

p53 suppresses type II endometrial carcinomas in mice and governs endometrial tumour aggressiveness in humans

Peter J. Wild^{1†}, Kristian Ikenberg^{1†}, Thomas J. Fuchs², Markus Rechsteiner¹, Strahil Georgiev³, Niklaus Fankhauser³, Aurelia Noske¹, Matthias Roessle¹, Rosmarie Caduff¹, Athanassios Dellas⁴, Daniel Fink⁵, Holger Moch¹, Wilhelm Krek³, Ian J. Frew^{6*†}

Keywords: clear cell; endometrial carcinoma; mouse model; p53; serous

DOI 10.1002/emmm.201101063

Received November 08, 2011
Revised April 20, 2012
Accepted April 23, 2012

Type II endometrial carcinomas are a highly aggressive group of tumour subtypes that are frequently associated with inactivation of the *TP53* tumour suppressor gene. We show that mice with endometrium-specific deletion of *Trp53* initially exhibited histological changes that are identical to known precursor lesions of type II endometrial carcinomas in humans and later developed carcinomas representing all type II subtypes. The mTORC1 signalling pathway was frequently activated in these precursor lesions and tumours, suggesting a genetic cooperation between this pathway and *Trp53* deficiency in tumour initiation. Consistent with this idea, analyses of 521 human endometrial carcinomas identified frequent mTORC1 pathway activation in type I as well as type II endometrial carcinoma subtypes. mTORC1 pathway activation and p53 expression or mutation status each independently predicted poor patient survival. We suggest that molecular alterations in p53 and the mTORC1 pathway play different roles in the initiation of the different endometrial cancer subtypes, but that combined p53 inactivation and mTORC1 pathway activation are unifying pathogenic features among histologically diverse subtypes of late stage aggressive endometrial tumours.

INTRODUCTION

Carcinomas of the endometrium, the mucosa that lines the uterus, are the most frequently diagnosed malignancies of the female genital tract (Jemal et al, 2011). Endometrial carcinomas

are classified in a dualistic model based on histological appearance, clinical behaviour and molecular differences (Di Cristofano & Ellenson, 2007). Type I tumours are endometrioid endometrial adenocarcinomas (AdCas), which account for 80% of endometrial malignancies and are characterized by a relatively high-survival rate (85% at 5 years), due partly to their frequent early detection. These tumours frequently exhibit mutations or alterations in oncogenes and tumour suppressor genes, including *PTEN*, *PIK3CA*, *PIK3R1*, *PIK3R2*, *LKB1*, *TSC2* and *KRAS*, that result in activation of the PI3K-AKT-mTORC1 signalling pathway (Catusus et al, 2009; Cheung et al, 2011; Konopka et al, 2011; Lu et al, 2008; Oda et al, 2005; Salvesen et al, 2009; Tashiro et al, 1997; Urlick et al, 2011; Velasco et al, 2006). Type I tumours are associated with excessive oestrogen stimulation, frequently harbour mutations in *CTNNB1* and exhibit microsatellite instability (Di Cristofano & Ellenson, 2007). While roughly half of poorly differentiated, high-grade type I tumours exhibit *TP53* mutations, low-grade tumours rarely display *TP53* mutations, suggesting that loss of function of

- (1) Institute of Surgical Pathology, University Hospital Zurich, Zurich, Switzerland
- (2) Department of Electrical Engineering, California Institute of Technology, Pasadena, CA, USA
- (3) Institute of Cell Biology, ETH Zurich, Zurich, Switzerland
- (4) Department of Pathology, University Hospital Basel, University of Basel, Basel, Switzerland
- (5) Department of Gynaecology, University Hospital Zurich, Zurich, Switzerland
- (6) Zurich Center for Integrative Human Physiology, University of Zurich, Zurich, Switzerland

*Corresponding author: Tel: +41 44 635 50 04; Fax: +41 44 635 6814;
E-mail: ian.frew@access.uzh.ch

†These authors contributed equally to this work.

the p53 tumour suppressor protein is a late event in the progression of type I disease (Lax et al, 2000).

Type II endometrial tumours develop mainly in elderly women and comprise three different subtypes; serous AdCas, clear cell AdCas and undifferentiated carcinomas, each representing approximately 5–10% of all endometrial carcinoma cases (Di Cristofano & Ellenson, 2007; Prat et al, 2007). Despite their relative rarity, these tumours contribute to nearly half of the total number of endometrial cancer-related deaths due to their highly invasive and metastatic nature. Carcinosarcomas (also known as malignant mixed Müllerian tumours) represent approximately 1–3% of all endometrial malignancies and comprise both malignant epithelial and mesenchymal components. These tumours mostly arise as a result of de-differentiation of high-grade type I or type II AdCas and follow a highly aggressive clinical course that has a very poor outcome (Abeln et al, 1997; Wada et al, 1997).

Type II endometrial carcinomas and carcinosarcomas are associated with different sets of gene mutations to type I carcinomas. Mutations in *PTEN* or *KRAS* and microsatellite instability are rare in type II tumours (Catasus et al, 2009; Lax et al, 2000; Tashiro et al, 1997), but there is strong correlative evidence that mutations in *TP53* are involved at the earliest stage of tumour development. *TP53* mutations are found in up to 95% of cases of endometrial serous AdCa (Fadare & Zheng, 2009; Zheng et al, 2011). Endometrial serous AdCas are believed to arise from precursor lesions termed endometrial glandular dysplasia (EmGD) that progress to a non-invasive cancer termed endometrial intraepithelial carcinoma (EIC; Fadare & Zheng, 2009; Zheng et al, 2007, 2004, 2011). Approximately 50–75% of these lesions display *TP53* mutations (Jarboe et al, 2009; Jia et al, 2008; Liang et al, 2004; Zhang et al, 2009). Clusters of cells displaying strong p53 immunoreactivity can also be infrequently observed in some morphologically normal endometrial glands, suggesting that *TP53* mutation represents the earliest step of serous AdCa formation (Jarboe et al, 2009; Zhang et al, 2009). *TP53* mutations also occur at high frequency in endometrial clear cell AdCas (An et al, 2004; Fadare et al, 2006; Lax et al, 1998) and in carcinosarcomas (Abeln et al, 1997; Kounelis et al, 1998; Soong et al, 1999; Szukala et al, 1999; Taylor et al, 2006; Wada et al, 1997). Thus, in contrast to type I tumours, type II tumours are frequently characterized by early mutations in *TP53*.

We present functional genetic evidence showing that p53 suppresses the formation of type II endometrial tumours in mice and have employed a large cohort of human endometrial carcinomas to demonstrate molecular similarities between aggressive type I and type II tumours.

RESULTS

Analysis of mice with genitourinary tract-specific *Trp53* deletion

The *Ksp1.3-Cre* transgene drives Cre expression widely in the kidney epithelium and in the embryonic precursor structures that give rise to most of the epithelia of the adult genitourinary

system (Frew et al, 2008; Shao et al, 2002). In the uterus, *Ksp1.3-Cre* activity results in a mosaic pattern of gene deletion in the endometrial epithelial cells of the lumen and glands (Frew et al, 2008). We attempted to generate a mouse model of endometrial type II carcinomas by crossing *Trp53^{fl/fl}* mice (Jonkers et al, 2001), in which *loxP* sites are present in intron 1 and intron 10 of the *Trp53* gene, the mouse homologue of human *TP53*, to mice expressing the *Ksp1.3-Cre* transgene. PCR-based assays of genomic DNA from the kidney, uterus and epididymis of these *Ksp1.3-Cre/+;Trp53^{fl/fl}* mice confirmed the genotype and the presence of Cre-mediated recombination of the *loxP*-flanked *Trp53* allele (Fig 1A). Real-time PCR analysis demonstrated reduction in *Trp53* mRNA abundance in these tissues in Cre-expressing mice (Fig 1B). Cohorts of littermate *+/+; Trp53^{fl/fl}* (hereafter referred to as WT) and *Ksp1.3-Cre/+;Trp53^{fl/fl}* (hereafter referred to as *Trp53^{Δ/Δ}*) mice were analysed at 9–10 weeks (WT, *n* = 5; *Trp53^{Δ/Δ}*, *n* = 7), 24–35 weeks (WT, *n* = 8; *Trp53^{Δ/Δ}*, *n* = 21), 47–58 weeks (WT, *n* = 5; *Trp53^{Δ/Δ}*, *n* = 10) and 65–79 weeks (WT, *n* = 22; *Trp53^{Δ/Δ}*, *n* = 33). *Trp53^{Δ/Δ}* mice developed normally and at 10 weeks of age displayed no gross morphological alterations in the kidney (Fig 1D), uterus (Fig 1H and Supporting Information Fig 1D, G, M and P), ureter, epididymis, vas deferens or vesicular glands (unpublished observations). Moreover, both male and female *Trp53^{Δ/Δ}* mice were normally fecund and fertile.

Trp53^{Δ/Δ} mice displayed no pathological alterations in the kidney, ureter, bladder, vas deferens or vesicular glands up until at least 79 weeks of age. Regions of the epithelium of the corpus epididymis of male *Trp53^{Δ/Δ}* mice frequently developed a vacuolated appearance and displayed substantial nuclear atypia at around 6 months of age (Fig 1F). To our knowledge, these lesions are unlike any reported lesions in humans. Consistent with the very low frequency of tumour formation in the human epididymis, these lesions did not progress to tumours in any of the 14 male mice in the oldest cohort and were not further studied.

While no tumours were observed in female mice in the cohorts up until approximately 1 year of age, 16 of 19 female mice in the 65–79 week cohort developed a total of 26 independent endometrial tumours of various histological appearances (Table 1 and Fig 1I–N). Individual mice frequently exhibited multiple independent endometrial tumours of different histological appearances (Supporting Information Fig 2). Human serous AdCas of the uterus typically have complex, branching papillae with cellular budding and usually broad, thick fibrovascular cores, but occasionally thin to delicate cores. The nuclei are usually highly atypical (Silverberg et al, 2003). Many of the mouse lesions displayed almost identical morphologies with high-grade nuclei, papillary or micropapillary growth patterns and were regarded as serous AdCas. AdCas composed mainly of clear or hobnail cells arranged in solid, tubulo-cystic or papillary patterns were classified as clear cell AdCas. Twelve tumours arose in the lumen of the uterus and exhibited papillary morphology with characteristics of either serous AdCa (Fig 1I) or clear cell AdCa (Fig 1J), or in some instances a mixture of both. In contrast, 11 tumours that arose from the glandular component of the endometrium displayed an

