Evidence for different expression profiles for c-Met, EGFR, PTEN and the mTOR pathway in low and high grade endometrial carcinomas in a cohort of consecutive women. Occurrence of PIK3CA and K-Ras mutations and microsatellite instability

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Summary. Molecular and genetic investigations in endometrial carcinogenesis may have prognostic and therapeutic implications. We studied the expression of EGFR, c-Met, PTEN and the mTOR signalling pathway (phospho-AKT/phospho-mTOR/phospho-RPS6) in 69 consecutive tumours and 16 tissue microarrays. We also analysed PIK3CA, K-Ras mutations and microsatellite instability (MSI). We distinguished two groups: group 1 (grade 1 and 2 endometrioid cancers) and group 2 (grade 3 endometrioid and type II clear and serous cell cancers). We hypothesised that these histological groups might have different features. We found that a) survival was higher in group 1 with less aggressive tumours (P<0.03); b) EGFR (P=0.01), PTEN and the AKT/mTOR/RPS6 signalling pathway were increased in group 1 versus group 2 (P=0.05 for phospho-mTOR); c) conversely, c-Met was higher (P<0.03) in group 2 than in group 1; d) In group 1, EGFR was correlated with c-Met, phospho-mTOR, phospho-RPS6 and the global activity of the phospho-AKT/phospho-mTOR/phospho-RPS6 pathway. In group 2, EGFR was correlated only with the phospho-AKT/phospho-mTOR/phospho-RPS6 pathway, whereas c-Met was correlated with PTEN; e) survival was higher for tumours with more than 50% PTEN-positive cells; f) K-RAS and PIK3CA mutations occurred in 10-12% of the available tumours and MSI in 40.4%, with a loss of MLH1 and PMS2 expression. Our results for endometrial cancers provide the first evidence for a difference in status between groups 1 and 2. The patients may benefit from different targeted treatments, anti-EGFR agents and rapamycin derivatives (anti-mTOR) for group 1 and an anti c-MET/ligand complex for group 2.

Key words: EGFR, c-Met, PTEN, mTOR pathway, Endometrial cancer

Introduction

The incidence of endometrial cancer is increasing due to the lengthening in life expectancy in women. It is now the fourth most frequent cancer in women. Classical treatment is based on a combination of surgical intervention and radiotherapy for well differentiated and initial stages of endometrial cancers. Adjuvant chemotherapy is used in cases of poor prognosis, such as extensive and clear and serous cell carcinomas, and hormone treatment is used for hormone-sensitive tumours. However, neither of these approaches is particularly effective. There is therefore a need to identify biological dysregulations in cancer cells that could be used as new therapeutic targets or considered as eventual new prognosis factors.

Molecular profiling studies of endometrial malignancies have generated fragmented results. Some have focused on the expression of tyrosine kinase receptors (TKR) such as EGFR (Hayes et al., 2009; Konecny et al., 2009; Ai et al., 2010; Albitar et al., 2010) and the receptor c-Met of hepatocyte growth
factor (HGF) (Wagatsuma et al., 1998; Bishop et al., 2011). The tumour suppressor gene PTEN (Phosphatase and tensin homologue deleted on chromosome 10) is frequently inactivated, resulting in the loss of expression and/or activity of the corresponding protein. PTEN down-regulates cell proliferation and survival by modulating cell signalling, through the dephosphorylation of PIP3, the product of PI3K (phosphoinositide-3-kinase) (Uegaki et al., 2005; Okuda et al., 2010). In particular, the PI3K/AKT/mTOR pathway is implicated in endometrial carcinogenesis (Faivre et al., 2006) and is also involved in control of the cell cycle, mitosis, apoptosis, survival and cell migration. PI3K can be activated by TKR, the mutation of its catalytic site PIK3CA (Hayes et al., 2009), or activated RAS protein. In endometrial cancer, PIK3CA mutations mostly occur in exons 9 and 20 and during late stages of carcinogenesis (Velasco et al., 2006; Catasus et al., 2009; Samarnthai et al., 2010; Matias-Guiu and Prat., 2013). K-RAS encodes a member protein of the small GTPase superfamily and is involved in signal transduction pathways between cell surface receptors and the nucleus. K-RAS mutations have been identified in a fraction of endometrial carcinomas (Velasco et al., 2006; Okuda et al., 2010). PIP3 activates the serine/threonine kinase AKT and its downstream targets, including mTOR (mammalian Target Of Rapamycin). The mTOR protein forms two complexes. The first, m-TORC1, is sensitive to rapamycin and acts on several downstream targets, including p70S6K, which, when activated, phosphorylates the S6 ribosomal protein (phospho-RPS6) (Ruvinsky and Meyuhas, 2006). Conflicting data have been published concerning mTOR in endometrial carcinomas with reports of either high or low protein levels in severe cancers (Darb-Esfahani et al., 2009; Choi et al., 2010). Endometrial carcinogenesis also involves impaired DNA mismatch repair leading to microsatellite instability (MSI) and tumour development (Okuda et al., 2010; Samarnthai et al., 2010). Hormonal receptors also seem to play a role in clinical outcome of endometrial cancers (Kounelis et al., 2000), especially, estrogen-dependent tumours have a good prognosis and an endometrioid histology (Llaurado et al., 2012).

