APC* and *AXIN1* mutations, may also lead to stabilization and nuclear localization of β-catenin.[92] Loss of heterozygosity and promoter hypermethylation of *APC* were not associated with nuclear β-catenin expression.[93] Loss of E-cadherin expression, either due to *CDH1* mutations or promoter hypermethylation, results in the disruption of cell–cell adhesion, but does not lead to nuclear localization of β-catenin, as the destruction complex immediately phosphorylates β-catenin for subsequent degradation. Estrogens may also play a role in β-catenin/Wnt signaling. The role of estrogen is suggested because β-catenin is detected in the nucleus in the proliferative phase of the menstrual cycle, and in the cytoplasm and at the membrane in the secretory phase. In addition, most of the *CTNNB1*-mutant tumors are estrogen-driven.

Not all *CTNNB1*-mutant endometrial cancers exhibit nuclear staining. It is hypothesized that the amount of nuclear β-catenin in these tumors is sufficient to activate Wnt signaling but is not enough to be detected by immunohistochemistry.[91] Another hypothesis for endometrial cancers could be that the proteins responsible for the cytosol-nucleus transport of proteins without a nuclear localization sequence (such as β-catenin) are altered. The exact mechanisms controlling β-catenin nuclear localization are poorly understood, although many mechanisms have been reported.[94] A final hypothesis could be that a chaperone protein with a nuclear export sequence rapidly exports β-catenin from the nucleus to the cytosol.

L1CAM

L1CAM expression, in more than 10% of tumor cells, has been reported as a strong, independent prognostic factor for distant recurrences and overall survival in endometrial cancer.[95-99] This is consistent with findings in several other malignancies, including colorectal, breast, pancreas, and ovarian cancer.[100] It has been described that L1CAM downregulates

cadherins and interacts with molecules in the extracellular matrix, thereby enhancing cell motility and promoting invasiveness.[101] In view of their inferior prognosis, adjuvant systemic treatment may be considered for patients with L1CAM expressing tumors. However, L1CAM has been proven to confer chemotherapy resistance in glioblastoma and pancreatic cancer.[102,103] Antibody therapies targeting L1CAM have shown promising anti-tumor effects in preclinical models,[104,105] and should be further explored in metastatic L1CAM-positive endometrial cancer. Furthermore, the mechanism underlying the expression of L1CAM remains to be elucidated in endometrial cancer in order to understand and improve the therapeutic implications of L1CAM expression.

Several mechanisms leading to L1CAM expression in cancer have been described. DNA methylation of the L1CAM promoter appears to play a role in the regulation of L1CAM expression.[106-108] The L1CAM gene has two different promoter regions, among which promoter 1 was found to be the core promoter.[106,107] DNA hypomethylation at this promoter region correlated with L1CAM expression both in colorectal cancer and endometrial cancer.[106-108] In addition, the chromatin (DNA-protein complex) can also be modified and remodeled by histone acetyltransferases and histone methyltransferases. Endometrial cancer cell lines treated with an histone deacetylase inhibitor showed upregulation of L1CAM expression. Combination treatment with a DNA demethylating agent and histone deacetylase inhibitor showed even a stronger induction of L1CAM expression. Our preliminary observations also showed a correlation between *L1CAM* DNA promoter methylation status and *L1CAM* promoter histone methylation. Further studies on the interrelationships between DNA and histone modification will aid in the understanding of the epigenetic regulation.

Hypomethylation and changes in chromatin remodeling in the *L1CAM* promoter could influence the binding of transcription factors which regulate L1CAM expression. In colorectal cancer, it has been demonstrated that nuclear β-catenin activates *L1CAM* gene expression through binding to the *L1CAM* promoter region 2.[109] Overexpression of mutant β-catenin was associated with increased L1CAM expression in endometrial cancer cell lines.[110] However, no correlation between L1CAM and nuclear β-catenin expression was found in the molecular analysis of the PORTEC-1 and -2 study samples, in line with the finding that expression of L1CAM mostly depends on the core promoter region 1.[106,107] Another transcription factor implicated in L1CAM expression is SLUG, that functions upon transforming growth factor β1 (TGFβ1) induction.[111] *In vitro* studies in endometrial cancer cell lines have shown that SLUG binds to the *L1CAM* promoter region 1 upon TGFβ1 treatment or SLUG overexpression, thereby activating L1CAM expression.[95,110] Snail, a family member of SLUG, is also upregulated upon TGFβ1 induction and can lead to downregulation of the estrogen receptor.[112] Interestingly, similar to breast cancer studies, we found a strong inverse correlation between estrogen receptor and L1CAM expression, but not all L1CAM expressing cancers showed loss of the estrogen receptor.[95,113] Further studies are necessary to identify if there is a cause-effect relationship.