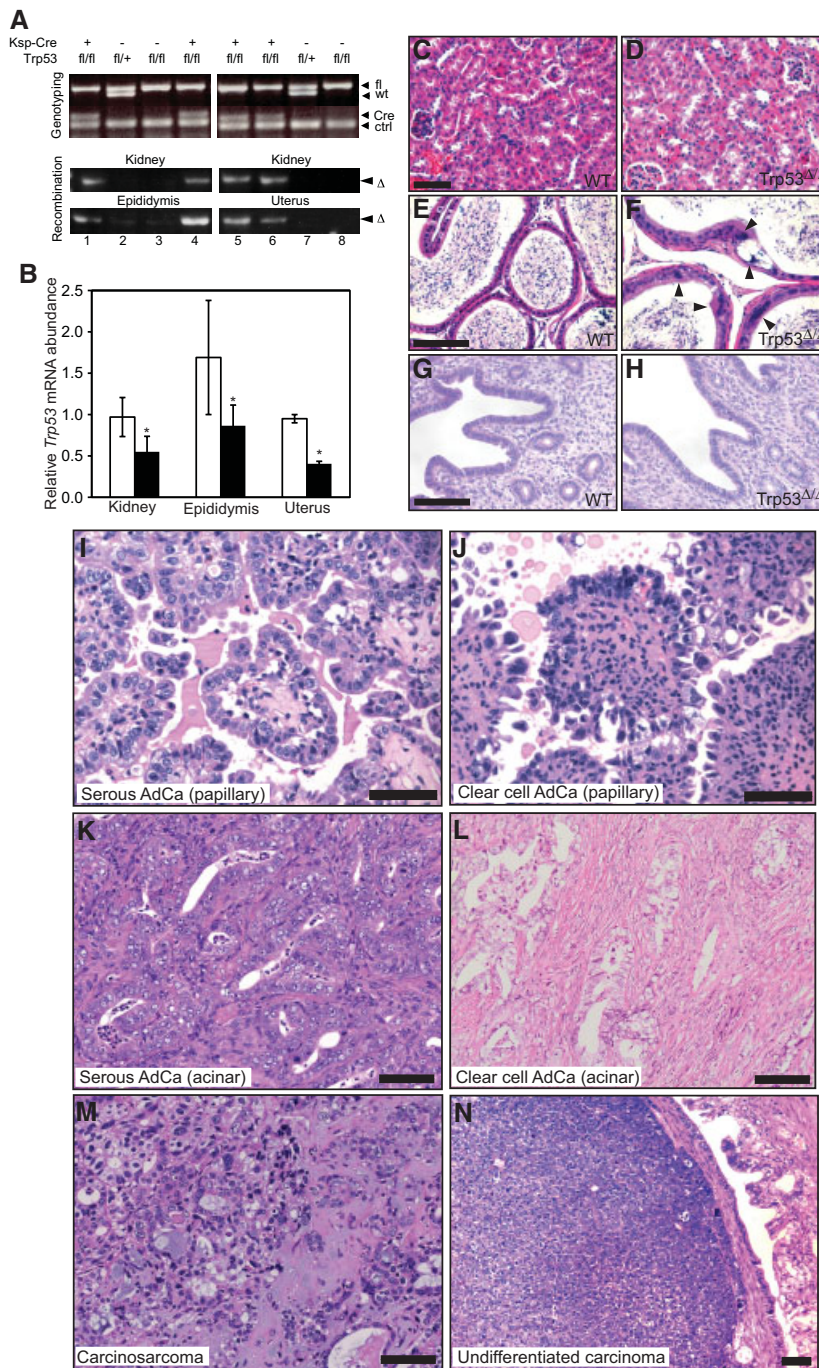


Figure 1. Type II endometrial tumours formed in older *Ksp1.3-Cre; Trp53^{fl/fl}* mice.

A. Upper panels: genotyping PCR of tail DNA to detect the *Ksp1.3-Cre* transgene (+ denotes present and – denotes absent) or the *Trp53* floxed (fl) or wild-type (+) allele. Lower panels: PCR to detect Cre-mediated recombination at the *Trp53* locus using DNA isolated from kidney, epididymis and uterus of the same mice used in the upper panels. The position of the recombined band is shown by Δ.

B. Real-time quantitative PCR analysis of *Trp53* mRNA abundance (normalized to *18S RNA* abundance) in indicated tissues from wild-type (white bars) or *Trp53^{Δ/Δ}* (black bars) mice.

C-H. Haematoxylin and eosin stain of sections of kidneys (C and D), epididymis (E and F) and uterus (G and H) from wild-type or *Trp53^{Δ/Δ}* mice aged 10 weeks (C, D, G and H) or 6 months (E and F). (I–N) Examples of tumours that arose in 14–16-month-old knockout mice. Arrowheads in (F) highlight atypical nuclei. All scale bars represent 50 μm.

I–N. Serous AdCa with papillary growth pattern (I), Clear cell AdCa with papillary growth pattern (J), Serous AdCa with acinar growth pattern (K), Clear cell AdCa with acinar growth pattern (L). Carcinosarcoma displaying mixed clear cell and (heterologous) chondrosarcoma components (M), Undifferentiated carcinoma (N).

acinar pattern of growth and displayed either serous or clear cell AdCa morphologies (Fig 1K and L). Three carcinosarcomas with mixed clear cell AdCa components and either chondrosarcoma (Fig 1M) or rhabdomyosarcoma components also arose. Additionally, two undifferentiated carcinomas, containing a mixture of regions of solid, tubulo-cystic and squamous growth patterns (Fig 1N) arose. Of these latter five lesions, four had invaded tissues beyond the uterus and one of these had metastasized throughout the peritoneal cavity, displaying nodules of tumour growth on the liver, intestines and peritoneal

wall. Carcinosarcomas and mixed carcinomas are believed to result from the de-differentiation of high-grade type I or type II carcinomas (Abeln et al, 1997; Wada et al, 1997). Supporting this idea, two of the three tumours of this type that arose in *Trp53^{Δ/Δ}* mice were very large tumours (Table 1 and Supporting Information Fig 3) that contained regions of either clear cell or undifferentiated carcinoma. One carcinosarcoma contained a very large region of ‘pure’ tubulo-cystic clear cell AdCa that likely represents the initial large lesion from which the carcinosarcoma component is derived (Supporting Information

Table 1. Summary of endometrial tumour incidence, histological subtype, growth pattern, nuclear grade, tumour size and staining intensity for antibodies against phospho-Tyr1248-ErbB2 (P-ErbB2), phospho-Thr202/Tyr204-ERK1/2 (P-ERK), phospho-Ser473-AKT (P-AKT), phospho-Thr421/Ser424-p70 S6 Kinase (P-S6K), phospho-Ser240/244-ribosomal S6 protein (P-S6) and phospho-Thr37/46-4E-BP1 (P-4E-BP1)

Mouse ID	Age (weeks)	Tumour ID	Tumour entity	Histological subtype (s)	Growth patterns	Nuclear grade	Diameter (mm)	P-ErbB2	P-ERK	P-AKT	P-S6K	P-S6	P-4E-BP1
310	65	21	EiC	Serous	Micropapillary, acinar, cribriform	High	0.5	+	+++	+	+	+	+
279	72	2	AdCa	Serous	Micropapillary, acinar, cribriform	High	4.0	+	+	+	+++	+++	+
279	72	5	AdCa	Serous, partly clear cell	Micropapillary, acinar, cribriform, hobnailing	High	5.5	+	+	+	+++	+++	+
306	69	17	AdCa	Serous, partly clear cell	Micropapillary, acinar, cribriform, diffuse	High	13.0	+	+	+	+++	+++	+
285	67	6	AdCa	Serous	Acinar, cribriform	High	3.7	+	+	+	+++	+++	+
281	72	11	AdCa	Serous	Micropapillary, acinar, cribriform	High	1.3	+	+	+	+	+	+
310	65	20	AdCa	Serous	Micropapillary, acinar, cribriform	High	1.7	+	+	+	+	+	+
281	72	9	AdCa	Serous	Acinar, cribriform	High	3.3	+	+	+	+	+	+
279	72	4b	EiC	Serous, partly clear cell	Papillary, hobnailing	High	0.3	+	+	+	+++	+++	+
287	67	15	AdCa	Clear cell	Papillary	Low	1.0	+	+	+	+++	+++	+++
287	67	16	AdCa	Serous	Micropapillary, acinar, cribriform	High	0.9	+	+	+	+++	+++	+
288	67	23a	EiC	Serous	Acinar, cribriform	High	0.4	+	+++	+	+++	+++	+
281	72	8	AdCa	Serous, partly clear cell	Papillary, micropapillary	High	3.1	+	+++	+	+++	+++	+++
323	68	14	AdCa	Serous, partly clear cell	Micropapillary, papillary, hobnailing	Low	2.3	+	+++	+	+++	+++	+++
388	71	25	AdCa	Serous, partly clear cell	Micropapillary, papillary, hobnailing	High	1.4	+	+++	+	+++	+++	+++
280	72	27	AdCa	Serous, partly clear cell	Micropapillary, papillary, hobnailing	High	1.1	+	+++	+	+++	+++	+++
282	72	19	AdCa	Serous, partly clear cell	Papillary, micropapillary	Low	4.5	+	+++	+	+++	+++	+++
279	72	4a	EiC	Serous	Micropapillary, acinar	High	0.5	+++	+	+	+	+++	+
320	68	12	EiC	Serous	Micropapillary, papillary, acinar	High	0.5	+	+	+	+++	+	+
389	71	7	AdCa	Serous	Micropapillary, acinar, cribriform	High	4.0	+	+	+	+	+	+
281	72	10	AdCa	Serous	Micropapillary, acinar, cribriform, diffuse	High	8.2	+	+	+	+	+	+++
323	68	13	Carcinosarcoma	With heterologous component	Acinar, cribriform, signet ring like, solid, diffuse	High	13.0	+	+++/+	+/-	+/-	+/-	+++/-
278	58	22	Carcinosarcoma	With heterologous component	Tubulo-cystic, solid, diffuse	High	16.0	+/-	+++/-	+/-	+/-	+	+++/-
288	67	23b	Carcinosarcoma	With heterologous component	Solid, diffuse	High	2.5	-	+/-	+/-	+/-	+/-	+++/-
275	75	18	Carcinoma	Undifferentiated	Solid, tubulo-cystic, squamous	High	11.0	+	+++/-	-	+	+	+/-
280	72	26	Carcinoma	Undifferentiated	Solid	High	15.0	-	+++/-	-	-	+	+++

AdCa, adenocarcinoma; EiC, endometrial intraepithelial carcinoma; +, same staining intensity as non-tumourous endometrium; +++, upregulated staining compared to non-tumourous endometrium; -, no staining; /, mixed staining within tumour.

Fig 3). PCR analysis of genomic DNA isolated from 4 of 4 macrodissected tumours displayed Cre-mediated recombination at the *Trp53* locus (unpublished observations), indicating that these tumours arose from *Trp53* knockout cells. Thus, three different histological subtypes of type II endometrial carcinomas, as well as carcinosarcomas, arose in *Trp53^{Δ/Δ}* mice at high frequency.

Identification of tumour precursor lesions in *Trp53^{Δ/Δ}* mice

Analysis of the non-tumorous endometrium of tumour-bearing mice identified a series of putative tumour precursor lesions in the surface epithelium of the lumen and in the epithelium of

endometrial glands. In longitudinal histological sections of uteri from 16 mice that contained 26 macroscopically visible tumours we identified 169 and 191 independent sites of EmGD in the epithelium lining the glands (Fig 2B) and the lumen (Fig 2F), respectively. EmGD represents the earliest morphologically identifiable serous carcinoma precancerous lesion in humans (Yi & Zheng, 2008; Zheng et al, 2004). The EmGD lesions in the lumen and glands of *Trp53^{Δ/Δ}* mice are histologically identical to human EmGD and are characterized by enlarged and hyperchromatic nuclei, prominent nucleoli, nuclear crowding and loss of basal nuclear localization. In humans, EIC is believed to arise from EmGD and appears to be the immediate precursor of serous AdCa (Ambros et al, 1995; Sherman et al, 1995). Serous EIC is specifically associated with concurrent serous AdCa of the endometrium but may also be observed in association with clear cell AdCa (Ambros et al, 1995; Lax et al, 1998). EIC exhibits single or multiple layers of malignant cells that have replaced the endometrial surface epithelium or gland. The human EIC cells have pleomorphic nuclei and frequent mitotic figures, resembling the cells of high-grade tumours (Sherman et al, 1995). Endometrial glands and lumen in *Trp53^{Δ/Δ}* mice also displayed numerous sites of EIC (46 and 54, respectively), characterized by extensive nuclear atypia, multi-layered cellular growths and the presence of micropapillary projections (Fig 2C and G). A novel apparent tumour precursor lesion that we term ‘EIC with papillary structures’ was also evident in the lumen surface epithelium. This lesion, which was identified at 20 discrete sites in these sections, is characterized by a papillary growth pattern with a fibrovascular core (Fig 2H). The epithelial components of some of these papillary EIC lesions exhibited typical clear cell morphology with tubulocystic growth pattern, cuboidal cells with clear cytoplasm and ‘hobnail’ cells with apical nuclei that bulge into the lumen. Other lesions displayed serous morphology with micropapillary features, large vesicular nuclei with coarse chromatin and prominent nucleoli. Thus, these EIC lesions appear to be precursor lesions of clear cell and serous AdCAs with papillary growth patterns. An equivalent intermediate lesion, that we call ‘microinvasive adenocarcinoma’, was observed 10 times in the glandular compartment of the endometrium (Fig 2D). Based on their morphologies and relative frequencies of occurrence, it appears that these lesions represent a spectrum of histological changes that occur in the progression from a normal epithelium to a carcinoma, consistent with the model proposed for human endometrial serous AdCa (Zheng et al, 2004). AdCAs with a papillary growth pattern in our model appear to form exclusively from the surface

