7

Improved risk assessment of endometrial cancer by combined analysis of MSI, PI3K-AKT, Wnt/ß-catenin and P53 pathway activation

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Abstract

Objective: To investigate if analysis of genetic alterations in the main pathways involved in endometrioid type carcinogenesis (PI3K-AKT, Wnt/β-catenin, P53-activation and MSI) improves the current risk assessment based on clinicopathological factors.

Methods: Formalin fixed paraffin embedded (FFPE) primary tumor samples of 65 patients with FIGO-stage I endometrioid type endometrial cancer (EEC) were selected from the randomized PORTEC-2 trial. Tumors were stained by immunohistochemistry for P53, PTEN and ß-catenin. Tumor DNA was isolated for sequence analysis of *TP53* (exons 4 to 8), hotspot mutation analysis of *KRAS* (exon 1) and *PI3K* (exon 9 and 20) and microsatellite-instability (MSI) analysis including *MLH1* promotor-methylation status. Univariate and multivariate analyses for disease-free survival (DFS) using Cox regression models were performed.

Results: P53 status (HR 6.7, 95%CI 1.75 – 26.0, p=0.006) and MSI were the strongest single genetic prognostic factors for decreased DFS, while high PI3K-AKT pathway activation showed a trend and ß-catenin was not prognostic. The combination of multiple activated pathways was the most powerful prognostic factor for decreased DFS (HR 5.0; 95%CI 1.59 – 15.6 p=0.006). Multiple pathway activation, found in 8% of patients, was strongly associated with aggressive clinical course. In contrast, 40% of patients had no alterations in the investigated pathways and had a very low risk of disease progression.

Conclusions: Activation of multiple oncogenic pathways in EEC was the most powerful prognostic factor for decreased DFS, resulting in an individual risk assessment superior to the current approach based on clinicopathological factors.

Introduction

Endometrial cancer (EC) is the most common gynecological cancer in Western countries.[1] Surgery alone (hysterectomy and bilateral salpingo-oophorectomy) is a curative treatment for the majority of patients.[2,3]

This study focuses on type 1 EC (EEC), the most frequent subtype (80-85%), characterized by endometrioid morphology. The remaining 15-20% are type 2 cancers with a non-endometrioid morphology (NEEC), mostly serous or clear cell cancers having a far more aggressive clinical behavior than type 1 cancers. Adjuvant treatment for EEC has become increasingly tailored to clinicopathological risk factors.[4] In low-risk EEC patients adjuvant treatment is not indicated, while high-risk EEC patients receive adjuvant radiotherapy and/or chemotherapy. The (high)intermediate risk category of EEC patients has at least two high-intermediate risk (HIR) features; advanced age, grade 3, deep invasion, and LVSI.[5,6] The PORTEC-2 trial, in which HIR patients were randomly allocated to external beam radiotherapy or vaginal brachytherapy, concluded that vaginal brachytherapy is an effective therapy to prevent local recurrence with <2% vaginal recurrence at 5 years and 8% distant metastasis.[7] Despite tailoring adjuvant therapy to clinicopathological factors, considerable over- and undertreatment still exists. Approximately 7 HIR patients need to receive brachytherapy to prevent 1 vaginal recurrence.

The signaling pathways currently known to drive EEC development are activation of the phosphatidylinositol 3-kinase (PI3K)-AKT pathway, Wnt/β-catenin-signaling, microsatellite-instability (MSI) and, although typically described in NEEC, mutational activation of TP53.[8-10] The PI3K-AKT pathway can be deregulated by many different mechanisms, including inactivation of PTEN or mutations in PI3K and KRAS.[11] A recently investigated marker of PI3K-AKT pathway activation, Stathmin (STMN1), was shown to have independent prognostic capacity and may reflect the degree of PI3K-AKT pathway activation.[12] As in other cancers, endometrial carcinogenesis is likely to be the result of a complex interaction of pathway alterations. Concomitant PI3K-AKT and P53 alterations were found to be associated with poor prognosis.[13] Most studies

have however focused on single oncogenic pathway alterations in EEC and were performed in a heterogenic population containing both EEC and NEEC and both early and advanced FIGO stage tumors.

The aim of the present study was to investigate whether combined analysis of genetic alterations in the main pathways involved in endometrioid type carcinogenesis can help to improve the current clinicopathological risk assessment of patients with EEC.

Methods

Patient and tissue selection

The current analysis was undertaken in a sample size of 65 EEC patients selected from the PORTEC-2 trial population (427 participants). Written informed consent was obtained from all patients and included consent for collection of a tumor sample. The trial protocol was approved by the Dutch Cancer Society (CKTO 2001-04) and the ethics committees of all participating centers. Eligible trial patients had EEC with HIR factors based on the original pathology report of the treatment center. At central pathology review some EC were diagnosed to have low-risk (LR, 6%) or high-risk (HR, 8%) features.[7] In order to detect a trend in the incidence of alterations in oncogenic pathways in the LR, HIR and HR risk groups, all patients who were found to have LR (N=23) or HR features (N=16) at review and of whom tissue samples were available, were included in the current selection. In addition, sufficient disease-related events were required to correlate the pathway alterations with disease recurrence. For this purpose, from the subgroup of patients with HIR features, with grade 1-2 tumors with deep (>50%) myometrial invasion, confirmed after review (true-HIR, 366 patients) tissue samples of 26 patients were selected with the aim to include 50% of patients with disease recurrence during follow-up.