Bokhman first described two different types of endometrial carcinoma in 1983. Type I, endometrioid represents the majority of sporadic cases. Type II, corresponding to serous and clear cell tumours, is less frequent (5-10%) and more aggressive (Silverberg et al., 2003). There is evidence for a link between mutations of certain genes such as PTEN, PIK3CA, K-RAS and MSI and the type I endometrioid cancers (Okuda et al., 2010). No mutation of these genes seems to play a major role in the type II carcinomas (Samarnthai et al., 2010). According to the 2003 WHO Classification of Tumours (Silverberg et al., 2003), grade 3 endometrioid carcinoma with bizarre nuclear atypia may also represent type II serous differentiation. However, endometrioid and non-endometrioid carcinomas can exhibit overlapping features (Matias-Guiu et al., 2001; Soslow, 2013) and diagnostic problems were created by mixed tumours or with ambiguous histology (Darvishian et al., 2004; Gilks et al., 2013). Some authors propose a gene panel to aid in the morphological classification of problematic cases (McConechy et al., 2012).

Herein, the aim was to examine all the parameters described above in a single cohort of women with endometrial cancer. We investigated a) the immunohistochemical expression of EGFR, c-Met, PTEN and the phospho-AKT (p-AKT)/phospho-mTOR (p-mTOR)/phospho-RPS6 (p-RPS6) signalling pathway; b) the possible correlations between the various factors; c) activating mutations of PIK3CA and K-RAS and d) MSI. We analysed the relationship between these findings and patient survival. In addition, we extended the study by comparing two different histological groups of cancers: low-grade group 1 comprising grade 1 and 2 endometrioid cancers and high-grade group 2 gathering grade 3 endometrioid with type II clear cell and serous cancers. We hypothesised that these groups might have different features.

Materials and methods

Patients and tissues

All 69 consecutive women were included in a retrospective study performed from 1998 to 2003 at Bichat-Claude Bernard Hospital, Paris, France. They were followed up until 2008, resulting in a follow-up period of 5 to 10 years. Their average age was 65 years (range 42-93 years). Sixty nine endometrial adenocarcinomas were obtained from 86 lesion specimens (66 from hysterectomies, 20 endometrial biopsies). Normal endometrial biopsies removed at various phases of the ovarian cycle and from menopausal women were used as controls. Tumours were examined by 5 pathologists and classified. They were defined as pure tumours (exclusive of a given type) or with largely predominant histological component. The characteristics of the tumours are summarised in Table 1. In accordance with WHO (Silverberg et al., 2003) and like others (Rowlands et al., 2011), we distinguished group 1 comprising grade 1 and 2 endometrioid cancers (53 tumours) and group 2 gathering poorly or undifferentiated grade 3 endometrioid with type II clear cell and serous cancers (16 tumours). Type II accounted for 7.2% of tumours, consistent with published findings (Silverberg et al., 2003). Sixteen type II cancer tissue microarrays were also examined. All patients gave consent for diagnosis and research on tissue specimen before inclusion. The study was approved by the Human Research Committee of the Bichat-Claude Bernard Hospital.