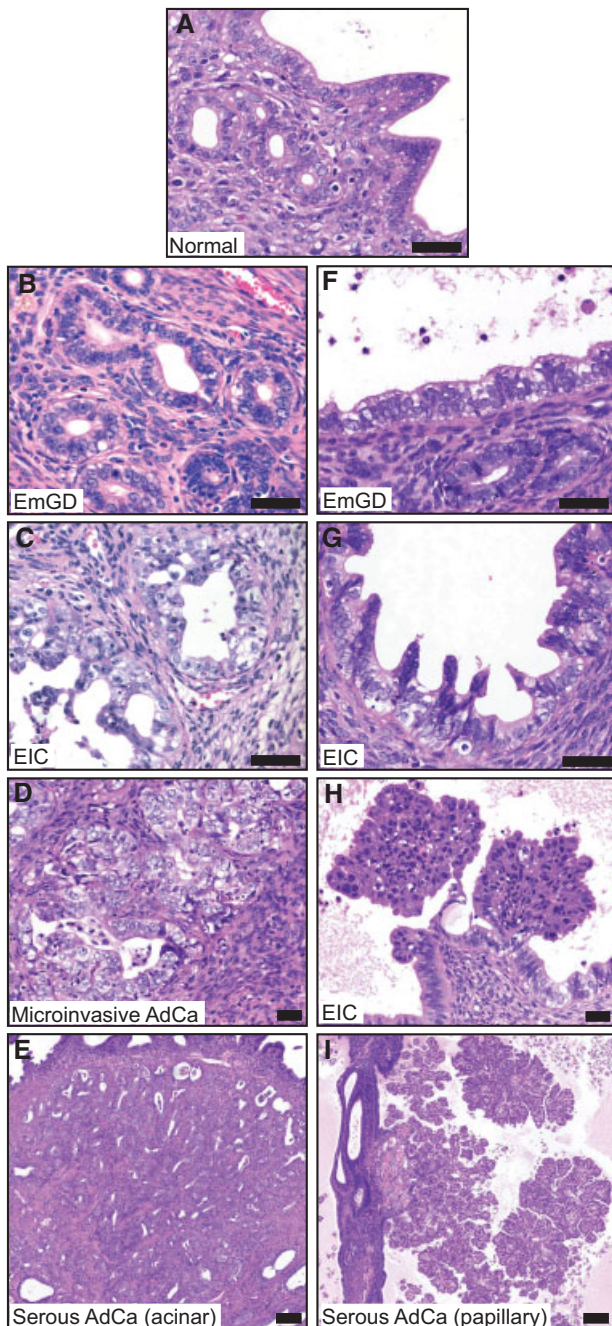


Figure 2. Precursor lesions and progression model of tumour formation.

A. Normal endometrial glands and luminal surface epithelium.
B-E. Examples of lesions illustrating the proposed progression model from EmGD arising in glands (B), to EIC in the glands (C) to microinvasive AdCa (D) to serous AdCa with acinar growth pattern (E).
F-I. Examples of lesions illustrating the proposed progression model from EmGD arising in luminal surface epithelium (F), to EIC in the lumen (G) to EIC with papillary growth (H) to serous AdCa with papillary growth pattern (I). Scale bars in A–H depict 50 μm and in E and I depicts 200 μm.

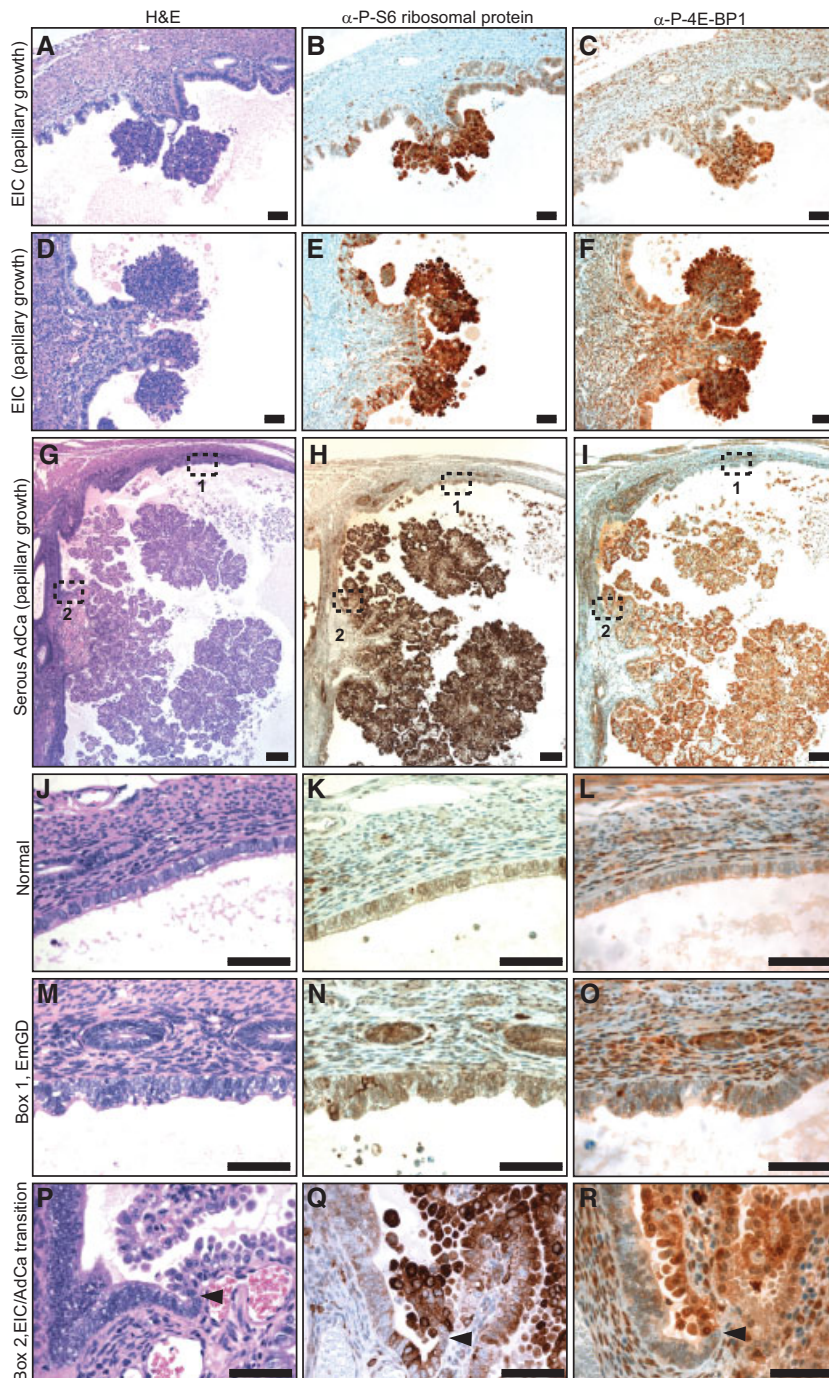


Figure 3. Upregulation of P-S6 and P-4E-BP1 at transition zones of serous AdCa. (A, D, G, J, M, and P) Haematoxylin and eosin stain (H&E), (B, E, H, K, N and Q) P-S6 IHC stain and (C, F, I, L, O and R) P-4E-BP1 IHC stain.

A-F. Two examples of EIC with papillary growth.
 G-I. Low-power image of a serous AdCa and dysplastic adjacent epithelia.
 J-L. Non-dysplastic endometrial surface epithelium from a region that is continuous with the upper epithelium but outside of the fields shown in (G-I).
 M-O. Zoom of boxed region 1 from (G) to (I) showing EmGD.
 P-R. Zoom of boxed region 2 from (G) to (I) showing the transition from EmGD (multi-layered epithelium below/left of the arrowhead) to EIC/serous AdCa (epithelium above/right of the arrowhead). Scale bars in (G-I) represent 200 μ m, in all other panels 50 μ m.

epithelium of the lumen (Fig 2I), whereas AdCas with an acinar growth pattern arise from endometrial glands (Fig 2E). Supporting the progression model, and consistent with observations in human disease (Zheng et al, 2004), the endometrial epithelium directly surrounding several papillary AdCas (Fig 3G shows an example) displayed a continuous transition from normal epithelium (Fig 3J) to EmGD (Fig 3M) to EIC (Fig 3P) to a papillary AdCa growth pattern (Fig 3P). Furthermore, all mice in the 24–29-week-old cohort displayed some regions of EmGD (Supporting Information Fig 1E, H, N

and Q) and all mice in the 47–58-week-old cohort frequently displayed widespread regions of EmGD and/or EIC (Supporting Information Fig 1F, I, O and R) but did not display any tumours. Thus, as they age, *Trp53*-deficient mice progressively display a spectrum of endometrial histological alterations ranging from low-nuclear grade dysplasia through to invasive high-grade carcinomas. These mice therefore represent the first model that accurately reproduces the progression and hallmark morphological features of human type II endometrial carcinomas.

Molecular analyses of *Trp53* mutant endometrial tumours

Since the *Ksp1.3-Cre* transgene drives gene deletion during embryogenesis (Frew et al, 2008; Shao et al, 2002) but tumours do not arise until approximately 14–16 months of age, *Trp53* appears to be largely dispensable for the normal formation and proliferative homeostasis of the endometrium. It is likely that additional mutations must occur that cooperate with *Trp53* mutation to initiate and drive tumour progression. Since 17–29% of type II endometrial tumours in humans are characterized by amplifications of the *ERBB2* gene (Konecny et al, 2009; Morrison et al, 2006; Nofech-Mozes et al, 2008) we analysed ErbB2 protein expression and activation status using immunohistochemical (IHC) staining of sections of mouse uteri containing a total of 26 type II tumours. One small glandular clear cell EIC displayed intense staining for ErbB2 and for phospho-Tyr1248-ErbB2, demonstrating that the expression of this oncogenic tyrosine kinase receptor is highly upregulated and activated (Table 1 and Supporting Information Fig 4). However, no other tumours showed upregulation of ErbB2 expression or activity, implicating pathways other than ErbB2 as playing a predominant pathogenic role in these tumours.

To investigate the activation status of other signalling pathways that may be dysregulated, IHC staining was conducted using antibodies that recognize phosphorylated forms of various signalling molecules. A positive score was given to a tumour either if the intensity of staining or the frequency of staining in the epithelial cells was higher than non-tumourous endometrium in the same section (see Supporting Information Fig 5 for an example). Markers of activation of the AKT-mTORC1 signalling axis, including phospho-Ser473-AKT (P-AKT), phospho-Thr421/Ser424-p70 S6 Kinase (P-S6K), phospho-Ser240/244-ribosomal S6 protein (P-S6) and phospho-Thr37/46-4E-BP1 (P-4E-BP1) were upregulated in a large percentage of the tumours (Table 1). 21 of 26 tumours displayed positive staining for at least one marker of the AKT-mTORC1 signalling pathway and 9 of these were positive for two or more markers. In contrast, only 5 of 24 tumours were positive for phospho-Thr202/Tyr204-ERK1/2 (Table 1), one was positive for phospho-Ser217/221-MEK1/2 and none for phospho-Thr183/Tyr185-SAPK/JNK (unpublished observations). Thus, type II endometrial tumours in *Trp53^{Δ/Δ}* mice frequently display over-activation of the AKT-mTOR C1 signalling cascade but more rarely of the MAPK signalling cascades. A particularly striking correlation between activation of the AKT-mTORC1 signalling pathway and tumour subtype was observed in the group of papillary lesions, including serous and clear cell AdCas as well as larger EIC lesions (Table 1).