Formalin fixed paraffin-embedded (FFPE) blocks containing representative tumor were selected. Hematoxylin-eosin stained slides were viewed by an experienced gynecopathologist (V.S.), in order to select an area of tumor tissue containing at least 70% tumor cells. From this area two to three 0.6 mm cores

were extracted and used to isolate tumor DNA after proteinase K digestion. For the immunohistochemistry (P53, β-catenin, PTEN, Stathmin) procedures 4µm whole slide sections were used.

Mutational analysis

All samples were analyzed using a custom made panel of hydrolysis probe assays, designed to detect hotspot mutations in PIK3CA (*PI3K)* and *KRAS*.[14] The hotspot mutations investigated for *PI3K* were exon 9, c.1624G>A; p.E542K and c.1633G>A; p.E545K and in exon 20 the c.3140A>G; p.H1047R and for *KRAS* exon 1, c.34G>A; p.G12S, c.34G>C; p.G12R, c.34G>T; p.G12C, c.35G>A; p.G12D, c.35G>C; p.G12A, c.35G>T; p.G12V and c.38G>A; p.G13D. Real time qPCR was performed by allelic discrimination using primers and probes designed and ordered by Applied Biosystems (Applied Biosystems, Nieuwerkerk aan de IJssel, the Netherlands).

Microsatellite instability (MSI) and Methylation-specific PCR

The microsatellite status of each tumor was determined using the Promega MSI analysis system (version 1.2, Promega, Madison, WI, US) , following the recommendation of the National Cancer Institute/ICG-HNPCC.[15] Tumors were classified as microsatellite instable (MSI) when two or more markers showed an instable pattern. The MSI tumors were selected for further testing for methylation status of the 5' regulatory region of MLH1, using methylation-specific PCR (MSP), with primers that have been previously described.[16]

TP53 Mutation Analysis

Primers were designed to amplify exons 4-8 of *TP53* by PCR. *TP53* primers were designed to avoid amplification of a pseudogene, and have been described previously.[17] Sanger sequencing was performed on purified PCR products (Macrogen, Amsterdam, the Netherlands). Sequences were analyzed with Mutation Surveyor™ DNA variant analysis software (version 3.97 Softgenetics). A mutation was only accepted once it was identified in both forward and reverse strands.

Immunohistochemistry

Slides were deparafinated in xylene, rehydrated though a graded ethanol series, and washed with phosphate-buffered saline. Antigen retrieval was achieved by microwave oven treatment for 10 min in 10mmol/L citrate buffer, pH6.0 (ß-catenin in 10 mmol/L Tris-EDTA, pH9.0). Sections were incubated overnight with monoclonal p53 antibody (clone D0-7, 1:1000 dilution; NeoMarkers), polyclonal Stathmin antibody (3352, 1:100 dilution, Cell Signaling), PTEN (clone 6.H2.1, 1:200 dilution; DAKO), ß-catenin (cat. 610154; 1:1600; BD Transduction) and monoclonal MLH-1 (clone ES05, 1:200 dilution; DAKO). The sections were incubated and stained with a secondary antibody (Poly-HRP-GAM/R/R; DPV0110HRP; ImmunoLogic). Diaminobenzidine tetrahydrocholoride was used as a chromogen for all antibodies. The slides were counterstained with hematoxylin.

Evaluation of staining

Slides were evaluated by two pathologists (T.B. and V.S), blinded for patient characteristics and outcome. Evaluations were done independently, and discrepancies were resolved at simultaneous viewing. P53 was scored positive if >50% of the tumor cells showed strong positive nuclear staining, or when discrete geographical patterns showed >50% tumor cell positivity.[18] Activated Wnt-signaling was defined as nuclear staining of ß-catenin. For Stathmin and PTEN a semi quantitative grading system incorporating staining intensity (score 0-3) and area of tumor with positive staining was used, as described by Trovik et. al.[19]: 0, no staining; 1, <10%; 2, 10-50%, and 3, >50% of tumor cells. Staining index was calculated as the product of staining intensity and staining area, range 0-9. Values defined by the upper quartile for the data set were considered positive.

Statistics

The distribution of patient and tumor characteristics of the different risk groups was tested for significance using the Chi-square test for categorical variables and the student T-test for continuous variables. Disease free survival (DFS) was defined as the time between date of randomization and date of

disease recurrence or death from any cause; all other patients were censored at the date of last follow-up. DFS was calculated with the Kaplan Meier method including Log rank test. Multivariate analysis of prognostic factors for disease free survival was performed using Cox regression models. All variables with a univariate Log rank p-value less than 0.1 were included in the model. SPSS software version 17.0 was used for statistical analysis.

Results

Patient characteristics

Patient characteristics are shown in Table 1. Median follow-up was 88 months (range 4 – 106 months) and was not significantly different from the main trial population (p=0.29).