Several microRNAs (miRNAs) have also been shown to inhibit L1CAM expression. miR-34a was identified as a binder to the L1CAM-3'UTR region in endometrial cancer which leads to inhibition of L1CAM expression.[100] A functional link between wildtype p53 by upregulation of miR-34a and loss of L1CAM expression has been identified.[114] A significant association was found between mutant p53 and L1CAM expression, but the majority of endometrial cancers with mutant p53 expression displayed low L1CAM expression.[24,98,114] In early-stage endometrial cancer, we have shown that L1CAM is a strong prognostic marker, often coexisting with but independent from p53 expression.[24] Another way to increase miR-34a levels is by DNA demethylation.[114] Treatment with demethylating agents in L1CAM expressing endometrial cancer cells leads to upregulation of miR-34a and decreased L1CAM expression. In conclusion, the (epi)genetic mechanisms leading to upregulation of L1CAM expression in cancer are complex. Non-endometrioid endometrial cancers are more frequently L1CAM positive than pure endometrioid cancers (43-100% vs. 6-33%).[95-99,115,116] Therefore, it may be hypothesized that L1CAM expression is differently regulated in non-endometrioid histological subtypes.

p53

The gene *TP53*, encoding the protein p53, is frequently mutated in endometrial cancer. *TP53* mutations are mainly identified in non-endometrioid cancers (>90%) and in ~10-20% of endometrioid cancers (mainly grade 3).[2] Therefore, *TP53* mutations cannot be used as marker to reliably distinguish between histological subtypes. Further, *TP53*-mutant serous cancers frequently harbor *PPP2R1a* mutations, whereas *PTEN* mutations are frequently identified in *TP53*-mutant endometrioid endometrial cancers.[117,118] The majority of *TP53* mutated cancers are defective for apoptosis and cell cycle arrest upon stressed conditions, thereby contributing to tumor development. *TP53* mutation is a strong predictor of an aggressive clinical course, even in a selected cohort of high-grade and high-risk endometrial cancers.[2,12,119]

We and others have suggested that routine evaluation of p53 abnormalities might refine prognostic risk assessment of endometrial cancers.[11,24] p53 immunohistochemistry, which can be easily implemented in daily diagnostics, can be used as a surrogate marker for *TP53* mutations, but this requires correct interpretation of the staining. *TP53* abnormalities correlate with certain p53 immunostaining patterns including diffuse intense nuclear staining and totally absent staining, whereas wildtype staining is focal, weak and heterogeneous.[120,121] Interestingly, in the PORTEC-1 and -2 molecular studies, 15% of p53-mutant endometrial cancers displayed subclonal p53-mutant expression. It remains to be determined whether subclonal p53-mutant expression has the same unfavorable prognostic impact as p53-mutant expression in all tumor cells. In chronic lymphocytic leukemia it was found that p53 abnormalities in a fraction or in all tumor cells have the same impact on clinical outcome.[122]

Furthermore, pathologists need to be aware of the rare cases (~1%, Table 2) that present with p53 abnormalities together with the favorable *POLE* mutation. Some studies have classified these tumors within the *POLE*-mutant subgroup, however, further analysis on a larger subset is required to determine the prognostic significance, mutational load and copy-number status of these rare cases with multiple classifiers.

Adjuvant chemotherapy may be considered for p53-mutant endometrial cancers, although the efficacy of adjuvant chemotherapy still needs to be proven for patients with endometrial cancer. However, *TP53* mutations have been strongly associated with chemoresistance in several cancers.[123,124] Since *TP53* is frequently mutated in cancer, approaches have been undertaken to target p53 for the development of precision cancer medicine.[125-127] Strategies that are currently being explored to target mutant *TP53* include: promotion of mutant p53 degradation, restoration of transcriptional activity of mutant p53, restoration of wildtype p53 and induction of synthetic lethality. Some drugs reactivating wildtype p53 or depleting mutant p53 are already in phase I/II clinical trials, including ovarian cancer (ClinicalTrials.gov). However, several issues (e.g. unwanted side effects, acquired resistance after prolonged exposure) have been described that need to be resolved before the provision of p53 as targeted therapy.[125]

Current issues

There are some challenges to overcome before an individual molecular profile predicting recurrence risk in patients with endometrial cancer can be clinically implemented. Ideally, a combination of clinicopathological risk factors and the above discussed molecular alterations should be used. Prognostic validation of the molecular integrated risk assessment is essential,[11,12] although the prognostic significance of the molecular alterations and classification have been previously reported. A randomized phase III trial, PORTEC-4[a], has been initiated to assess if a molecular integrated risk assessment improves prognostication and recommendations for adjuvant radiotherapy for early-stage endometrial cancer.[128] The molecular integrated risk profile guided recommendation for adjuvant treatment (favorable: observation; intermediate: vaginal brachytherapy; unfavorable: external beam radiotherapy) will be compared to standard postoperative vaginal brachytherapy based on high-intermediate risk factors. PORTEC-4[a] will mainly address the issue of overtreatment because the exclusion of serous cancers affect the number of p53-mutant endometrial cancers entering the trial. Still, some patients will receive external beam RT instead of brachytherapy in view of unfavorable risk features. The prognostic significance of the molecular classification in patients with clinicopathological high-risk features still needs to be confirmed. This can be analyzed in endometrial cancer tissue samples from patients who participated in the PORTEC-3 randomized phase III trial (see General Introduction Table 4 for details on PORTEC-3).[129]

The molecular risk assessment can be achieved using clinically applicable methods on formalin-fixed paraffin-embedded endometrial cancer samples. The feasibility of determining the molecular profile within tight time limits will also be prospectively established in the PORTEC-4[a] trial. If risk assessment and decision-making is desired before surgery, we and other have suggested the possibility to determine the molecular profile on endometrial biopsy or curettage specimens obtained at the time of diagnosis.[130-132] The high concordance of molecular alterations between pre-operative and hysterectomy specimens also implies that intratumor heterogeneity is not a major issue for the molecular classifiers. This is supported by the finding that alterations in MMR and p53 protein expression are mostly seen in all tumor cells of the analyzed tumor tissue slide.[61,133-137] Finally, this has also been confirmed in our follow-up study in which multiple tumor samples of one endometrial cancer were analyzed for molecular alterations.[138] Analyzing one tumor sample was found to be sufficient in >90% of endometrial cancers to ensure correct molecular classification.