Immunohistochemical staining and evaluation

All tissue samples (pathological and tissue controls) were routinely formalin-fixed, paraffin-embedded and
blocks cut into 4 µm-thick sections. The first sections were stained with hematoxylin-eosin-saffron for histological diagnosis. Subsequent sections were used for immunohistochemistry with the different antibodies against TKR (EGFR, c-Met), m-TOR pathway (p-AKT, p-mTOR, p-RPS6), PTEN and MSI proteins (MLH1, MSH2, MSH6, PMS2). Estrogen and progesterone receptors (ER and PR respectively), and Ki67 antibody as indicator of cell proliferation were also studied. Immunostaining was performed using a Menarini Bond Max automat (Rungis, France). Sections were first incubated with a primary antibody (see Table 2) then with the corresponding biotinylated secondary antibody, followed by a polymer detection kit (Menarini). Immunoreactivity was detected with diaminobenzidine and nuclei were counterstained with hematoxylin. The specificity of immunostaining was checked by omitting the primary antibodies and by using positive tissue controls recommended by suppliers, depending on these antibodies.

Reactions were observed under x 400 magnification as follows: i) the intensity of immunostaining in normal endometrial mucosa and in each lesion was evaluated independently by two researchers, using a semiquantitative method: negative reaction (class 0); just detectable or weak (class 1); moderate (class 2); strong (class 3); intense (class 4). In most cases, the two evaluations were concordant. If negative or doubtful results were obtained for a lesion, immunoreactions were repeated; ii) the percentage of immunoreactive cells on cancer sections was estimated. In addition, for EGFR, c-Met, PTEN and the m-TOR pathway, we calculated an immunostaining score for each tumour by multiplying the percentage of immunostained cancer by the staining intensity class (maximal score 400) (Walker et al., 2009; Voss et al., 2012).

**Genomic DNA extraction**

DNA was extracted from 3 paraffin sections (20 µm-thick) of each tumour, some normal endometrial tissues from non cancerous patients and normal adjacent tissues to the endometrial tumours. These sections were incubated in ATL lysis buffer with proteinase K for 10 min at 70°C and DNA was extracted using the QIAamp DNA Mini tissue kit according to the manufacturer’s indications (Qiagen GmBH, Hilden, Germany). DNA was successfully extracted from 43 available tumours.

**K-RAS and PIK3CA mutational analysis in tumour samples**

We searched for somatic mutations in tumours. PCR primers were chosen in order to obtain short amplicons because it is well known that DNA extracted from paraffin could be fragmented. Mutation analysis of the PIK3CA (Genbank mRNA : NM_006218.2, Genbank protein : NP_006209.2) and K-RAS genes (Genbank mRNA : NM_04985.3, Genbank protein : NP_004976.2) was performed by bidirectional direct sequencing. As in colorectal cancer (Moroni et al., 2005), database analysis (COSMIC) showed that, in endometrial cancers, the PIK3CA gene mutations mainly occur in exons 9 and 20, codons 542, 545, 546 and codons 1007-1069, respectively, and the K-RAS mutations in exon 2, codons 12 and 13. Moreover, the primers to amplify exon 9 of PIK3CA were designed to amplify specifically exon 9 of the gene and not the pseudogene of PIK3CA sharing 97% of identity with the gene. The list of primers and PCR conditions are available from the authors upon request. These exons of PIK3CA and K-RAS were amplified by PCR. After purification of PCR products (PCR purification kit, Qiagen CA, USA), both strands were sequenced using a Big Dye Terminator Cycle Sequencing kit (Applied Biosystems, Life Technologies, Carlsbad, CA, USA), purified (Sephadex G50, GE Healthcare, Piscataway, NJ, USA) and sequencing products were analysed using a 3130xl Genetic Analyzer (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) and the Seqscape analysis software (v2.6.0) (Applied Biosystems, Life Technologies, Carlsbad, CA, USA). Search on the Sanger Centre COSMIC, PubMed databases, Exome Variant Server and 1000 Genomes was performed to determine which of the somatic mutations identified had already been described. The missense mutations were studied in silico with bioinformatics tools predicting the functional impact of the substitution i.e; polyphen, SIFT and SNP3D softwares (Ng and Henikoff, 2003).

**Molecular biological analysis of microsatellite instability**

According to revised Bethesda guidelines, MSI analysis (Wong et al., 2006) was performed with five consensus panel markers (BAT25, BAT26, NR21, NR...
Techniques are routinely made with negative (normal colonic tissues) and positive (colon adenocarcinomas exhibiting the five positive markers cited above) controls. The Gene Mapper v4.0 software allows the analysis of MSI marker migration. A MSI was considered to be present if at least three markers displayed instability (RER+ and MSI high phenotype). When two markers were modified, the result was doubtful (MSI low phenotype).