Despite the disease free survival (DFS) rate being lower in the true-HIR population (N=26) due to selection of patients with recurrent disease, DFS in the total population (N=65) did not differ significantly from that of the original trial population (N=427) (Appendix 1). In total 15 of 65 (23.1%) patients recurred during follow-up, while 12 of 26 (46.2%) true-HIR patients recurred during follow-up (Table 1).

In univariate analysis both age (p=0.03) and deep myometrial invasion (p=0.02) were prognostic factors for decreased DFS, and were included in the subsequent Cox regression analysis, in contrary to grade (p=0.26) and lymph vascular invasion (p=0.15).

Table 1. Patient, tumor and treatment characteristics of endometrioid type tumors.

	Low Risk		High-intermediate Risk		High Risk		Total		
	No.	%	No.	%	No.	%	No.	%	p-value*
Total	23	35.4	26	40.0	16	24.6	65	100	
Age at Diagnosis									
Mean	64.7		70.1		68.8		67.9		**0.04**
Range	51.6 - 84.6		56.0 - 82.0		60.7 - 82.7		51.6 - 84.6		
Myometrial Invasion									
<50%	23	100	0	0	0	0	23	35.4	**<0.001**
>50%	0	0	26	61.9	16	38.1	42	64.6	
Grade									
1	21	55.3	17	44.7	0	0	38	58.5	**<0.001**
2	2	18.2	9	81.8	0	0	11	16.9	
3	0	0	0	0	16	100	16	24.6	
Lymph Vascular Invasion									
Abstent	21	39.6	22	41.5	10	18.9	53	81.5	*0.07*
Present	2	16.7	4	33.3	6	50.0	12	18.5	
Treatment arm									
EBRT	10	32.3	13	41.9	8	25.8	31	47.7	0.88
VBT	13	38.2	13	38.2	8	23.5	34	52.3	
Site of First Recurrence									
No Recurrence	22	44.0	14	28.0	14	28.0	50	76.9	**0.01**
Pelvic Lymph Node Recurrence	0	0	1	100	0	0	1	1.5	
Distant Metastasis	1	7.1	11	78.6	2	14.3	14	21.5	

EBRT: external beam radiotherapy; VBT: vaginal brachytherapy.
**p-value by χ2 method, p-values <0.05 in bold and between 0.05 and 0.10 in italic.*

PI3K-AKT pathway activation

PI3K mutations were found in 7 tumors (11%) of which 5 had a mutation in exon 20 (H1047R). KRAS mutations in exon 1 were found in 14 tumors (22%) of which 11 were located at position 35. Loss of PTEN expression was found in 45% of the tumors. Both PI3K and KRAS mutation status alone were not predictive for decreased DFS, while loss of PTEN expression showed a trend for decreased DFS (Table 2).

PI3K-AKT pathway activation was classified as high when two or more altered genetic factors (PI3K and KRAS mutation status and loss of PTEN) were found (12/65 tumors, 19%), moderate in case of one altered genetic factor and no pathway activation if none of the factors were altered (26/65 tumors, 40%). High PI3K-AKT pathway activation was more frequently seen in patients with low and high-intermediate risk tumors and was mainly caused by a combination of KRAS mutation and complete loss of PTEN expression (Figure 1). Although not significant, DFS of patients with high PI3K-AKT pathway activation seemed decreased compared to those with no or moderate activation (Figure 1). High Stathmin expression was more frequently found in high risk tumors without an association with DFS. No correlation between the degree of Stathmin expression and the degree of PI3K-AKT pathway activation was found (Table 2 and Figure 1).

A

PI3K-AKT pathway activation			
No. Patients	*PI3K*	*KRAS*	Loss of PTEN
PI3K	5	1	1
KRAS		3	10
Loss of PTEN			18
Total	7	14	29

B

PI3K-Akt pathway	Stathmin negative		Stathmin positive		Total
	No.	%	No.	%	No.
No activation	18	66.7	9	33.3	27
Moderate activation	16	61.5	10	38.5	26
High activation	8	66.7	4	33.3	12
Total	42	64.6	23	35.4	65

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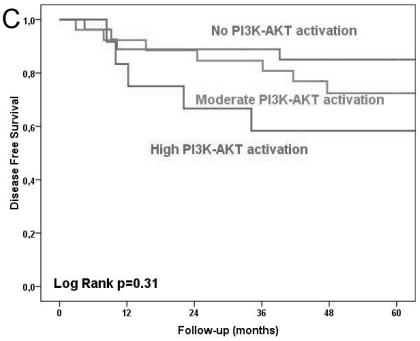

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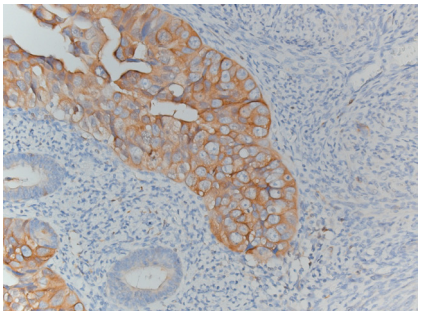


Figure 1A-D. PI3K-Akt pathway activation and the role of Stathmin. (A) Cross correlation table of components of the PI3K-AKT pathway. High PI3K-AKT pathway activation (red fields): tumors having 2 or more altered components of the pathway, moderate activation: 1 altered component and no activation: none of the components showed alterations. In 7 patients a PI3K mutation was found of whom 1 had a simultaneous mutation in KRAS and 1 patient had loss of PTEN. (B) No correlation between the degree of PI3K-AKT pathway activation and Stathmin expression on immunohistochemistry. (C) Degree of PI3K-AKT pathway activation is not significantly associated with DFS, with trend that high Pi3K-AKT pathway activation is indicative for a worse DFS. (D) Stathmin protein expression shows positive cytoplasmic staining of the tumor cells and negative normal endometrial glands for comparison.