We took advantage of this correlation as well as the strong and specific IHC staining pattern of P-S6 and P-4E-BP1 as sensitive downstream markers of activation of mTORC1 to further investigate the sequence of events that leads to the formation of papillary AdCas. Small clusters of elevated staining for P-S6 were frequently observed in regions of EmGD lesions in glands (Supporting Information Fig 6A and B) and in the lumen (Supporting Information Fig 6C and D) of endometria from *Trp53^{Δ/Δ}* mice. However, regions of EmGD were also observed that did not display elevated staining for P-S6 (Supporting

Information Fig 6E and F). Sites of elevated staining corresponded to 18 and 12%, in the glands and lumen, respectively, of the total number of identified EmGD lesions in the same samples, suggesting that mTORC1 pathway activation is not required for the transition from a normal epithelium to EmGD. Strikingly however, all papillary EIC lesions, even those papillary outgrowths that comprised only a few cells (Supporting Information Fig 6G and H), were strongly positive for P-S6 (Fig 3B and E) and P-4E-BP1 (Fig 3C and F). Moreover, in three large papillary tumours where it was possible to identify a continuous transition in the epithelium from normal to EmGD to EIC to serous or clear cell AdCa, elevated staining for P-S6 (Fig 3H and Q) and for P-4E-BP1 (Fig 3I and R) was observed precisely at the transition between EIC and papillary AdCa. These data demonstrate that mTORC1 pathway activation is not necessary for the formation of EmGD or EIC, but suggest that they are associated with, or drive, papillary outgrowth, a frequent feature of aggressive clear cell and serous endometrial AdCas.

Activation of the PI3K-AKT-mTORC1 pathway occurs frequently in all subtypes of human endometrial tumours

We constructed two human endometrial carcinoma tissue microarrays (TMA; see Supporting Information Table 1), in total comprising tumour punches from 463 endometrioid AdCas (type I), 34 serous AdCas (type II), 19 clear cell AdCas (type II), 16 undifferentiated carcinomas (type II) and 16 carcinosarcomas. A poor patient outcome was independently associated with type II carcinomas or carcinosarcomas (Supporting Information Fig 7A), older age at diagnosis (Supporting Information Fig 7B), high-grade (see later in Fig 6G) or high-FIGO stage (see later in Fig 6J), validating the TMAs against known clinical parameters of these tumour types. We immunohistochemically stained the TMAs using antibodies against proteins or epitopes that reveal the activation status of the PI3K and/or mTORC1 signalling pathways (p110 α , PTEN, P-AKT, phospho-Ser9-GSK3 β (P-GSK3 β), phospho-Ser2448-mTOR (P-mTOR), TSC1, TSC2, P-S6K and P-4E-BP1), or have been previously proposed to be differentially expressed between different subtypes of endometrial carcinoma (PAX8, IMP3 and p16; Mhawech-Fauceglia et al, 2010; Tong et al, 2010; Yemelyanova et al, 2009) or have been shown to predict a poor prognosis (p53, ErbB2; Catusus et al, 2009; Konecny et al, 2009; Morrison et al, 2006). Examples of tumour spots displaying different IHC scores for p53 or displaying negative (0) and strong (3) IHC scores for the other antibodies are shown in Supporting Information Fig 8.

Consistent with our mouse genetic observations that *Trp53* suppresses type II endometrial tumours, tumours displaying frequent and strong nuclear p53 expression, an indicator of *TP53* mutation or aberrant stabilization, occurred more frequently in serous AdCas, clear cell AdCas, carcinosarcomas and undifferentiated carcinomas than in endometrioid AdCas (Fig 4A). High levels of p16 expression were more frequent in serous AdCas and carcinosarcomas than in endometrioid AdCas (Fig 4B). High levels of ErbB2 expression were more frequent in serous AdCas and clear cell AdCas than in endometrioid AdCas (Fig 4C). PAX8 and IMP3 expression are more frequently high in serous AdCas,

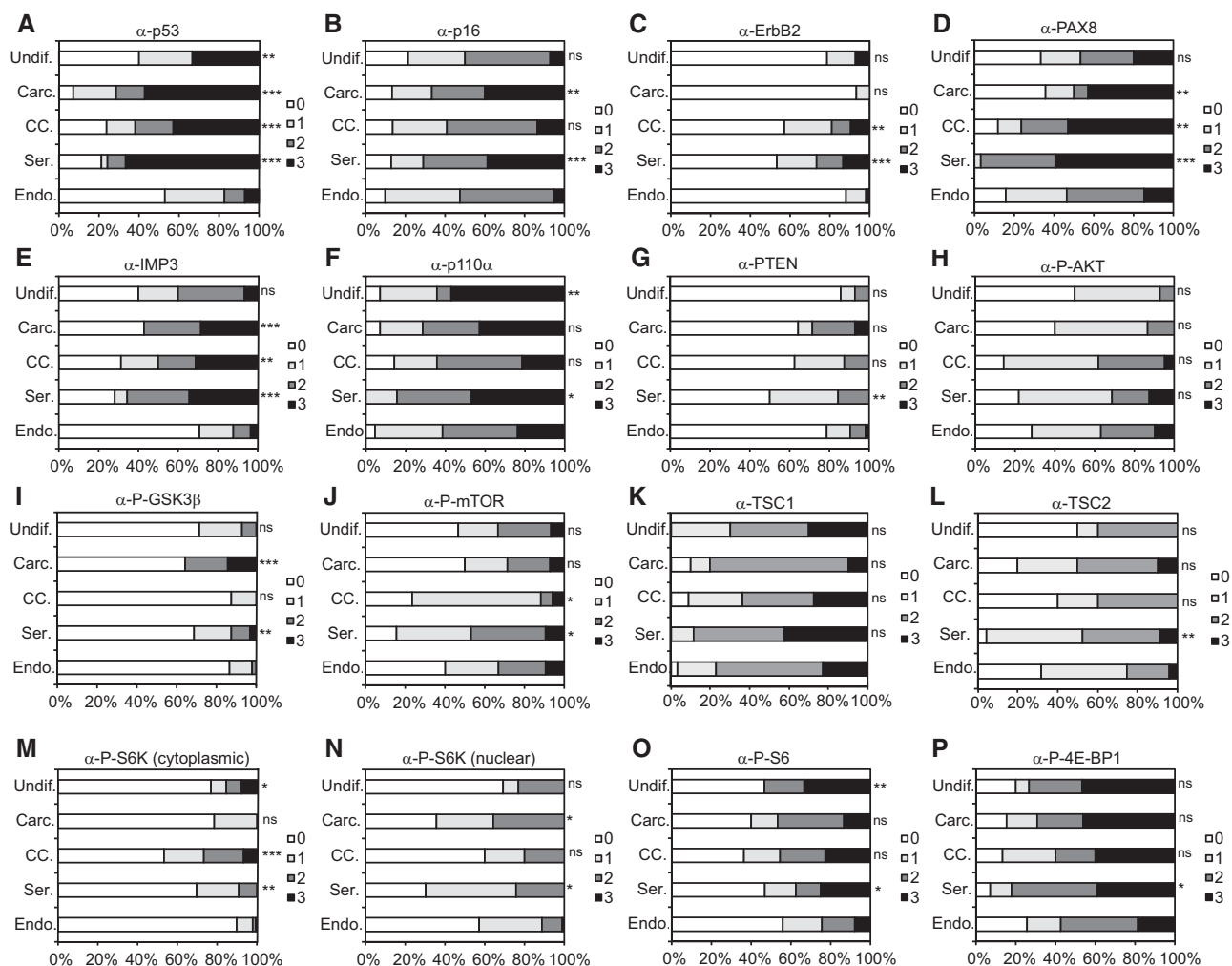


Figure 4. Identification of molecular discriminants of tumour subtype. TMA were stained with antibodies against (A) p53, (B) p16, (C) ErbB2, (D) PAX8, (E) IMP3, (F) p110 α , (G) PTEN, (H) P-AKT, (I) P-GSK3 β , (J) P-mTOR, (K) TSC1, (L) TSC2 (M) P-S6K (cytoplasmic staining), (N) P-S6K (nuclear staining), (O) P-S6 and (P) P-4E-BP1. Graphs depict cumulative frequency of staining score for each tumour subtype. Fisher's exact test was used to compare frequency of expression of each marker in each subtype of endometrial cancer pairwise with endometrioid carcinoma. * $p < 0.05$, ** $p < 0.01$ or *** $p < 0.001$, ns, no significant difference. Endo, endometrioid adenocarcinoma; Ser, serous adenocarcinoma; CC, clear cell adenocarcinoma; Carc, carcinosarcoma; Undif, undifferentiated carcinoma.

clear cell AdCas and carcinosarcomas than in endometrioid tumours (Fig 4D and E). Thus, subsets of type I and type II tumours displayed different, as well as overlapping, molecular profiles.

Consistent with the known frequent mutational activation of the PI3K-AKT-mTORC1 signalling pathway in type I endometrioid AdCas this group of tumours frequently displayed moderate or strong expression of p110 α (Fig 4F), P-AKT (Fig 4H), P-GSK3 β (Fig 4I), P-mTOR (Fig 4J), P-S6K (Fig 4M and N), P-S6 (Fig 4O) and P-4E-BP1 (Fig 4P), as well as frequent lack or low level of PTEN staining (Fig 4G). Consistent with the findings in our mouse model, serous AdCas, clear cell AdCas, carcinosarcomas and undifferentiated carcinomas also frequently displayed a similar PI3K pathway activation signature. Indeed, the frequency of type II tumours that displayed high levels of expression of PI3K pathway activation markers (or low

levels of expression of PTEN) were always either statistically indistinguishable from the frequency displayed by type I tumours or in many cases were more frequent. Thus, dysregulation of the PI3K-AKT-mTORC1 signalling pathway is a unifying molecular feature of many type I and type II endometrial tumours.

p53 status strongly predicts patient survival in endometrial carcinomas

Given the overlapping molecular features between subsets of type I and type II tumours, we sought to identify pathogenic factors and diagnostic markers for aggressive cases of endometrial carcinomas in general, irrespective of histological subtype. Kaplan-Meier survival curves for each marker are presented in Fig 5. High levels or frequency of expression of p53 (Fig 5A), p16 (Fig 5B), IMP3 (Fig 5E), p110 α (Fig 5F), P-GSK3 β