The PORTEC-4[a] feasibility data will also focus on the practical issues such as the logistics of specimen processing, molecular assessment and analysis of the results. The proportion of samples with successful molecular analysis will be determined. In case of quality issues, e.g. suboptimal tissue fixation, pathologists can rely on immunohistochemical analysis of the pre-operative endometrial cancer specimen. Another issue which has already been pointed out in the above sections is how to deal with the rare occurrence of double classifiers (e.g. co-occurrence of *POLE* and *TP53* mutation in one tumor). In view of the very small numbers of cases with double classifiers, (inter)national collaborations are essential to further study these uncommon cases and determine which molecular alteration has the strongest impact on outcome.

The integrated risk assessment combining clinicopathological and molecular prognostic factors is mostly based on objective variables, such as mutational status of *POLE*. Immunohistochemical analyses tend to be less accurate and less objective than mutational analyses. However, training sessions for pathologists will improve the performance of interpretation of staining results. For the interpretation of MMR protein expression, there is knowledge and experience from the colorectal cancer diagnostic pathology. In addition, *TP53* mutation analysis may be helpful in reaching correct assessment of p53 staining in ambiguous cases. A recent study showed a good agreement between the different pathologists' interpretations on L1CAM protein expression (kappa of 0.82).[99] Future studies need to prove whether the cut-off of 10% for L1CAM positivity also applies for high-risk endometrial cancers.[98,99] In addition to the integration of molecular alterations in the clinicopathological risk assessment, quantitative grading (focal versus extensive) of lymphovascular space invasion further improves the prediction of patients' recurrence risk.[86] The prognostic significance of lymphovascular space invasion has been firmly established and is incorporated in clinical decision making by its implementation in the ESMO-ESTRO-ESGO guidelines for treatment

of endometrial cancer. Recent work established that pathologists can reliably distinguish lymphovascular space invasion from its mimics, but that training and experience are required to improve reproducibility of the quantification of lymphovascular space invasion.[139]

Future perspectives

The performance of the molecular integrated risk assessment is considered to be a good model (AUC >0.8), but not yet excellent.[24] In addition, the largest subgroup of The Cancer Genome Atlas molecular classification, the copy-number low group, cannot be identified by a specific molecular classifier and is still a heterogeneous subgroup.[2] Therefore, there is still room for improvement of the molecular classification and refinement of the molecular integrated risk model. The copy-number low, endometrioid endometrial cancers can be characterized by *CTNNB1* mutations, chromosome 1q amplification, and increased hormone receptor expression. *CTNNB1* mutations have already been included in the molecular integrated risk assessment, as *CTNNB1* was found to be an independent risk factor for the copy-number low group of early-stage endometrial cancer.

Amplifications of chromosome arm 1q were previously identified as prognostic factor in single biomarker studies.[140,141] Analysis of 1q amplifications in the PORTEC-1 and -2 endometrial cancers have confirmed the prognostic significance of 1q. Although 1q amplifications have been frequently noted in cancer, the candidate genes on chromosome 1q that contribute to tumorigenesis remain to elucidated.[142] More than 600 genes are located on the chromosome 1q which may play a role in tumorigenesis, including genes involved in calcium-binding (*S100-family*), phosphoinositide signaling pathway (*PI3KC2B*, *AKT3*), chromatin modifying (*SETDB1*).[142] *MDM4*, located on chromosome 1q32, may also be a candidate gene because of its role in p53 inactivation. However, we were unable to correlate *MDM4* gene amplification with increased protein levels using immunohistochemistry. We observed that *MDM4* gene amplification co-occurs with p53 mutant-like expression in endometrial cancer, although, an inverse correlation was observed in breast cancer.[143] MDM4 oncogenic functions independent of p53 have also been described.[144] Future studies may confirm this p53 independent tumorigenesis in endometrial cancer, which will make *MDM4* a potential targetable alteration.

Patients with high-risk endometrial cancer have an inferior prognosis due to their higher risk of distant spread. Recently, studies investigating the efficacy of combined adjuvant chemotherapy and radiotherapy compared to radiotherapy or chemotherapy alone in these patients (PORTEC-3, GOG-258) have completed accrual and results are awaited. The identification of molecular subgroups with distinct clinical outcomes among the broad group of high-risk endometrial cancers would improve patient selection based on their molecular and immunological risk factors and enable tailored treatment. First findings require validation

in a larger series of high-risk endometrial cancers before they can be introduced in clinical decision making.[2,12,145] In addition, the identification of molecular alterations predicting chemotherapy response or resistance is mandatory to reduce unnecessary exposure to toxic chemotherapeutic agents. Patients receiving combined chemotherapy and radiotherapy in the PORTEC-3 trial had significant more severe adverse events and reduced health-related quality of life during and after treatment compared with radiotherapy alone.[146] More importantly, the identification of actionable molecular alterations may lead to tailored targeted therapies (e.g. checkpoint inhibitors, or drugs targeting p53) for endometrial cancer patients with high-risk features.