ß-catenin / Wnt-signaling pathway

Nuclear staining of ß-catenin as a marker for an activated Wnt-signaling pathway was found in 9 tumors (14%) and infrequently found in combination with other alterations (Figure 4). Staining was only seen in low and high-intermediate risk tumors without any relation to DFS.

P53 pathway

P53 protein overexpression, assessed by immunohistochemical staining, was found in 17% of the tumors and was highly predictive for decreased DFS (Table 2 and Figure 2). Sequencing of TP53 (exons 4-8) succeeded in 48 patients and revealed 9 (14%) functional and 4 (6%) non-functional mutations (Figure 2 and Table Web Appendix 2). There was high agreement between immunohistochemical staining and sequencing of TP53 (Kappa 0.86).

Microsatellite Instability (MSI)

MSI was found in 12 tumors (19%), with a significantly higher frequency of MSI with increasing depth of myometrial invasion and increasing tumor grade (Table 2). The methylation status of the MLH-1 promoter region was successfully assessed in 9 patients and was hypermethylated in 8 patients. One patient was found to have a MSH6 germline mutation (confirming Lynch syndrome) and developed a colon carcinoma during follow-up. MSI was predictive for decreased DFS (Figure 3), and was found to be mutually exclusive with P53 overexpression (Figure 4).

Accumulation of alterations in oncogenic pathways

An alteration in one of the four main mechanisms of endometrioid type carcinogenesis (PI3K-AKT, Wnt/ß-catenin, P53 pathways and MSI) was found in 60% (39/65). In a Cox regression model that included age and deep myometrial invasion, P53 status was found to be the strongest (HR 6.7, 95%CI 1.75 – 26.0, p=0.006) single alteration to predict for a decreased disease free survival, followed by MSI (Figure 4).

In order to analyze the prognostic impact of the accumulation of alterations in the oncogenic pathways, three groups were defined: no altered pathway; one altered pathway; two or more altered pathways. When the factor accumulated altered oncogenic pathways was entered in the regression analysis (including age and deep myometrial invasion) followed by P53 overexpression and MSI, it was the only factor that remained significantly predictive for decreased DFS (HR 5.0; 95%CI 1.59 – 15.6 p=0.006, Figure 4). This finding was confirmed in the subgroup of true-HIR patients (Figure Appendix). Five out of 65 (8%) EEC patients had two or more activated pathways, and all died due to early disease recurrence (Figure 4). In contrast, 24 patients (40%) had no activated pathway of whom only one patient developed a recurrence.

Table 2. Alterations in the most common mechanisms of endometrioid type tumor carcinogenesis.

	Low Risk		High-intermediate Risk		High Risk			Total		Recurrence		Disease Free Survival
	No.	%	No.	%	No.	%	p-value*	No.	%	No.	%	Log rank p-value
Total	23	35.4	26	40.0	16	24.6		65	100	15	23.1%	
PI3K-Akt pathway alterations												
PI3K												
Wildtype	20	34.5	22	37.9	16	27.6	0.27	58	89.2	13	22.4	0.81
Mutated	3	42.9	4	57.1	0	0		7	10.8	2	28.6	
KRAS												
Wildtype	17	33.3	21	41.2	13	25.5	0.80	51	78.5	11	21.6	0.84
Mutated	6	42.9	5	35.7	3	21.4		14	21.5	4	28.6	
PTEN												
Functional PTEN	17	50.0	9	26.5	8	23.5	0.12	34	52.3	4	13.8	*0.065*
Loss of PTEN	6	20.7	15	51.7	8	27.6		29	44.6	10	34.5	
Missing	0	0	2	100	0	0		2	3.1	1	50.0	
PI3K-Akt pathway												
No Activation	13	48.1	8	29.6	6	22.2	0.14	27	41.5	4	14.8	0.31
Moderate Activation	5	19.2	12	46.2	9	34.6		26	40.0	6	23.1	
High Activation	5	41.7	6	50.0	1	8.3		12	18.5	5	41.7	
Stathmin (STMN1)												
Absent / Low	13	38.2	18	52.9	3	8.8	**<0.001**	34	52.3	8	23.5	0.82
Intermediate	4	50.0	4	50.0	0	0		8	12.3	2	25.0	
High	6	26.1	4	17.4	13	56.5		23	35.4	5	21,.7	
P53 alterations												
P53 by Immunohistochemistry												
Negative	21	39.6	18	34.0	14	26.4	0.18	53	81.5	8	15.1	**0.003**
Positive	2	18.2	7	63.6	2	18.2		11	16.9	6	54.5	
Missing	0	0	1	100	0	0		1	1.5	1	21.9	
P53 by Sequencing‡												
Wildtype	14	40.0	13	37.1	8	22.9	0.15	35	53.8	5	14.3	**<0.001**
Functional Mutation	1	11.1	7	77.8	1	11.1		9	13.8	6	66.7	
Non-functional Mutation	1	25.0	1	25.0	2	50.0		4	6.2	1	25.0	
Missing	7	41.2	5	29.4	5	29.4		17	26.2	3	17.6	
Micro Satelite Instability												
Micro Satelite Stable	22	42.3	21	40.4	9	17.3	**0.007**	52	80.0	9	17.3	**0.02**
Micro Satelite Instable	1	8.3	4	33.3	7	58.3		12	18.5	5	41.7	
Missing	0	0	1	100	0	0		1	1.5	1	100	
Wnt signaling pathway alterations												
β-Catenin: nuclear staining												
Absent	18	32.1	22	39.3	16	28.6	0.15	56	86.2	12	21.4	0.54
Present	5	55.6	4	44.4	0	0		9	13.8	3	33.3	
Accumulation of Altered Oncogenic Mechanisms												
No Altered Mechanims	10	38.5	10	38.5	6	23.1	*0.08*	26	40.0	1	3.8	**<0.001**
1 Altered Mechanism	13	38.2	11	32.4	10	29.4		34	52.3	9	26.5	
≥ 2 Altered Mechanisms	0	0	5	100	0	0		5	7.7	5	100	