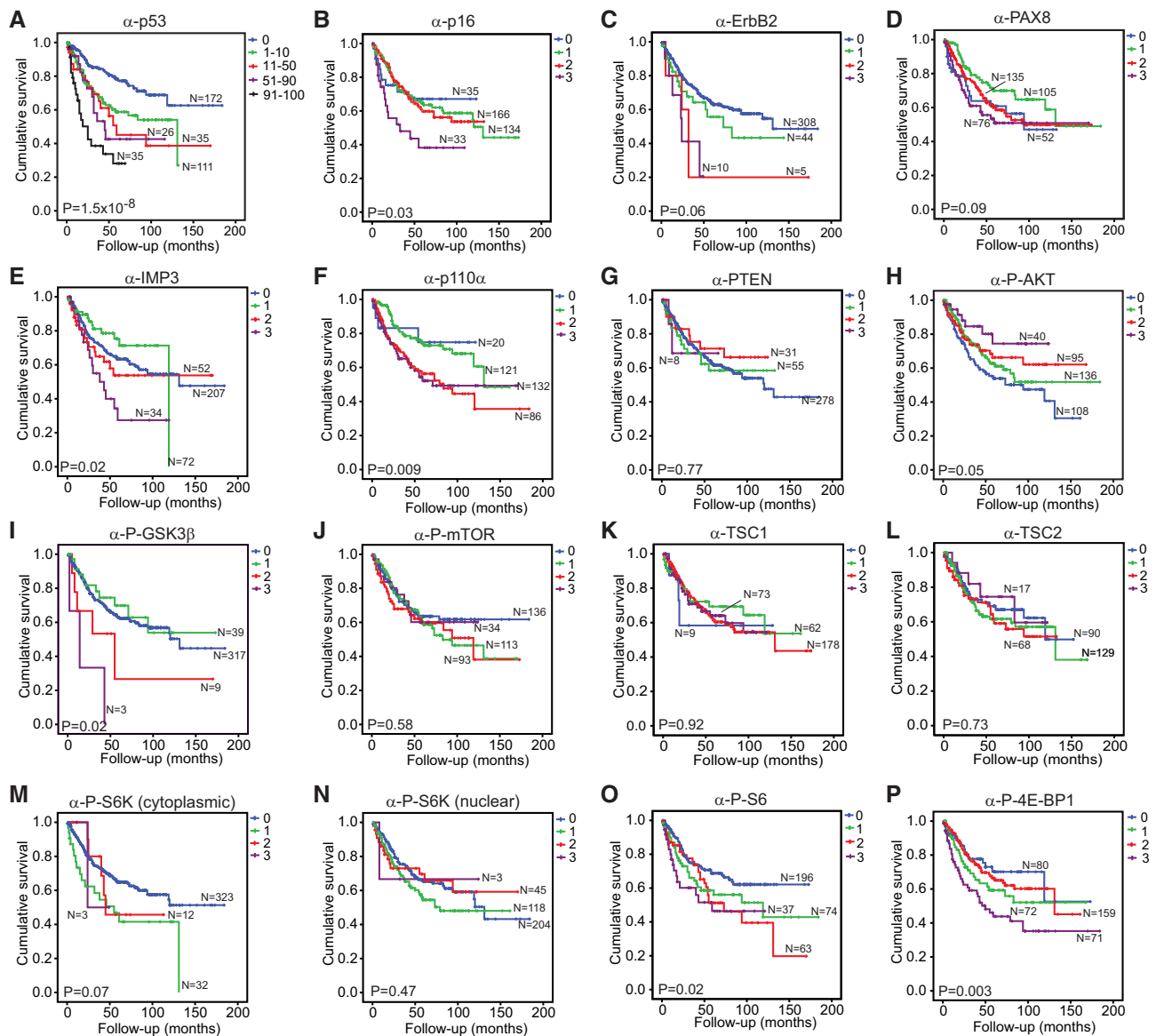


Figure 5. Identification of individual molecular predictors of patient outcome in endometrial carcinoma. Kaplan–Meier plots of patient survival over time after diagnosis when patients were grouped according to IHC staining frequency (% strongly positive cells) for (A) p53 or IHC score for (B) p16, (C) ErbB2, (D) PAX8, (E) IMP3, (F) p110α, (G) PTEN, (H) P-AKT, (I) P-GSK3β, (J) P-mTOR, (K) TSC1, (L) TSC2, (M) P-S6K (cytoplasmic staining), (N) P-S6K (nuclear staining), (O) P-S6 or (P) P-4E-BP1. Log Rank (Mantel–Cox) tests were conducted to test for equality in the survival expectation of each group. *N*-values represent the number of patients in each group.

(Fig 5I), P-S6 (Fig 5O) or P-4E-BP1 (Fig 5P) all independently predicted poor survival, emphasizing the importance of p53 and the PI3K-mTORC1 pathway in controlling the aggressiveness of endometrial tumours.

We systematically assessed if multiple combinations of any of these IHC markers may represent a ‘signature’ that is a superior predictor of survival. To overcome the frequent problem of missing values in TMA datasets we developed a novel statistical approach based on a learning model that is invariant to missing values and results in an easily interpretable and practically applicable linear model. This learning model generated a hazard

score for each endometrial carcinoma patient based on combinations of marker stainings. The final model was able to split the patients into a high-risk group and a low-risk group based on staining patterns for p110α, P-S6, ErbB2 and p53 (Fig 6A). The difference between these groups was highly statistically significant and the individual contribution of each marker to this signature is shown by the coefficient confidence intervals depicted in Fig 6B. The model was validated by a 10-fold cross-validation strategy (Fig 6C). A multivariate Cox regression model was then learned to assess the influence of the risk score, age at diagnosis, histological tumour subtype,

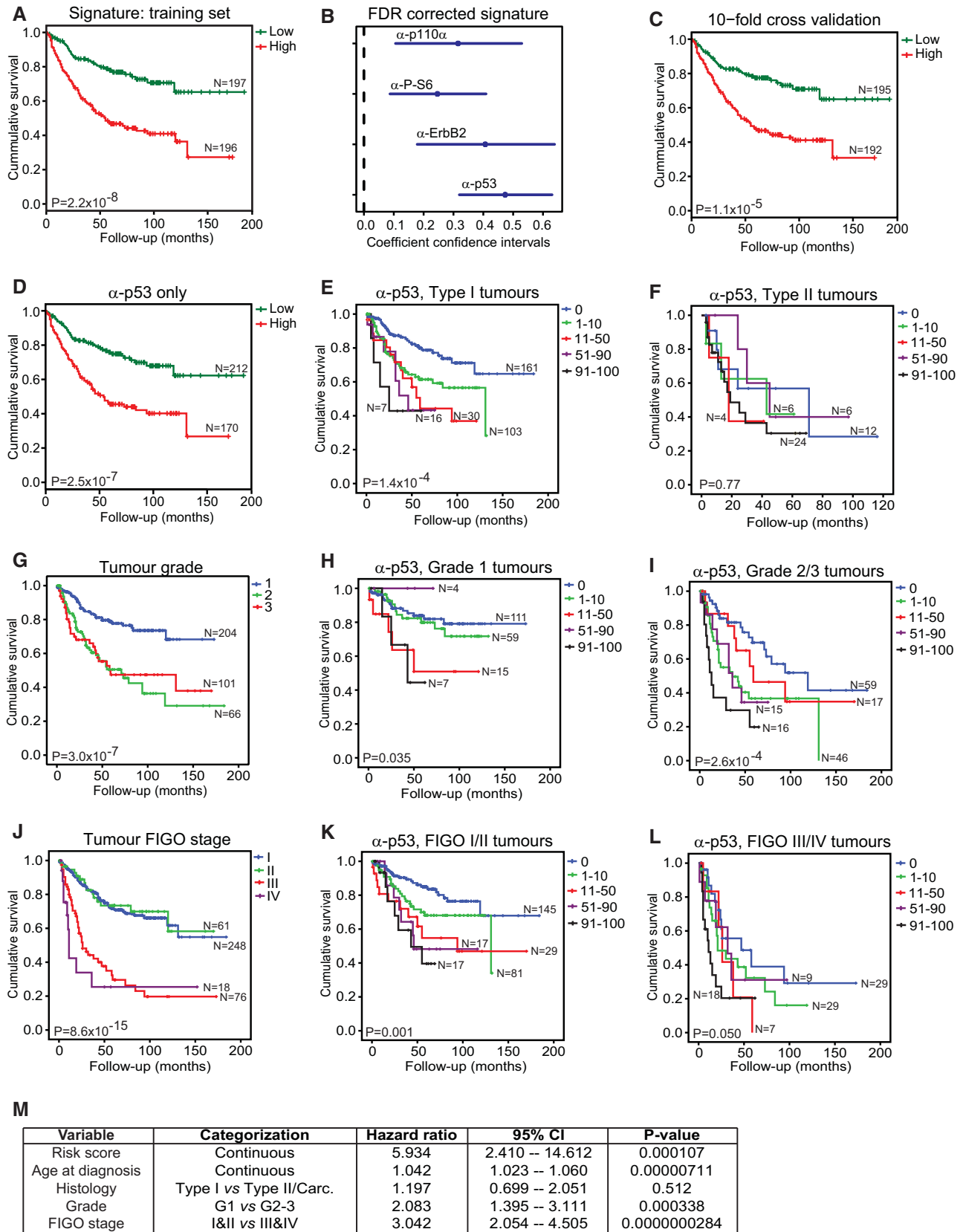


Figure 6.

tumour grade and FIGO stage on the overall survival rate (Fig 6M). In this model, the risk score was the best predictor of poor outcome ($p = 0.000107$) with a hazard ratio of 5.93 (95% confidence interval 2.41–14.61). Hence in cases with increased risk score, the probability of death was approximately six times higher than in patients with low-risk scores. Age at diagnosis, tumour grade and FIGO stage also had a significant effect on overall survival, albeit with lower hazard ratios than the risk score (Fig 6M). Detailed analysis of the signature model revealed that p53 is the dominating predictor and that the risk for an individual patient can be equally well assessed by using p53 staining status alone as by using the entire signature (Fig 6D). Thus, these analyses demonstrated that p53 status is by far the most important factor in predicting survival of patients with endometrial carcinomas.

Learning models were also separately applied to type I and type II tumours in order to predict a risk score for each endometrial carcinoma patient (Supporting Information Fig 9). The model for type I endometrial tumours was able to split the patients into a high-risk group and a low-risk group based on staining patterns for p110 α , P-AKT, ErbB2 and p53, with p53 again being the dominating predictor. In contrast, the model learned for type II tumours revealed that P-S6K was the only IHC-based marker necessary to predict the outcome of patients. p53 did not show a significant effect on overall survival in type II tumours.

We next subdivided the endometrial carcinoma patients into the categories that are currently used for endometrial carcinoma diagnosis, namely histological subtype, tumour grade and FIGO stage and investigated the prognostic power of our risk signature (p110 α , P-S6, ErbB2 and p53) and of p53 IHC staining alone in each of these groups. In each cohort of patients, either p53 staining alone (Fig 6D–L) or the four-marker signature (Supporting Information Fig 10) was able to predict poor patient outcome. Almost half of all type I endometrioid tumours, which are typically considered to be less aggressive tumours, displayed some degree of strong nuclear p53 staining and this strongly predicted a poor outcome for this subset of patients (Fig 6E). In contrast, p53 staining was not predictive of survival of patients with type II endometrial carcinomas (Fig 6F), likely because the vast majority of these tumours contain *TP53* mutations and perhaps because some of the non-p53 staining tumours may have lost p53 function by gene deletion or truncating mutation, which cannot be detected in our assay. Importantly, while conventional histopathological analysis

predicts a better clinical outcome for grade 1 tumours in comparison to grade 2 or 3 tumours (Fig 6G), or for FIGO stage I and II tumours in comparison to FIGO stage III and IV tumours (Fig 6J), grouping according to p53 IHC score within these subgroups was able to further stratify these patients according to risk of death and predicted a poor survival outcome in the early grade (Fig 6H) and stage (Fig 6K) tumours, as well as in the more advanced grade (Fig 6I) and stage (Fig 6L) tumours. In this study, p53 immunoreactivity therefore represents a highly sensitive and predictive marker that assists with risk stratification of endometrial carcinomas.

To validate that our p53 IHC score indeed reflects the presence of *TP53* mutations we performed deep sequencing analyses (Supporting Information Table 2) of *TP53* exons 5–8, in which mutations were detected previously with high frequency (<http://www.sanger.ac.uk/genetics/CGP/cosmic>). Tumour DNA was isolated from endometrioid ($n = 56$) or serous ($n = 7$) endometrial carcinomas that displayed different p53 IHC scores. An average of 2059 sequencing reads per amplicon provided insight into the heterogeneity of *TP53* mutations present in each individual tumour. The number of independent *TP53* gene mutations that cause coding alterations (Fig 7A) and the cumulative frequencies at which these mutations were represented in the DNA population (Fig 7B) were similar in all groups of tumours, regardless of subtype or p53 IHC score. However, the frequency of the most abundant (dominant) *TP53* mutation in each tumour correlated excellently with the p53 IHC score (Spearman's $\rho = 0.6113$, $p < 0.0001$). Groups of tumours that displayed an IHC score of over 50% contained dominant *TP53* mutations that were present at high frequencies in the tumour DNA population (Fig 7C). Bioinformatic assessment of the predicted effect of these mutations on the function of the p53 protein revealed that tumours with a high p53 IHC score more frequently contain dominant mutations that are predicted to have a highly detrimental effect on protein function (Fig 7D). Moreover, there is a statistically significant association between the predicted severity of the *TP53* mutation and the abundance of the mutation within the DNA population of an individual tumour (Fig 7E). Finally, the presence of a dominant *TP53* mutation at a frequency of greater than 33% predicted a poor patient outcome (Fig 7F). These findings demonstrate a strong correlation between the p53 IHC score and presence of *TP53* gene mutation and suggest that in both type I and serous endometrial carcinomas that *TP53* gene mutations that inhibit p53 protein function provide a selective advantage to tumour

◀ **Figure 6. p53 is the best predictor of patient survival in endometrial cancers.**

- A. Kaplan–Meier plots of low-risk and high-risk patients using a four-marker risk-model signature for endometrial carcinoma based on staining patterns for p110 α , P-S6, ErbB2 and p53.
- B. Contribution of each marker to the signature represented by coefficients and confidence intervals.
- C. Patient stratification based on 10-fold cross-validation of the linear risk score model.
- D–L. Patient stratification based on the median split of p53 expression frequency only (D). Kaplan–Meier survival plots of subgroups of patients based on p53 staining in type I tumours (E), p53 staining in type II tumours (F), tumour grade (G), p53 staining in grade 1 tumours (H), p53 staining in grade 2 and 3 tumours (I), tumour FIGO stage (J), p53 staining in FIGO stage I and II tumours (K) and p53 staining in FIGO stage III and IV tumours (L). Log Rank (Mantel–Cox) tests were conducted to test for equality in the survival expectation of each group. *N*-values represent the number of patients in each group.
- M. Multivariate Cox regression analysis of factors possibly influencing overall survival of patients with endometrial carcinoma ($n = 360$, number of events = 112, 50 observations deleted due to missing values). Carc., carcinosarcoma.