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Summary

Endometrial cancer is a cancer that arises from the inner lining of the uterus and is the 5th most common cancer in women worldwide. The incidence is increasing due to ageing of the population, increased life expectancy, increasing rates of obesity, and decreasing rate of hysterectomy for benign causes. Postmenopausal vaginal bleeding is the most common symptom, which often leads to diagnosis of endometrial cancer at an early stage before it has spread outside the uterus. The standard treatment consists of abdominal or laparoscopic hysterectomy with bilateral salpingo-oophorectomy, in case of risk factors followed by tailored adjuvant therapy.

Over the last two decades, the management of endometrial cancer has improved by refining indications for adjuvant therapy and introducing adjuvant treatment modalities with fewer side effects. Adjuvant therapy of early-stage endometrial cancer is tailored to the patient's risk of disease recurrence, generally based on three risk groups using clinicopathological factors such as age, stage, histological subtype, tumor grade, and lymphovascular space invasion. The PORTEC-1 and -2 (Post Operative Radiation Therapy for Endometrial Cancer) clinical trials have contributed evidence that adjuvant radiotherapy can be safely omitted in patients with low- and intermediate-risk features, and that endometrial cancer patients with high-intermediate risk features can effectively be treated with vaginal brachytherapy. Recently, two randomized trials, evaluating the role of adjuvant chemotherapy in combination with external beam radiotherapy for high-risk endometrial cancer (PORTEC-3 and GOG258) have completed accrual and results are awaited. Surgery followed by adjuvant treatment based on risk factors provides effective curative treatment for the majority of patients with endometrial cancer, but, considerable over- and undertreatment remains and patients could benefit from refinement of more individual risk assessment and tailored therapy.

Traditionally, endometrial cancer was classified into two subtypes based on epidemiology, histopathology and clinical behavior by the dualistic model of Bokhman. The histologic subtypes and molecular alterations expanded the dualistic model at a later stage – type I, mainly endometrioid, low grade, hormone receptor positive, prognostically favorable endometrial cancers and type II mainly non-endometrioid, high grade, receptor negative, unfavorable cancers. The Cancer Genome Atlas studies provided a molecular classification of endometrial cancers and identified four molecular subtypes based on comprehensive genomic, transcriptomic and proteomic analysis. These four molecular subgroups; polymerase-epsilon (*POLE*) ultramutated, microsatellite instability hypermutated, copy-number low with frequent *CTNNB1* mutations, and copy-number high with frequent *TP53* mutations, were associated with distinct clinical outcomes. Subsequent studies have shown that L1CAM is also an emerging prognostic marker, which has been confirmed in The Cancer Genome Atlas cohort using RNA expression data. These recent advances in the molecular understanding of

endometrial cancer may strongly improve the clinicopathological risk assessment. Moreover, new effective targeted drugs may be developed for patients with high-risk features or recurrent disease.

This thesis focuses on molecular alterations and their prognostic significance in endometrial cancer, investigated mainly in early-stage endometrial cancers with (high)-intermediate risk features, to refine clinicopathological risk assessment and direct adjuvant therapy. **Chapter 2** provides evidence that molecular alterations such as *CTNNB1* mutations and microsatellite instability can be successfully evaluated in pre-operative endometrial curettage specimens using DNA-based and immunohistochemical techniques. The paired curettage and hysterectomy specimens of 48 endometrial cancers showed 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 molecular alterations on hysterectomy specimens, as the results can safely be translated to diagnostic tissue samples and thus be used in pre-surgical clinical decision making. The prognostic value of *POLE* exonuclease domain mutations is described in **Chapter 3**. The Cancer Genome Atlas studies were the first to show the association of *POLE* exonuclease domain mutations with a high mutation burden and favorable clinical outcome. We confirmed the frequency and favorable prognosis of *POLE* exonuclease domain mutations in 788 early-stage endometrial cancer tissues from patients enrolled in the PORTEC-1 and -2 clinical trials and in 681 patients from three additional endometrial cancer series. Although the *POLE* subgroup is relatively small (5-10%), the strong association of *POLE* exonuclease domain mutations with grade 3 tumors, and the highly favorable prognostic outcome of this subgroup suggests that strategies to minimize therapy in these patients are worthy of investigation.

Several other molecular alterations, such as loss of hormone receptor expression, *CTNNB1* and *FGFR2* mutations, have been previously reported as having prognostic potential in single biomarker studies. **Chapter 4** reports on an integrated analysis of molecular and clinicopathological prognostic factors in the PORTEC-1 and -2 tissue samples resulting in a comprehensive overview of the prognostic potential of these factors. The clinical outcome of (high)-intermediate risk endometrial cancer patients was very heterogeneous, and our study showed that approximately 15% of these patients had unfavorable prognostic features (substantial lymphovascular space invasion, p53-mutant, and/or >10% L1CAM expression), 35% had intermediate features (microsatellite instability or *CTNNB1*-mutant) and 50% had favorable features (*POLE*-mutant, *CTNNB1*-wildtype and remaining tumors). This integrated risk model combining molecular and clinicopathological risk factors clearly improved risk prognostication. Cases with unfavorable prognostic features may need more intensive treatment to improve their prognosis, while overtreatment and unnecessary exposure to potential toxic treatment might be avoided in cases with favorable prognostic features.