*p-value by $\chi 2$ method, p-values <0.05 in bold and between 0.05 and 0.10 in italic. ‡One patient had a functional and non-functional mutaion (Web Appendix Table 2.).

Figure 2A-D.
TP53 mutations in endometrioid endometrial cancer. (A) HE stain of an EC with endometrioid grade 1 morphology; note the glandular architecture with nuclear stratification. (B) *This case showed strong nuclear P53 staining by immunohistochemistry, indicative for TP53 mutation.* (C) *TP53 mutation was confirmed by sequencing. The upper pane shows wild type sequence for reference and the lower pane shows the sample sequence. In this case a single-nucleotide substitution, cytosine (C) to thymine (T) at position 12523 in exon5 (↓) was found, resulting in a histidine (H) to tyrosine (Y) substitution at position 179 of TP53.* (D) *P53 staining by immunohistochemistry was the strongest single prognostic factor associated with a lower disease free survival on univariate analysis and multivariate analysis (HR 6.7, 95%CI 1.75 – 26.0, p=0.006).*

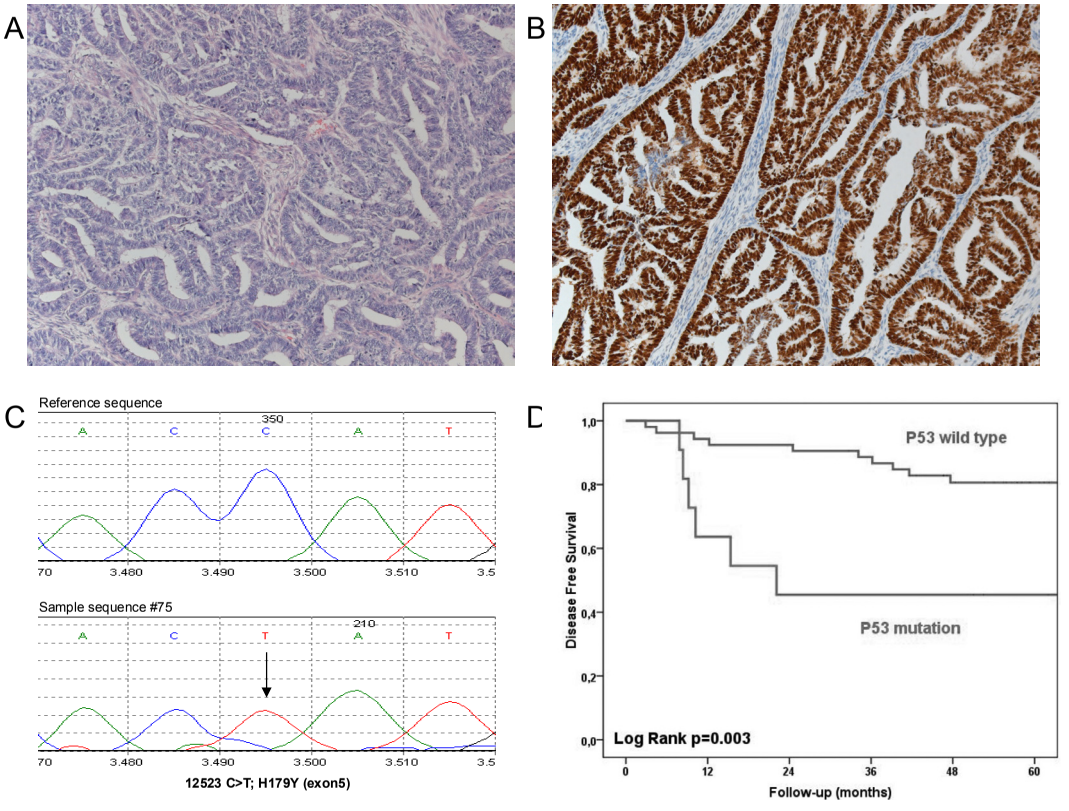
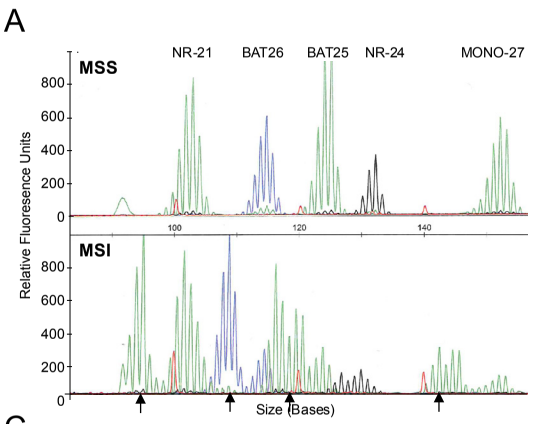
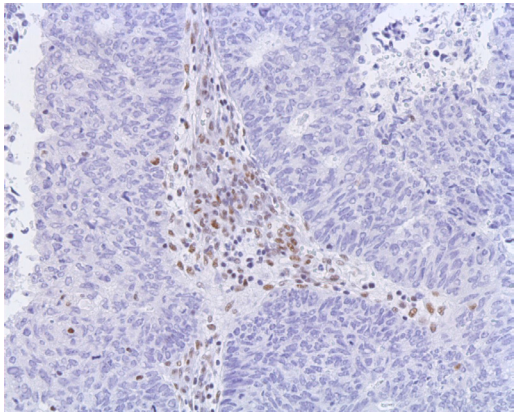
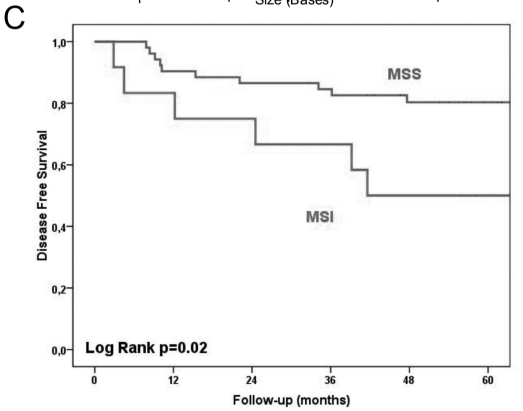


Figure 3A-C.
Microsatellite instability (MSI) and MLH-1 status. (A) Example of a MSI (lower pane) tumor sample analyzed by an ABI PRISM genetic analyzer, and the allelic pattern of the sample is shown. The presence of new alleles (↑) can be appreciated when compared to a microsatellite stable (MSS, upper pane) tumor sample. When 2 or more markers showed new alleles the sample was considered to be MSI. (B) MLH-1 immunohistochemistry confirmed MLH-1 downregulation in tumor cells; note the positive internal nuclear staining in the stroma cells. (C) Presence of MSI is prognostic for an inferior disease free survival on univariate analysis and in multivariate analysis (HR 5.3, 95%CI 1.36 – 20.9, p=0.016).







Discussion