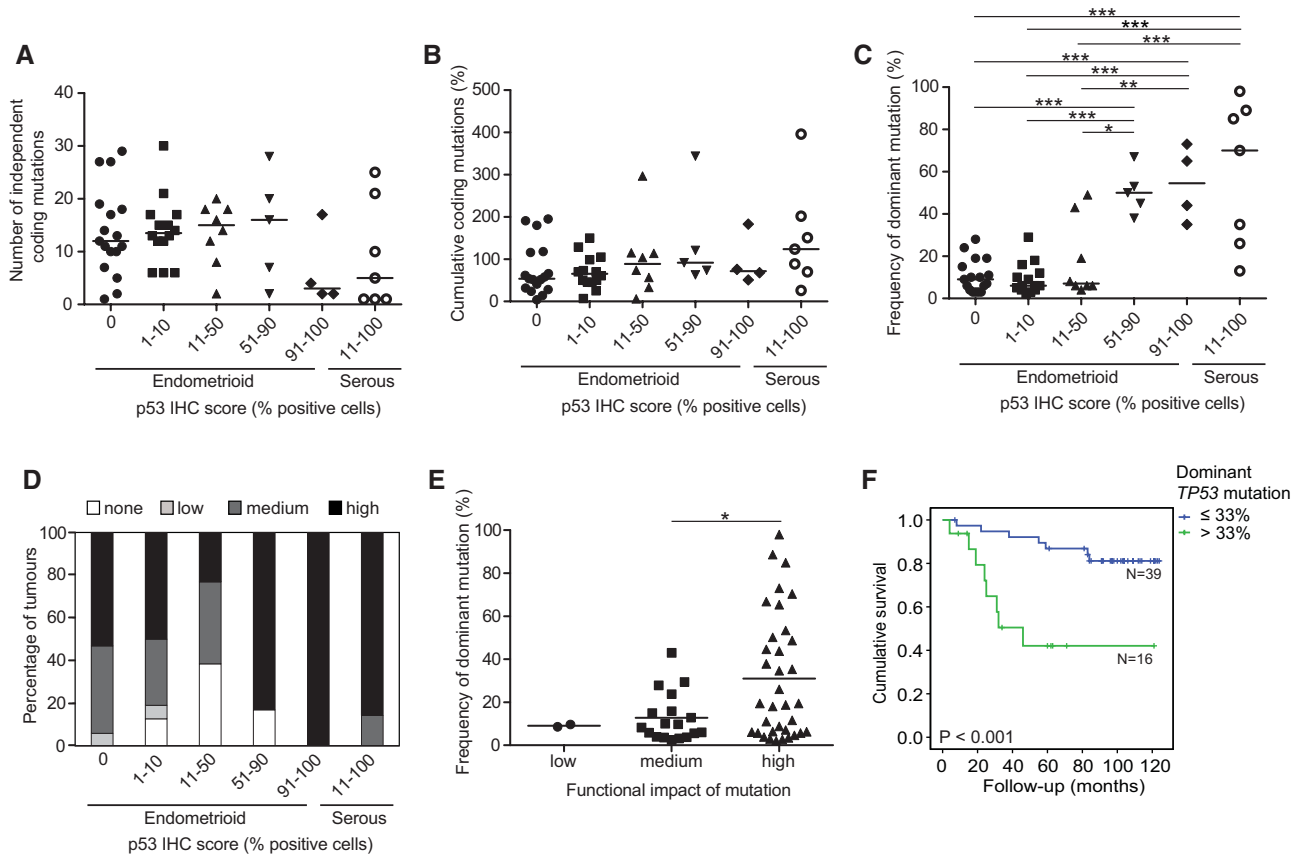


Figure 7. Deep sequencing of *TP53* exons 5–8 in endometrial carcinomas.

- A.** Total number of different mutations affecting the coding sequence in endometrioid or serous carcinomas grouped according to p53 IHC score. There are no significant differences between groups.
- B.** Cumulative frequency of sequencing reads exhibiting one or more mutations. Scores of over 100% indicate that multiple independent mutations occur in the tumour. There are no significant differences between groups.
- C.** Frequency at which the dominant mutation is represented in the tumour DNA.
- D.** Percentage of tumours for which the dominant mutations depicted in **C** are predicted to have no impact or a low, medium or high-negative functional impact on the p53 protein.
- E.** Frequency at which the dominant mutation is represented in an individual tumour, grouped according to predicted functional impact of the mutation. In **A**, **B**, **C** and **E**, statistically significant differences between groups were calculated using a one-way ANOVA and Bonferroni's multiple comparison test and depicted as * $p < 0.05$, ** $p < 0.01$ or *** $p < 0.001$.
- F.** Kaplan–Meier survival plots of patients based on the frequency of occurrence of the dominant *TP53* mutation.

cells, resulting in their enrichment in the tumour population and generation of a highly aggressive tumour.

DISCUSSION

We present an accurate mouse model of type II subtypes of endometrial carcinomas that will facilitate future molecular and clinical studies of these highly aggressive cancer subtypes. Molecular genetic studies have demonstrated that different subtypes of human type II endometrial carcinomas exhibit frequent and early inactivation or mutation of the *TP53* tumour suppressor gene. We show that deletion of the *Trp53* gene in the mouse endometrium results in the formation of tumours with very high penetrance at around 14–16 months of age, providing

strong functional genetic evidence that *Trp53* functions as a tumour suppressor gene in type II endometrial carcinomas. These tumours are not only histologically identical to their human counterparts, but arise from a series of similar morphologically identifiable precursor lesions. Lesions that are identical to the EmGD and EIC lesions that are known to be precursors of human endometrial serous AdCa are observed in our mouse model. Thus, this model allows the study of the sequence of molecular and cell biological alterations that occur during the development and progression of type II endometrial tumours.

Trp53 gene deletion under the control of the *Ksp1.3-Cre* transgene occurs during embryogenesis and induces gene deletion widely in the kidney and genitourinary tract, yet tumours arise only in the endometrium in old mice, suggesting

that additional genetic alterations must accumulate and cooperate with *Trp53* deficiency to initiate cancer. The absence of tumours in tissues other than the endometrium likely reflects the very low rate of cellular turnover in these tissues in comparison to the cyclic nature of cellular proliferation in the endometrium, which presumably allows mutations to frequently accumulate in the absence of p53 function. Indeed, we identified activation of components of the PI3K and/or mTORC1 signalling cascade in the majority of the type II endometrial tumours that arose in *Trp53* mutant mice. Taking advantage of the fact that our mouse model allows analysis of numerous apparent tumour precursor lesions we identified in some tumours that activation of the mTORC1 pathway is precisely associated with the transition from an intraepithelial proliferation pattern to a papillary growth pattern, suggesting that secondary genetic alterations that lead to activation of the mTORC1 pathway may act to drive progression of tumours from EmGD or EIC precursor lesions.

Prompted by these observations, TMA analysis of a large cohort of endometrial cancers demonstrated that activation of the PI3K-AKT-mTORC1 signalling pathway occurs not only in type I carcinomas, which are known to be characterized by frequent activation of this pathway (Catusus et al, 2009; Cheung et al, 2011; Konopka et al, 2011; Lu et al, 2008; Oda et al, 2005; Salvesen et al, 2009; Tashiro et al, 1997; Urick et al, 2011; Velasco et al, 2006), but also at an equivalent or often higher frequency in serous AdCas, clear cell AdCas, undifferentiated carcinomas and carcinosarcomas. Moreover, IHC markers of activation of this pathway independently predict poor patient survival, highlighting the importance of this signalling pathway in endometrial tumour behaviour. Thus, PI3K and/or mTORC1 pathway activation appears to be a frequent pathogenic feature of all endometrial carcinoma subtypes, not only of type I tumours. An important question for future research will be to characterize what causes the hyperactivation of PI3K-mTORC1 pathway signalling in type II tumours as *PTEN* mutations are known to be very rare in this group of tumours (An et al, 2004; Catusus et al, 2009; Salvesen et al, 2009; Tashiro et al, 1997). Interestingly, we have previously observed a high rate of loss of *PTEN* immunoreactivity in type II endometrial carcinomas (Dellas et al, 2009), suggesting that mechanisms other than deletion or mutation, for example promoter hypermethylation or microRNA-mediated silencing, may potentially contribute to *PTEN* inactivation in type II cancers. Several studies have also identified amplifications or activating mutations in the genes encoding the catalytic and regulatory subunits of PI3K, namely *PIK3CA*, *PIK3R1* and *PIK3R2* in both type I and type II tumours (Catusus et al, 2009; Cheung et al, 2011; Salvesen et al, 2009; Urick et al, 2011).

While numerous previous smaller studies investigating the significance of *TP53* mutation status or p53 immunoreactivity have demonstrated that p53-deficient endometrial carcinomas tend to have a poor survival outcome, these studies in most cases failed to demonstrate a prognostic importance of p53 in multivariate survival analyses (reviewed in Lee et al, 2009). By analysing the largest patient cohort to date we show that the immunoreactivity level of p53, which has been best described

as being involved in the pathogenesis of aggressive type II tumours, is also a powerful predictor of poor patient survival in type I tumours. Deep sequencing of exons 5–8 of the *TP53* gene showed a strong correlation between the p53 IHC staining score and *TP53* gene mutation in many tumours. The absence of highly abundant *TP53* mutations in a sub-fraction of tumours that exhibit a strong p53 IHC score is likely attributable to *TP53* mutations in other exons that were not sequenced here or due to aberrant stabilization of p53 due to alterations in one of the many regulatory pathways that control p53 protein stability and function. We demonstrate that *TP53* mutations that are predicted to have a strong detrimental functional impact on the p53 protein are frequently enriched in the population of tumour cells in type I or type II endometrial tumours, consistent with the idea that these mutations provide a proliferative advantage to the individual tumour cells that harbour these mutations. In summary, irrespective of endometrial carcinoma subtype, grade or FIGO stage, we find that strong p53 nuclear immunoreactivity represents an excellent marker to stratify patients into poor outcome groups, even in low-grade and low-FIGO stage tumours. We suggest that these patients, who would otherwise be stratified into a relatively low-risk group, should actually be considered as high-risk patients who would benefit from additional monitoring and therapy, possibly including the use of drugs to inhibit the PI3K pathway and/or strategies to kill p53 deficient tumour cells (Lane et al, 2010). We suggest that further clinical studies should investigate the possible value of performing p53 immunohistochemistry as a routine procedure to assist with risk stratification of endometrial carcinomas.

Given that aggressive type I and type II endometrial carcinomas exhibit similar patterns of p53 inactivation and PI3K pathway activation, we argue that the differences between these two subtypes may in fact be minimal and encourage that these aggressive tumour types should be viewed as being 'molecularly similar' rather than 'histologically different'. One of the major differences between the pathogenesis of type I and type II endometrial cancers may lie in their origin and the order of occurrence of mutations in *TP53* and in the PI3K pathway. Precursor lesions of type I endometrioid carcinomas, termed complex atypical hyperplasia, frequently exhibit loss of expression or mutational inactivation of *PTEN*, demonstrating that PI3K pathway activation is a very early event in type I tumour formation (Hayes et al, 2006; Tantibirojn et al, 2008). The presence of functional wild-type p53 in these early lesions likely suppresses aggressive tumour formation. Our clinical data strongly support the notion that the mutation of *TP53* in type I tumours converts them to an aggressive tumour type, reflected in the poor survival of patients whose type I tumours exhibited frequent strong p53 nuclear immunoreactivity. In contrast, type II tumours arise from precursor lesions that already lack p53 function, making them highly prone to very rapidly form aggressive tumours in response to 'second hit' mutations, such as those in the PI3K pathway or *ERBB2*, leading to a very poor clinical outcome for these patients. Consistent with these ideas, uterine-specific double knockout of *Pten* and *Trp53* caused tumours to form more rapidly than in mice harbouring deletion

The paper explained

PROBLEM:

Endometrial carcinomas of the uterus are the most common tumour type that develops in the female genital tract. The most frequent form of these tumours are so-called type I endometrioid carcinomas which typically have a relatively good prognosis. On the other hand, the rarer but more aggressive type II endometrial carcinomas, comprising serous carcinomas, clear cell carcinomas and undifferentiated carcinomas, account for a disproportionate number of endometrial cancer-associated deaths. The molecular causes of these different forms of cancer remain incompletely understood.