In **Chapter 5**, a series of 116 endometrial cancers with high-risk features was molecularly analyzed to explore whether high-risk endometrial cancers can also be classified in molecular prognostic subgroups. Our findings indicate that molecular analysis can discriminate high-risk patients with a favorable (*POLE*-mutant, or microsatellite instability) and unfavorable (p53-mutant and remaining tumors with no specific molecular classifier) clinical outcome. Moreover, within the latter group of truly high-risk patients, targetable alterations were identified which may serve as targets for individualized treatment. The identified mutations in the PI3K-AKT pathway, mutations in *FBXW7* and *FGFR2* and hormone receptor positivity are potentially targetable with PI3K-AKT-mTOR inhibitors, HDAC inhibitors, FGFR inhibitors or hormonal therapies. To conclude, our findings emphasize that molecular classification does not replace but clearly improves the clinicopathological risk assessment by refining the prediction of tumor behavior and identifying targets for therapy.

DNA mismatch repair-deficiency, characteristic of one of the prognostic molecular subgroups, was detected by microsatellite instability testing in our studies, while others analyzed the protein expression of typically four mismatch repair proteins. **Chapter 6** focuses on the optimal approach for mismatch repair-deficiency testing in endometrial cancer. Microsatellite instability analysis and mismatch repair protein expression analysis using a two- (PMS2 and MSH6) or four-antibody immunohistochemical approach were highly concordant, also in <3% of the cases with subclonal loss of mismatch repair protein expression. These findings, based on comprehensive analysis of 696 endometrial cancers, provide sufficient evidence that a two-antibody immunohistochemical approach is both practical and feasible for mismatch repair-deficiency testing in routine clinical pathology. The mismatch repair-deficiency affects microsatellites in genes causing frameshift mutations and functional inactivation of affected proteins, thereby promoting (driver mutation) or not promoting (passenger mutation) tumor survival. **Chapter 7** describes the high frequency of *JAK1* frameshift mutations in endometrial cancers with microsatellite instability and its possible mechanism contributing to tumorigenesis. *JAK1* frameshift mutations were not associated with survival, but showed an association with impaired upregulation of LMP7 and HLA class I. This impaired upregulation may interfere with tumor lysis by cytotoxic T-cells, and therefore *JAK1* frameshift mutations may facilitate immune escape.

Taken together, better understanding of the molecular profiles in endometrial cancer may greatly improve the clinical decision-making for endometrial cancer patients, reducing over- and undertreatment by integrating clinicopathological and molecular risk factors to direct adjuvant therapy. **Chapter 8** describes current issues for clinical implementation, highlights remaining questions about the established molecular markers and discusses future perspectives. The clinical utility for tailoring adjuvant therapy and the feasibility of determining the molecular integrated profile within tight time limits needs to be prospectively established. In addition, better understanding of the molecular mechanisms is needed to improve

therapeutic outcome. For instance, *POLE* mutations may guide alternative management and surveillance choices for endometrial cancer patients; however, the mechanism(s) by which these mutations lead to the excellent prognosis of these patients is unknown. Endometrial cancers with microsatellite instability are associated with genome-wide DNA promoter hypermethylation, which makes epigenetic therapy an attractive approach for cancer treatment. However, the cause and relevance of epigenetics in endometrial cancer remains to be elucidated. L1CAM might be a target for antibody-therapy, but the mechanism underlying the expression of L1CAM are not clearly established. Finally, the performance of the molecular integrated risk assessment is considered to be a good model, but may be further refined to an excellent model. The identification of actionable molecular alterations may provide an even more personalized approach to cancer therapy.

Nederlandse samenvatting

Baarmoederkanker, ofwel endometriumcarcinoom, is een kwaadaardig gezwel dat ontstaat in het slijmvlies dat de binnenkant van de baarmoeder bekleed. Het is wereldwijd de vijfde meest voorkomende kankersoort bij vrouwen. De incidentie van endometriumcarcinoom neemt toe als gevolg van de vergrijzing van de bevolking, verlengde levensverwachting, obesitas, en het minder vaak verwijderen van de baarmoeder bij goedaardige aandoeningen. Het meest voorkomende symptoom van endometriumcarcinoom is het optreden van postmenopauzaal vaginaal bloedverlies, waardoor de aandoening veelal in een vroeg stadium wordt ontdekt waarin de tumor beperkt is tot de baarmoeder. De eerst aangewezen behandeling van het endometriumcarcinoom is een operatie, waarbij de baarmoeder wordt verwijderd (hysterectomie) en doorgaans ook de eierstokken en eileiders, eventueel gevolgd door aanvullende behandeling afhankelijk van risicofactoren.