Over the past decades prospective randomized trials have provided a solid basis for the use of clinicopathological prognostic factors such as age, grade and myometrial invasion for risk-based adjuvant therapy.[5,6] The vast majority of patients with EEC have either low (55%) or high-intermediate risk features (30%) and have a good prognosis. The present study was undertaken to investigate whether combined analysis of genetic alterations in the main involved pathways of endometrioid type carcinogenesis improved the current risk assessment on an individual basis, with the ultimate goal to further reduce both over- and undertreatment.

Although all PORTEC-2 trial patients were randomized under the condition of having HIR features at central pathology review diagnosed a subset of 14% was diagnosed with low-risk (LR) or high-risk (HR) features.[7] Incorporation of these patients into this study enabled the detection of alterations in oncogenic mechanisms in all three risk groups (LR, true-HIR, HR). In addition, patients with HIR features confirmed at central review (true-HIR) were selected for disease-related events in order to correlate the alterations in oncogenic pathways with disease recurrence. An obvious advantage of using this trial population is the relative homogeneity of the study cohort. All patients were diagnosed with EEC and were treated in a comparable manner with a long and well documented follow-up (median 7.3 years).

Although TP53 mutations have frequently (80-90%) been found in non-endometrioid endometrial cancer (NEEC), they are found in 10-15% of the EEC.[9,10] In this study 17% of the EEC tumors showed P53 overexpression. For validation purposes, TP53 (exons 4-8) was sequenced and showed a high agreement with the immunohistochemical staining (Kappa 0.86). P53 overexpression was the strongest independent prognostic pathway for decreased DFS. This finding supports the assumption that P53-positive endometrial cancers, independent of their morphology, should be considered as intrinsically aggressive tumors.