RESULTS:

We find that deletion of the *Trp53* tumour suppressor gene in the endometrium of mice causes the formation of all subtypes of

type II endometrial carcinomas. The accuracy of this mouse model is reflected by the fact that tumours arise from dysplastic precursor lesions that are identical to those that are believed to represent the first steps in the development of human endometrial cancers. We identify that dysregulation of the PI3K-mTOR signaling pathway plays an important role in the development of type II tumours in mice and in humans and that the status of the p53 tumour suppressor protein is a very powerful predictor of the aggressiveness of both type I and type II subtypes of human endometrial tumours.

IMPACT:

This highly accurate mouse model of type II endometrial carcinomas will facilitate future studies of the causes of these cancers.

of *Pten* alone (Daikoku et al, 2008). Unfortunately, the histological appearance of these double knockout tumours was not reported. Moreover, sequencing of *PTEN*, *PIK3CA* and *TP53* in human endometrial carcinomas revealed that mutations in either *PTEN* or *PIK3CA* together with concomitant mutation in *TP53* predicted a worse patient outcome than for tumours with *TP53* mutation alone (Catusus et al, 2009).

In summary, our data derived from *Trp53* deficient mice and from human tumour samples form the basis of a new understanding of tumour formation in the endometrium. We suggest that PI3K-mTOR pathway activation and *TP53* inactivation play different roles in the initiation of type I and type II endometrial cancer initiation but that combined signalling pathway alterations in both PI3K and/or mTORC1 and p53 are a frequent unifying pathogenic feature of late stage tumours of both subtypes.

MATERIALS AND METHODS

Mouse genetics and analyses

Trp53^{fl/fl} mice (FVB.129-*Trp53^{tm1Bnn}*; Jonkers et al, 2001) from the NCI Frederick Mouse Repository were crossed to C57BL/6J *Ksp1.3-Cre* mice (Shao et al, 2002) obtained from Dr. Peter Igarashi (UT Southwestern, USA) to generate *+/+;Trp53^{fl/fl}* (WT) and *Ksp1.3-Cre/+;Trp53^{fl/fl}* (*Trp53^{ΔΔ}*) mice. The *Ksp1.3-Cre* transgene was genotyped as described (Shao et al, 2002). Genotyping of the *Trp53^{fl}* allele and detection of Cre-mediated recombination at the *Trp53* locus were performed as described (Jonkers et al, 2001). Real-time PCR was performed using the following primer pairs for *Trp53* (5'-GCGTAAACGCTTCGAGATGT-3' and 5'-TTTTATGGCGGGAAGTAGACTG-3') and for 18S rRNA (5'-GTTCCGACCATAAACGATGCC-3' and 5'-TGTTGGTGCCCTCCGTC-3'). Littermate controls were used in all experiments. Mouse experiments were conducted in accordance with the ethical guidelines of the Canton of Zurich.

Antibodies

The following antibodies were used: PI3K p110 α (Cell Signaling Technology, #4255), PTEN (Dako, M3627), phospho-Ser473-AKT (Cell Signaling Technology, #4051), phospho-Ser9-GSK3 β , phospho-Ser2448-mTOR (Cell Signaling Technology, #2976), phospho-Thr421/Ser424-p70 S6 Kinase (Cell Signaling Technology, #9204), phospho-Ser240/244-ribosomal S6 protein (Cell Signaling Technology, #2215), phospho-Thr37/46-4E-BP1 (Cell Signaling Technology, #2855), p53 (Dako, M7001), p16 (Santa Cruz Biotechnology, sc-56330), human ERBB2 (Novacastra Laboratories, NCL-L-CBE-356), mouse ERBB2 (Abcam, ab2428), phospho-Tyr1248-ERBB2 (Sigma Aldrich, SAB4300061), phospho-Thr202/Tyr204-ERK1/2 (Cell Signaling Technology, #4376), phospho-Ser217/221-MEK1/2 (Cell Signaling Technology, #9121), phospho-Thr183/Tyr185-SAPK/JNK (Cell Signaling Technology, #9251), PAX8 (Protein Tech Group, 10336-1-AP), IMP3 (Dako, M3626), TSC1 (Abcam, ab59273) and TSC2 (Abcam, ab32936).

Tissue microarrays

TMAAs containing 339 (Basel TMA) and 182 (Zurich TMA) formalin-fixed, paraffin-embedded endometrial cancer tissues were constructed as previously described (Dellas et al, 2009; Kononen et al, 1998; Lebeau et al, 2008). Median follow-up of the cohort from Basel was 31.5 months (1–184), and 44.5 months (1–124) for the cohort from Zurich. The study for both cohorts was approved by the scientific ethics committee from Basel and Zurich (approval no.: KEK-ZH-NR: 2010-0358). The endometrial carcinoma patients were treated in academic centers and outpatient clinics. Patients with endometrial cancer who had localized disease were treated by hysterectomy and bilateral salpingo-oophorectomy (with or without pelvic and para-aortic lymphadenectomy). Adjuvant intravaginal radiation therapy was postoperatively given when invasion of the myometrium or histologic grade 3 tumour was found. All carcinomas were staged according to the 7th edition of the International Union Against Cancer (UICC) and American Joint Committee on Cancer (AJCC) TNM system (Edge et al, 2010). Histopathologic subtype was determined by two

authors (H. M. and R. C.) with the aid of IHC staining in questionable cases. Histologic grading was according to the FIGO grading system based on the ratio of glandular or papillary structures versus solid tumour growth (grade 1, <5% solid tumour; grade 2, 6–50% solid; and grade 3, >50% solid; Creasman, 2009). Follow-up information about metastasis, and causes of death were obtained from the participating departments of gynecology at the University Hospital Basel, University Hospital Zurich, Cantonal Hospital in Liestal/Base-County and Women's Hospitals in Loerrach and Rheinfelden, and from the Cancer Registries of Basel and Zurich. Survival time was calculated from the time of biopsy diagnosis to the death of patients.

Immunohistochemistry

Three micrometres of TMA or mouse tissue sections were subjected to antigen retrieval (microwave oven for 10 min at 250 W) and immunohistochemistry was carried out in a NEXES immunostainer (Ventana, Tucson, AZ, USA). Two surgical pathologists (P. J. W. and K. I.) performed a blinded evaluation of the immunostained slides without knowledge of clinical data. For the analysis of p53 staining, tumours were scored in five groups based on the extent of p53 positivity by determining the frequency of tumour cells displaying strong nuclear staining (0, 1–10, 11–50, 51–90 and 91–100%). Focal weak nuclear p53 staining was regarded as background and scored as 0%. ERBB2 expression was scored according to the DAKO HercepTest criteria. Cytoplasmic immunoreactivity of p16, IMP3, P-GSK3 β , P-S6, P-S6K, P-4E-BP1, PTEN, P-AKT, P-mTOR and nuclear immunoreactivity of p110 α , PAX8 and P-S6K was estimated using a semi-quantitative four-step scoring system (0–3): 0, negative; 1, weak positive; 2, strong positive; 3, very strong positive.

Deep sequencing analysis

DNA was isolated using the DNeasy Blood & Tissue Kit (Qiagen) from punches taken from the same region on the paraffin blocks as used for TMA construction. Fifty nanograms of DNA was used as input for *TP53* PCR amplification (AmpliTaq Gold, Roche) with fusion primers including multiplex identifiers (MID; see Supporting Information). Amplicons were checked on a 1% agarose gel for proper amplification. Amplicon processing was conducted as described by the Amplicon Library Preparation and emPCR (Lib-A) Method GS Junior Titanium Series manual from Roche. Shortly, amplicons were purified using Agencourt AMPure XP beads (Beckman Coulter) and quantified with the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen). Forty-five amplicons (9 patients with five amplicons each) were mixed in equimolar ratio per sequencing run. Emulsion PCR (emPCR) was performed with 10⁶ molecules and 5,00,000 enriched beads were loaded on a 454 Junior Sequencer from Roche. Demultiplexing and variant calling was done with the Amplicon Variant Analyser (AVA) software from Roche. Variants with zero reads in either forward or reverse direction were discarded. Additionally, only variants with at least 50 reads were included in the analysis. Variants were further analysed using the Mutation Assessor software (<http://mutationassessor.org/>) and scored as 3, 2 and 1 with high, medium and low-functional impact, respectively. Deletions, insertions and missense mutations generating a STOP codon were scored as high-impact mutations (score 3). Statistical analysis involved a one-way ANOVA with subsequent Bonferroni's multiple comparison test.

Statistical analyses

The univariate proportional hazard models used to generate risk scores are described in detail in Supporting Information. A multi-variable Cox regression model was learned for testing the independent prognostic relevance of the risk score and other variables. The proportionality assumption for all variables was assessed with log-negative-log survival distribution functions. Non-parametric Kaplan–Meier estimators were used to analyse overall survival. Differences between survival estimates were assessed with the log-rank test (LRT). A statistical association between clinic-pathological and molecular parameters was tested using a two-sided Fisher's exact test. In case of multiple statistical tests the Bonferroni–Holm procedure was applied.

Author contributions

IJF, PJW and WK designed the experiments; IJF, PJW, KI, TJF, SG, MR and NF conducted and analysed the experiments; PJW, AN, MR, RC and HM conducted the histopathological analyses; AD, DF and HM generated the TMAs; IJF and PJW wrote the manuscript with the assistance of all authors.

Acknowledgements

We thank Silvia Behnke, Martina Storz, Susanne Dettwiler, Annette Bohnert, Sonja Brun-Schmid, Adriana von Teichman and Marcel Glöckler for excellent technical assistance and Simone Holdermann for critical reading of the manuscript. This work was supported by an SNF Förderungsprofessur grant to IJF and a Baugarten Foundation grant to PJW.

Supporting Information is available at EMBO Molecular Medicine Online.

The authors declare that they have no conflict of interest.

For more information

General information about endometrial cancers

<http://www.cancer.gov/cancertopics/types/endometrial>

Mutation database for human type II endometrial carcinomas:

http://www.sanger.ac.uk/perl/genetics/CGP/cosmic?action=by-hist&s=4&hn=carcinoma&sn=endometrium&sh=mixed_endometrioid_and_clear_cell_carcinoma&sh=mixed_serous_and_clear_cell_carcinoma&sh=carcinosarcoma-malignant_mesodermal_mixed_tumour&sh=-mixed_serous_and_endometrioid_and_clear_cell_carcinoma&sh=clear_cell_carcinoma&sh=mixed_serous_and_endometrioid_carcinoma&sh=serous_carcinoma&sh=undifferentiated_carcinoma

Corresponding author laboratory webpage

<http://www.physiol.uzh.ch/research/GrFrew.html>

References

- Abeln EC, Smit VT, Wessels JW, de Leeuw WJ, Cornelisse CJ, Fleuren GJ (1997) Molecular genetic evidence for the conversion hypothesis of the origin of malignant mixed müllerian tumours. *J Pathol* 183: 424–431
- Ambros RA, Sherman ME, Zahn CM, Bitterman P, Kurman RJ (1995) Endometrial intraepithelial carcinoma: a distinctive lesion specifically associated with tumors displaying serous differentiation. *Hum Pathol* 26: 1260–1267