De afgelopen jaren hebben verbeteringen plaatsgevonden in de behandeling van endometriumcarcinoom, door betere mogelijkheden in het voorspellen van een recidief en door nieuwe behandelopties met minder bijwerkingen. De noodzaak tot aanvullende behandeling met inwendige of uitwendige radiotherapie bij patiënten met vroeg stadium endometriumcarcinoom is afhankelijk van de recidiefkans die bepaald wordt door een combinatie van de volgende klinische en pathologische risicofactoren: leeftijd, FIGO-stadium, histologisch type, differentiatiegraad, en aanwezigheid van tumorgroei in lymfevaten. Op basis van deze risicofactoren kunnen patiënten worden ingedeeld in drie risicogroepen. De PORTEC-1 en -2 (Post Operatieve RadioTherapie bij EndometriumCarcinoom) klinische studies toonden dat de patiënten met een laag en intermediair risicoprofiel geen aanvullende behandeling nodig hebben, en dat bij patiënten met een hoog-intermediair risicoprofiel maximale locoregionale controle met zo min mogelijk bijwerkingen wordt bereikt met een korte behandeling van 3 inwendige bestralingen, ook wel vaginale brachytherapie genoemd. In de recentelijk gesloten PORTEC-3 en GOG258 klinische trials, waarvan de resultaten worden afgewacht, is bij de meer zeldzame patiëntengroep met hoog-risico endometriumcarcinoom gekeken naar het effect van de combinatie van uitwendige radiotherapie met aanvullende chemotherapie, vergeleken met radiotherapie of chemotherapie alleen.

Operatie met eventueel aanvullende radiotherapie en/of chemotherapie is in de meeste gevallen een curatieve behandeling, maar ondanks het gebruik van de klinische en pathologische risicobepaling is er nog altijd sprake van overbehandeling van patiënten met een goede prognose die geen extra behandeling nodig hebben, en onderbehandeling van patiënten waarvan de tumor een slechtere prognose heeft dan ogenschijnlijk lijkt. Een meer geïndividualiseerde risicobepaling kan patiënten gerichter behandelen.

Van oudsher wordt endometriumcarcinoom onderverdeeld in twee type maligniteiten gebaseerd op klinische en pathologische kenmerken, zoals beschreven in het dualistische model van Bokhman. De histologische subtypes en moleculaire afwijkingen werden later ook ondergebracht in deze tweedeling – het type 1 carcinoom, endometrioid histologisch subtype, laaggradig, hormoon receptor-positief en met een gunstig klinisch beloop en het type 2 carcinoom, non-endometrioid histologisch subtype, hooggradig, hormoon receptor-negatief en met een ongunstig klinisch beloop. Uitgebreid genomisch onderzoek van de "The Cancer Genome Atlas" studies heeft vier moleculaire subgroepen binnen het endometriumcarcinoom onderscheiden, waardoor de klassieke tweedeling verder wordt aangescherpt en opgedeeld. De vier moleculaire subgroepen; (1) polymerase epsilon (*POLE*)-gemuteerd met extreem veel mutaties, (2) microsatelliet instabiel met veel mutaties, (3) weinig copynumbervariaties (deletie of duplicatie van bepaalde stukken DNA op een chromosoom) en frequent *CTNNB1* mutaties, en (4) veel copynumbervariaties en frequent *TP53* mutaties, presenteerden zich met ieder een eigen prognose. Andere onderzoeken toonden dat L1CAM expressie in het endometriumcarcinoom ook een belangrijke prognostische factor is. Deze bevinding werd gevalideerd in de "The Cancer Genome Atlas" studies met behulp van de L1CAM RNA expressie gegevens. De toegenomen kennis betreffende de moleculaire veranderingen in het endometriumcarcinoom draagt mogelijk bij aan een verbetering van de risico-inschatting van het biologisch gedrag van endometriumcarcinoom. Bovendien kan de nieuwe kennis worden gebruikt om doelgerichte, geïndividualiseerde therapieën te ontwikkelen voor patiënten met hoog risicofactoren of recidiverende ziekte.

Het onderzoek in dit proefschrift richt zich op moleculaire afwijkingen in voornamelijk patiënten met vroeg stadium endometriumcarcinoom en (hoog)-intermediaire risicofactoren, die het klinisch beloop met betrekking tot locoregionaal terugkeren of metastasering van de kankercellen en overleving van de patiënt kunnen voorspellen. Het doel is om de huidige risico-inschatting op basis van klinische en pathologische kenmerken en indicatiestelling voor aanvullende behandeling te verbeteren. In **Hoofdstuk 2** wordt beschreven dat moleculaire afwijkingen, zoals *CTNNB1* mutaties en microsatelliet instabiliteit, met behulp van DNA- en immunohistochemische technieken al in het preoperatieve curettageweefsel kan worden bepaald. De 48 gepaarde preoperatieve curettage- en hysterectomieweefsels toonden dat moleculair onderzoek in het preoperatieve weefsel betrouwbaar de afwijkingen kan voorspellen in het hysterectomieweefsel van het endometriumcarcinoom. Deze studie laat hiermee zien dat moleculaire afwijkingen in het hysterectomieweefsel direct vertaald kunnen worden naar preoperatief weefsel en kunnen helpen bij de besluitvorming van (aanvullende) behandeling. In **Hoofdstuk 3** wordt de prognostische waarde van *POLE* proofreading domein mutaties beschreven, die door "The Cancer Genome Atlas" studies voor het eerst werden geassocieerd met extreem veel mutaties en een gunstige prognose. De frequentie van *POLE* proofreading domein mutaties en de relatie met een gunstig ziektebeloop werd bevestigd in 788 patiënten met vroeg stadium endometriumcarcinoom, die geparticipeerd hebben