Microsatellite instability (MSI) was demonstrated in 19% of the tumors and correlated with depth of myometrial invasion and grade. Although this frequency is in line with other studies (20%-45%)[9,10], its relation with other pathological factors remains controversial. In many previous MSI studies[20-23], patient cohorts including both early and advanced FIGO stages, and cancers of different

histological subtypes, may have blurred the evaluation of the prognostic value of MSI. In our cohort, MSI was mainly caused by hypermethylation of the promoter region of MLH-1. MSI as an oncogenic mechanism was found to be mutually exclusive with P53 overexpression and clearly associated in our study with decreased DFS. A similar inverse relationship between TP53 mutations and MSI status was recently found in gastric cancer.[24] The authors hypothesized that this may be explained by alterations in an emerging tumor suppressor gene, ARID1a (component of the SWI/SNF chromatin remodeling complex), that may constitute an alternative pathway of carcinogenesis strongly associated with MSI and independent of TP53 that drives cancer development through epigenetic modifications. Recent studies have shown frequent ARID1a mutations in gynecologic cancers, including endometrial cancer.[25]

The accumulation of molecular alterations in the PI3K-AKT pathway showed a trend toward a worse clinical outcome (Figure 1). It did not reach statistical significance probably due to the small sample size. The PI3K-AKT pathway is one of the most frequent deregulated pathways in cancer and has been associated with aggressive tumor behavior in endometrial cancers.[12] The well known oncogen KRAS and tumorsuppressor PTEN converge on the PI3K-AKT pathway, resulting in growth, proliferation and survival signaling.[26,27] In addition, 'hot spot' mutations in the kinase and helical domains of PI3K confer constitutive kinase activity and thereby directly activate PI3K-AKT signaling.[28] Our data show considerable coexistence between these molecular alterations, suggesting that individual mutations are not completely redundant. These independent alterations activate the PI3K-AKT pathway differently, and cumulative molecular alterations may have a selective advantage.[29] It may be possible that loss of PTEN results in circumvention of negative feedback loops which result in an additive effect on PI3K-AKT pathway activation, as was found in knock-out mice experiments.[30] Based on these data, it is likely that the magnitude of PI3K-AKT pathway activation is influenced by the underlying molecular alteration(s), and that this impacts on oncogenicity.