- An HJ, Logani S, Isacson C, Ellenson LH (2004) Molecular characterization of uterine clear cell carcinoma. *Mod Pathol* 17: 530-537
- Catasus L, Gallardo A, Cuatrecasas M, Prat J (2009) Concomitant PI3K-AKT and p53 alterations in endometrial carcinomas are associated with poor prognosis. *Mod Pathol* 22: 522-529
- Cheung LWT, Hennessy BT, Li J, Yu S, Myers AP, Djordjevic B, Lu Y, Stemke-Hale K, Dyer MD, Zhang F, et al (2011) High frequency of PIK3R1 and PIK3R2 mutations in endometrial cancer elucidates a novel mechanism for regulation of PTEN protein stability. *Cancer Discov* 1: 170-185
- Creasman W (2009) Revised FIGO staging for carcinoma of the endometrium. *Int J Gynaecol Obstet* 105: 109
- Daikoku T, Hirota Y, Tranguch S, Joshi AR, DeMayo FJ, Lydon JP, Ellenson LH, Dey SK (2008) Conditional loss of uterine Pten unfaithfully and rapidly induces endometrial cancer in mice. *Cancer Res* 68: 5619-5627
- Dellas A, Jundt G, Sartorius G, Schneider M, Moch H (2009) Combined PTEN and p27kip1 protein expression patterns are associated with obesity and prognosis in endometrial carcinomas. *Clin Cancer Res* 15: 2456-2462
- Di Cristofano A, Ellenson LH (2007) Endometrial carcinoma. *Annu Rev Pathol* 2: 57-85
- Edge SB, Byrd DR, Compton CC, Fritz AG, Greene FL, Trotti A (eds) (2010) Gynecologic sites. In: *AJCC Cancer Staging Manual*, pp 403-418. Springer, New York, USA
- Fadare O, Zheng W (2009) Insights into endometrial serous carcinogenesis and progression. *Int J Clin Exp Pathol* 2: 411-432
- Fadare O, Liang SX, Ulukus EC, Chambers SK, Zheng W (2006) Precursors of endometrial clear cell carcinoma. *Am J Surg Pathol* 30: 1519-1530
- Frew IJ, Minola A, Georgiev S, Hitz M, Moch H, Richard S, Vortmeyer AO, Krek W (2008) Combined VHLH and PTEN mutation causes genital tract cystadenoma and squamous metaplasia. *Mol Cell Biol* 28: 4536-4548
- Hayes MP, Wang H, Espinal-Witter R, Douglas W, Solomon GJ, Baker SJ, Ellenson LH (2006) PIK3CA and PTEN mutations in uterine endometrioid carcinoma and complex atypical hyperplasia. *Clin Cancer Res* 12: 5932-5935
- Jarboe EA, Pizer ES, Miron A, Monte N, Mutter GL, Crum CP (2009) Evidence for a latent precursor (p53 signature) that may precede serous endometrial intraepithelial carcinoma. *Mod Pathol* 22: 345-350
- Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D (2011) Global cancer statistics. *CA Cancer J Clin* 61: 69-90
- Jia L, Liu Y, Yi X, Miron A, Crum CP, Kong B, Zheng W (2008) Endometrial glandular dysplasia with frequent p53 gene mutation: a genetic evidence supporting its precancer nature for endometrial serous carcinoma. *Clin Cancer Res* 14: 2263-2269
- Jonkers J, Meuwissen R, van der Gulden H, Peterse H, van der Valk M, Berns A (2001) Synergistic tumor suppressor activity of BRCA2 and p53 in a conditional mouse model for breast cancer. *Nat Genet* 29: 418-425
- Konecny GE, Santos L, Winterhoff B, Hatmal M, Keeney GL, Mariani A, Jones M, Neuper C, Thomas B, Muderspach L, et al (2009) HER2 gene amplification and EGFR expression in a large cohort of surgically staged patients with nonendometrioid (type II) endometrial cancer. *Br J Cancer* 100: 89-95
- Kononen J, Bubendorf L, Kallioniemi A, Barlund M, Schraml P, Leighton S, Torhorst J, Mihatsch MJ, Sauter G, Kallioniemi OP (1998) Tissue microarrays for high-throughput molecular profiling of tumor specimens. *Nat Med* 4: 844-847
- Konopka B, Janiec-Jankowska A, Kwiatkowska E, Najmola U, Bidzinski M, Olszewski W, Goluda C (2011) PIK3CA mutations and amplification in endometrioid endometrial carcinomas: relation to other genetic defects and clinicopathologic status of the tumors. *Hum Pathol* 42: 1710-1719
- Kounelis S, Jones MW, Papadaki H, Bakker A, Swalsky P, Finkelstein SD (1998) Carcinosarcomas (malignant mixed mullerian tumors) of the female genital tract: comparative molecular analysis of epithelial and mesenchymal components. *Hum Pathol* 29: 82-87
- Lane DP, Cheok CF, Lain S (2010) p53-based cancer therapy. *Cold Spring Harb Perspect Biol* 2: a001222
- Lax SF, Pizer ES, Ronnett BM, Kurman RJ (1998) Clear cell carcinoma of the endometrium is characterized by a distinctive profile of p53, Ki-67, estrogen, and progesterone receptor expression. *Hum Pathol* 29: 551-558
- Lax SF, Kendall B, Tashiro H, Slebos RJ, Hedrick L (2000) The frequency of p53, K-ras mutations, and microsatellite instability differs in uterine endometrioid and serous carcinoma: evidence of distinct molecular genetic pathways. *Cancer* 88: 814-824
- Lebeau A, Grob T, Holst F, Seyedi-Fazlollahi N, Moch H, Terracciano L, Turzynski A, Choschzick M, Sauter G, Simon R (2008) Oestrogen receptor gene (ESR1) amplification is frequent in endometrial carcinoma and its precursor lesions. *J Pathol* 216: 151-157
- Lee EJ, Kim TJ, Kim DS, Choi CH, Lee JW, Lee JH, Bae DS, Kim BG (2009) p53 alteration independently predicts poor outcomes in patients with endometrial cancer: a clinicopathologic study of 131 cases and literature review. *Gynecol Oncol* 116: 533-538
- Liang SX, Chambers SK, Cheng L, Zhang S, Zhou Y, Zheng W (2004) Endometrial glandular dysplasia: a putative precursor lesion of uterine papillary serous carcinoma. Part II: molecular features. *Int J Surg Pathol* 12: 319-331
- Lu KH, Wu W, Dave B, Slomovitz BM, Burke TW, Munsell MF, Broaddus RR, Walker CL (2008) Loss of tuberous sclerosis complex-2 function and activation of mammalian target of rapamycin signaling in endometrial carcinoma. *Clin Cancer Res* 14: 2543-2550
- Mhawech-Fauceglia P, Herrmann FR, Rai H, Tchabo N, Lele S, Izevbye I, Odunsi K, Cheney RT (2010) IMP3 distinguishes uterine serous carcinoma from endometrial endometrioid adenocarcinoma. *Am J Clin Pathol* 133: 899-908
- Morrison C, Zanagnolo V, Ramirez N, Cohn DE, Kelbick N, Copeland L, Maxwell GL, Fowler JM (2006) HER-2 is an independent prognostic factor in endometrial cancer: association with outcome in a large cohort of surgically staged patients. *J Clin Oncol* 24: 2376-2385
- Nofech-Mozes S, Khalifa MA, Ismiil N, Saad RS, Hanna WM, Covens A, Ghorab Z (2008) Immunophenotyping of serous carcinoma of the female genital tract. *Mod Pathol* 21: 1147-1155
- Oda K, Stokoe D, Taketani Y, McCormick F (2005) High frequency of coexistent mutations of PIK3CA and PTEN genes in endometrial carcinoma. *Cancer Res* 65: 10669-10673
- Prat J, Gallardo A, Cuatrecasas M, Catasus L (2007) Endometrial carcinoma: pathology and genetics. *Pathology* 39: 72-87
- Salvesen HB, Carter SL, Mannelqvist M, Dutt A, Getz G, Stefansson IM, Raeder MB, Sos ML, Engelsen IB, Trovik J, et al (2009) Integrated genomic profiling of endometrial carcinoma associates aggressive tumors with indicators of PI3 kinase activation. *Proc Natl Acad Sci USA* 106: 4834-4839
- Shao X, Somlo S, Igarashi P (2002) Epithelial-specific Cre/lox recombination in the developing kidney and genitourinary tract. *J Am Soc Nephrol* 13: 1837-1846
- Sherman ME, Bur ME, Kurman RJ (1995) p53 in endometrial cancer and its putative precursors: evidence for diverse pathways of tumorigenesis. *Hum Pathol* 26: 1268-1274
- Silverberg SG, Mutter GL, Kurman RJ, Kubik-Huch RA, Nogales F, Tavassoli FA (2003) Tumors of the uterine corpus: epithelial tumours and related lesions. In *World Health Organization Classification of Tumours Pathology and Genetics of Tumours of the Breast and Female Genital Organs*, Tavassoli FA, Devilee P (eds) pp 217-232. Lyon: IARC Press
- Soong R, Knowles S, Hammond IG, Michael C, Iacopetta BJ (1999) p53 protein overexpression and gene mutation in mixed Mullerian tumors of the uterus. *Cancer Detect Prev* 23: 8-12
- Szukala SA, Marks JR, Burchette JL, Elbendary AA, Krigman HR (1999) Co-expression of p53 by epithelial and stromal elements in carcinosarcoma of the female genital tract: an immunohistochemical study of 19 cases. *Int J Gynecol Cancer* 9: 131-136
- Tantbirojn P, Triratanachai S, Trivijitsilp P, Niruthisard S (2008) Detection of PTEN immunoreactivity in endometrial hyperplasia and adenocarcinoma. *J Med Assoc Thai* 91: 1161-1165
- Tashiro H, Blazes MS, Wu R, Cho KR, Bose S, Wang SI, Li J, Parsons R, Ellenson LH (1997) Mutations in PTEN are frequent in endometrial carcinoma but

- rare in other common gynecological malignancies. *Cancer Res* 57: 3935-3940
- Taylor NP, Zigelboim I, Huettnner PC, Powell MA, Gibb RK, Rader JS, Mutch DG, Edmonston TB, Goodfellow PJ (2006) DNA mismatch repair and TP53 defects are early events in uterine carcinosarcoma tumorigenesis. *Mod Pathol* 19: 1333-1338
- Tong GX, Devaraj K, Hamele-Bena D, Yu WM, Turk A, Chen X, Wright JD, Greenebaum E (2010) PAX8: A marker for carcinoma of Mullerian origin in serous effusions. *Diagn Cytopathol* 39: 567-574
- Urick ME, Rudd ML, Godwin AK, Sgroi D, Merino M, Bell DW (2011) PIK3R1 (p85alpha) is somatically mutated at high frequency in primary endometrial cancer. *Cancer Res* 71: 4061-4067
- Velasco A, Bussaglia E, Pallares J, Dolcet X, Llobet D, Encinas M, Llecha N, Palacios J, Prat J, Matias-Guiu X (2006) PIK3CA gene mutations in endometrial carcinoma: correlation with PTEN and K-RAS alterations. *Hum Pathol* 37: 1465-1472
- Wada H, Enomoto T, Fujita M, Yoshino K, Nakashima R, Kurachi H, Haba T, Wakasa K, Shroyer KR, Tsujimoto M, *et al.* (1997) Molecular evidence that most but not all carcinosarcomas of the uterus are combination tumors. *Cancer Res* 57: 5379-5385
- Yemelyanova A, Ji H, Shih Ie M, Wang TL, Wu LS, Ronnett BM (2009) Utility of p16 expression for distinction of uterine serous carcinomas from endometrial endometrioid and endocervical adenocarcinomas: immunohistochemical analysis of 201 cases. *Am J Surg Pathol* 33: 1504-1514
- Yi X, Zheng W (2008) Endometrial glandular dysplasia and endometrial intraepithelial neoplasia. *Curr Opin Obstet Gynecol* 20: 20-25
- Zhang X, Liang SX, Jia L, Chen N, Fadare O, Schwartz PE, Kong B, Zheng W (2009) Molecular identification of "latent precancers" for endometrial serous carcinoma in benign-appearing endometrium. *Am J Pathol* 174: 2000-2006
- Zheng W, Liang SX, Yu H, Rutherford T, Chambers SK, Schwartz PE (2004) Endometrial glandular dysplasia: a newly defined precursor lesion of uterine papillary serous carcinoma. Part I: morphologic features. *Int J Surg Pathol* 12: 207-223
- Zheng W, Liang SX, Yi X, Ulukus EC, Davis JR, Chambers SK (2007) Occurrence of endometrial glandular dysplasia precedes uterine papillary serous carcinoma. *Int J Gynecol Pathol* 26: 38-52
- Zheng W, Xiang L, Fadare O, Kong B (2011) A proposed model for endometrial serous carcinogenesis. *Am J Surg Pathol* 35: e1-e14