in die PORTEC-1 en -2 klinische studies en in drie onafhankelijke cohorten van samen 681 patiënten met endometriumcarcinoom. *POLE* proofreading domein mutaties komen frequenter voor in patiënten met hooggradig endometriumcarcinoom en voorspellen ook in deze patiënten een gunstig klinisch beloop. Deze bevinding suggereert dat patiënten met een *POLE*-gemuteerde endometriumcarcinoom in de huidige situatie overbehandeld worden en vervolgstudies nodig zijn om te onderzoeken of voor deze patiënten aanvullende behandeling met de daarmee gepaard gaande bijwerkingen weggelaten kan worden.

Andere moleculaire afwijkingen zoals verlies van hormoon receptor expressie, *CTNNB1* en *FGFR2* mutaties zijn ook eerder beschreven als prognostische factoren in studies, waarin maar naar één specifiek biologisch kenmerk (biomarker) is gekeken. **Hoofdstuk 4** beschrijft een geïntegreerde analyse van moleculaire afwijkingen, klinische en pathologische factoren die is uitgevoerd op endometrium-carcinoomweefsel van patiënten, die hebben geparticipeerd in de PORTEC-1 en -2 klinische studies. Het klinisch beloop van patiënten met endometriumcarcinoom en (hoog)-intermediaire risicofactoren was heterogeen; ~15% toonde ongunstige prognostische factoren (uitgebreide aanwezigheid van tumorgroei in lymfevaten, p53-mutante expressie, en/of >10% L1CAM expressie), 35% toonde een gemiddeld risico (microsatelliet instabiliteit of *CTNNB1*-gemuteerd), en 50% toonde gunstige factoren (*POLE*-gemuteerd, *CTNNB1*-wildtype en resterende tumoren zonder specifieke moleculaire classifier). Dit geïntegreerde model van moleculaire afwijkingen, klinische en pathologische factoren verbeterde sterk de risico-inschatting van het biologisch gedrag van het endometriumcarcinoom. Patiënten met endometriumcarcinoom en ongunstige prognostische factoren zouden mogelijk geïntensiveerde nabehandeling moeten krijgen om hun klinische uitkomst te verbeteren, terwijl overbehandeling en onnodige toxiciteit waarschijnlijk vermeden kunnen worden in patiënten met gunstige prognostische factoren.

In **Hoofdstuk 5** wordt de prognostische waarde van onder andere de vier moleculaire subgroepen beschreven in een meer zeldzame groep van 116 patiënten met endometriumcarcinoom en hoog risicofactoren. Een populatie van patiënten met hoog risicofactoren bleek ook onderverdeeld te kunnen worden aan de hand van moleculaire afwijkingen in een gunstig (*POLE*-gemuteerd of microsatelliet instabiel) en ongunstig (p53-mutante expressie en resterende tumoren zonder specifieke moleculaire classifier) klinisch beloop. Bovendien werden bij de patiënten met een ongunstig klinisch beloop moleculaire afwijkingen gevonden, waarvoor in de toekomst doelgerichte therapie gegeven kan worden. De gevonden mutaties in de PI3K-AKT signaleringsroute, mutaties in *FBXW7* en *FGFR2*, en de expressie van hormoon receptoren komen mogelijk in aanmerking voor doelgerichte therapie met PI3K-AKT-mTOR, HDAC of FGFR--remmers of hormonale therapie. Onze bevindingen benadrukken dat de moleculaire classificatie niet de huidige risicobepaling vervangt, maar dat juist de combinatie van klinisch-pathologische en moleculaire factoren de risico-inschatting van het biologisch gedrag van het endometriumcarcinoom sterk verbetert en factoren identificeert voor doelgerichte therapie.