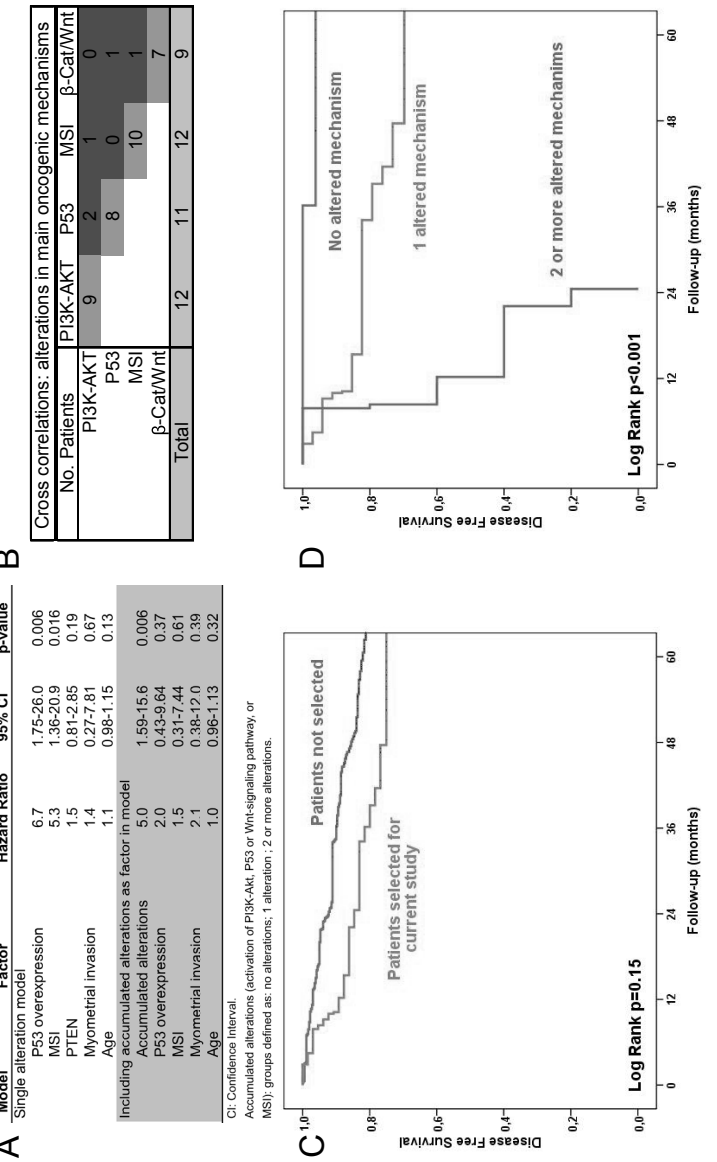


Figure 4 A-D. Accumulation of altered oncogenic mechanisms. (A) Cox regression model for effect on disease free survival of alterations in single oncogenic mechanisms. In a second model the accumulation of altered oncogenic mechanisms was entered together with oncogenic mechanisms (P53 and MSI) that were significantly associated with a decreased disease free survival on univariate analysis (Log rank p<0.1), showing that the factor accumulation of oncogenic mechanisms remained significant. (B) Table showing cross correlations between investigated oncogenic mechanisms, in red patients with 2 or more altered mechanisms and in green patients with 1 altered mechanism. PI3K-AKT pathway was activated in 12 patients of whom 2 had a P53 overexpression and 1 MSI. (C) DFS of patients selected for the current study (green, N=65) and of all unselected PORTEC-2 patients (blue, N=362). (D) DFS by accumulation of genetic alterations in PI3K-AKT, Wnt/β-catenin and P53 pathways and MSI including all patients selected for the current study (N=65).

In light of this discussion, indentifying a marker for PI3K-AKT pathway activation would be of major interest. Recently Stathmin (STMN1), a microtubule destabilizing protein, was postulated as a putative surrogate marker of the PI3K-AKT pathway with independent prognostic significance.[12,19,31] Our data do not support a clear relationship between Stathmin overexpression and PI3K-AKT pathway activation. This discrepancy may be partly explained by the difference in definition of PI3K-AKT activation. Another explanation may be that Stathmin is not only expressed in the context of activated PI3K-AKT, but also overexpressed when the TP53 tumor suppressor function is lost. This is supported by studies that show that wild-type P53 transcriptionally represses STMN1, and mutant TP53 can impair this negative regulation, leading to increased Stathmin levels.[32-34]

Mutations of multiple genes that participate in different pathways or functions may be additive or even synergistic in conferring a survival advantage to the tumor.[35] Patients with multiple activated pathways had significantly worse DFS (Figure 4). The most frequent co-occurrence was the combination of P53 and PI3K-AKT activation, which previously has been reported to be associated with a poor prognosis.[13] Taken together, these combined carcinogenic pathway alterations may better reflect the oncogenicity of tumors than their morphology, and prove to be the strongest prognostic factors.

In summary, analysis of multiple molecular genetic alterations in tissue samples of patients with EEC showed that P53 and MSI status are important independent prognostic factors for decreased DFS. The simultaneous activation of multiple oncogenic pathways was the most powerful factor predicting decreased DFS, resulting in a superior individual risk prediction as compared to the current clinicopathological approach. Although larger (prospective) series are necessary to validate these findings, our results support a biologically driven approach for individual risk assessment and treatment selection.

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Appendix 1.

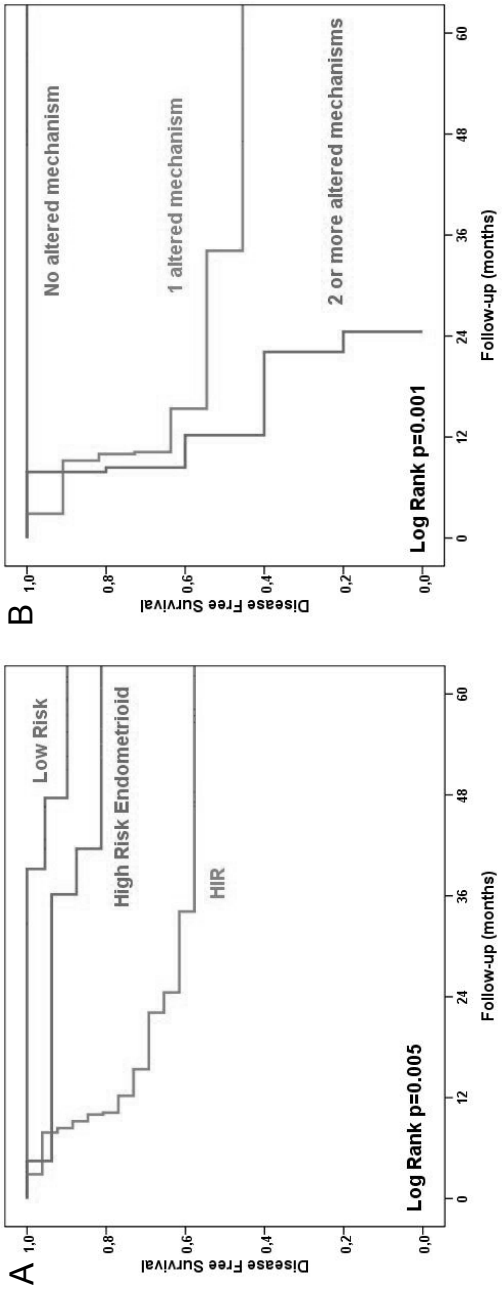

Figure 1A-B. Disease Free Survival. (A) DFS of all patients selected for the current study (N=65) by risk group based on clinicopathologic factors, see text for definitions. For this study High-intermediate risk (HIR) patients with recurrence during follow-up were selected (approximately 50%), explaining the lower than expected disease free survival of HIR patients compared to low and high risk patients in this figure. (B) DFS by accumulation of alterations in PI3K-AKT, Wnt/β-catenin and P53 pathways and MSI in patients whose HIR status was confirmed by central pathology review (N=26).