DNA mismatch repair deficiëntie, karakteristiek van één van de prognostische moleculaire subgroepen, werd in onze studies vastgesteld met behulp van de microsatelliet instabiliteitsanalyse, maar kan ook worden vastgesteld door immunohistochemische analyse van vier mismatch repair eiwitten. De optimale bepaling voor het vaststellen van mismatch repair deficiëntie is beschreven in **Hoofdstuk 6**. De uitkomsten van de microsatelliet instabiliteitsanalyse kwamen sterk overeen met de immunohistochemische analyse van zowel twee (PMS2 en MSH6) als alle vier de mismatch repair eiwitten. Dit was ook het geval in de <3% van de tumoren met verlies van mismatch repair eiwitexpressie in een deel van de tumor. Deze bevindingen, gebaseerd op een uitgebreide analyse van 696 baarmoedertumoren, tonen voldoende bewijs dat immunohistochemische kleuringen van ten minste de twee mismatch repair eiwitten PMS2 en MSH6 praktisch en haalbaar is voor het vaststellen van DNA mismatch repair deficiëntie in de dagelijkse pathologische diagnostiek. Een defect DNA mismatch repair systeem leidt tot frameshiftmutaties (insertie of deletie van nucleotiden) in repeterende sequenties, die in een coderende sequentie van een gen kan leiden tot een functioneel inactief eiwit. De frameshift mutaties kunnen worden onderscheiden in mutaties die de tumor een selectieve voorsprong geven (driver-mutaties), en mutaties die niet betrokken zijn bij het ontstaan van de tumor (passenger-mutaties). **Hoofdstuk 7** beschrijft de frequentie en de mogelijke bijdrage aan tumorprogressie van frameshift mutaties in het *JAK1* gen in patiënten met een microsatelliet instabiel endometriumcarcinoom. *JAK1* frameshift mutaties hadden geen effect op de overleving, maar toonden wel een verband met verminderde opregulatie van LMP7 en HLA-klasse I. Deze deregulatie verstoord de celdoding van tumor cellen door cytotoxische T-cellen, waardoor de tumor mogelijk kan ontsnappen aan het immuunsysteem.

Samenvattend kan de kennis over moleculaire veranderingen in het endometriumcarcinoom de klinische besluitvorming voor aanvullende behandeling verbeteren en hiermee over- en onderbehandeling verminderen. **Hoofdstuk 8** beschrijft de huidige kwesties die van belang zijn voor klinische implementatie, de openstaande vragen betreffende de prognostische moleculaire afwijkingen en bespreekt de toekomstperspectieven. Allereerst moet de klinische toepasbaarheid van de moleculair geïntegreerde risico-inschatting voor aanvullende therapie worden gevalideerd in een prospectieve klinische studie, en moet de bepaling van het moleculaire profiel hiervoor in een kort tijdsbestek kunnen plaatsvinden. Verder is een beter begrip van de moleculaire mechanismen van de prognostische moleculaire afwijkingen nodig voor een betere therapeutische benadering. Bijvoorbeeld: de aanwezigheid van *POLE* mutaties kunnen een indicatie zijn voor het onthouden van aanvullende behandeling: het is echter onbekend via welke mechanismen *POLE* mutaties aanleiding geven tot een goede prognose. De associatie tussen microsatelliet instabiliteit en genoombrede toename van DNA methylering in promoter-regio's van genen in het endometriumcarcinoom suggereert dat DNA methylering een aantrekkelijke kandidaat is voor epigenetische therapieën. De oorzaak en relevantie van de epigenetische veranderingen in het endometriumcarcinoom moeten echter nog worden onderzocht. L1CAM expressie is mogelijk een doelwit voor doelgerichte

therapie, maar het mechanisme, dat leidt tot de expressie van L1CAM is nog niet geheel opgehelderd. Tenslotte: het moleculair geïntegreerde model heeft een goede voorspellende waarde voor het klinisch beloop bij een patiënt, maar er is nog ruimte voor verbetering naar een uitstekend model. Uiteindelijk zal de ontwikkeling van doelgerichte, geïndividualiseerde therapieën leiden tot nog betere 'therapie op maat'.

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Curriculum Vitae

Ellen Stelloo werd geboren op 27 april 1989 te Amersfoort. In 2006 behaalde zij haar Hoger Algemeen Voortgezet Onderwijs diploma aan 't Atrium te Amersfoort, waarna zij de studie Biologie en Medisch Laboratoriumonderzoek begon aan de Hogeschool Utrecht. Na het behalen van haar Bachelor of Science diploma in 2010, werd de studie Oncologie gevolgd aan de Vrije Universiteit Amsterdam. Haar wetenschappelijk doctoraal stage deed zij op de afdeling Toxicologie van Imperial College, Londen, Verenigd Koninkrijk, onder begeleiding van prof. dr. S. Grimm and G. AbuAli. Na het behalen van haar Master of Science diploma, begon zij in mei 2013 met het werk beschreven in dit proefschrift verricht op de afdeling Pathologie onder begeleiding van prof. dr. V.T.H.B.M. Smit, dr. T. Bosse en prof. dr. C.L. Creutzberg. Eén van de onderzoeken in dit proefschrift was in samenwerking met de afdeling Moleculaire Pathologie van de Universiteit van Urbino, Fano, Italië, waar Ellen een aantal maanden van haar promotietraject heeft doorgebracht. Op 5 februari 2014 kreeg zij hiervoor de René Vogels reisbeurs uitgereikt. Verder bezocht Ellen, in het kader van haar promotietraject, diverse congressen in het binnen- en buitenland waar ze posters en presentaties gaf. In 2015 kreeg zij de LUF reisbeurs voor het Keystone Symposia in Denver, Colorado, Verenigde Staten, en de beste presentatieprijs tijdens het Europees gynaeco-oncologisch congres in Nice, Frankrijk. In 2017 bezocht zij de Wellcome Trust Centre for Human Genetics in Oxford, Engeland voor een bioinformatische stage van twee maanden in de onderzoeksgroep van dr. D.N. Church. Ellen zal haar carrière voortzetten als postdoc in de onderzoeksgroep van dr. W. P. Kloosterman en dr. R.P. Zweemer op de afdeling Genetica van het Universitair Medisch Centrum Utrecht.

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