Statistical analysis

Kaplan-Meier survival curves were constructed in logrank tests. Immunohistochemical results were expressed as means ± 1 SEM. Differences in values between the two defined groups of cancer were evaluated with the Student’s t test or Mann and Whitney U test. Correlations were estimated with the Pearson linear regression or the non parametric Spearman’s rank correlation. A level of \( P \geq 0.05 \) was considered statistically significant.

Results

Expression of TKR, PTEN and the mTOR pathway

Expression of TKR (EGFR, c-Met)

EGFR was expressed in 87% of tumours and c-Met in all of them (Fig. 1A). EGFR immunostaining was intense in 4% of tumours (Fig. 1B), and was found at the plasma membranes and occasionally in the cytoplasm (Fig. 2A-B). The c-Met signal was mostly cytoplasmic (Fig. 2C). None of these receptors had a prognostic value for patient survival.

Expression of PTEN and activity of mTOR signalling pathway

PTEN protein was absent or present in less than 10% of cells in 52% of tumours. The percentage of tumours expressing phosphorylated AKT, mTOR and RPS6 increased downstream along the signalling pathway from 77%, 84% up to 92%, respectively (Fig. 1C). Immunostaining for p-mTOR and p-RPS6 was intense in some cancers (Fig. 1D). PTEN was found in the nuclei and the cytoplasm, p-AKT in the cytoplasm and often also in the nuclei and membranes, p-mTOR and p-RPS6 in the cytoplasm (Fig. 2 D-H). Women with tumours containing more than 50% of PTEN-positive cells survived for longer than those with tumours containing less than 10% of immunoreactive cells, but this difference did not attain statistical significance (\( P=0.12 \)) (Fig. 3A). Most of the former tumours (95%) belonged to group 1. None of the mTOR pathway effectors affected survival.

Expression profiles of TKR, PTEN and the mTOR pathway in the two groups of patients

As we expected in the two groups defined above, overall survival was clearly higher in group 1, with the least aggressive tumours (\( P<0.03 \), Fig. 3B). Mean immunohistochemical scores for EGFR, PTEN and the AKT/mTOR/RPS6 signalling pathway were all lower in group 2 - and in the two subgroups of group 2 (grade 3 endometrioid and type II carcinomas), the decrease in these scores being most marked for type II carcinomas – than in group 1. This difference was statistically significant for EGFR (\( P=0.01 \)) and p-mTOR (\( P=0.05 \), Table 3). Conversely, c-Met score was significantly higher in group 2 – and in its two subgroups - than in group 1 (\( P<0.03 \)). Noteworthy, the difference in c-Met score between type II and group 1 tumours was significant (\( P<0.05 \), Table 3). Type II cancer tissue microarrays gave similar immunohistochemical patterns to tumours of type II. We then investigated the possible correlations between individual immunohistochemical scores for TKR and other variables. The correlations observed highlighted differences in behaviour between

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Antibody Description</th>
<th>Dilution</th>
<th>Reference</th>
<th>Suppliers</th>
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</thead>
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<td>C-Met</td>
<td>Mouse MoAb Clone 8F11</td>
<td>1:20</td>
<td>35480</td>
<td>Novocastra, Newcastle, UK</td>
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<td>EGFR</td>
<td>Mouse MoAb Clone 25</td>
<td>undiluted</td>
<td>RTU-EGFR-384</td>
<td>Novocastra, Newcastle, UK</td>
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<td>p-AKT (Ser 473)</td>
<td>Rabbit MoAb Clone 736E11</td>
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<td>3787</td>
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<tr>
<td>p-mTOR (Ser 2448)</td>
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<td>1:50</td>
<td>2976</td>
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<tr>
<td>p-RPS6 (Ser 235/236)</td>
<td>Rabbit MoAb Clone: D57.2.2E</td>
<td>1:50</td>
<td>4858L</td>
<td>Cell Signalling Techn. Ozyme, France</td>
</tr>
<tr>
<td>PTEN</td>
<td>Mouse MoAb Clone 6H2.1</td>
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<td>M3627</td>
<td>Dakopatt, Glostrup, Denmark</td>
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<tr>
<td>MLH1</td>
<td>Mouse MoAb Clone: G168-728</td>
<td>1:400</td>
<td>554073</td>
<td>Becton Dickinson, USA</td>
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<tr>
<td>MSH2</td>
<td>Mouse MoAb Clone FE11</td>
<td>1:25</td>
<td>NA27</td>
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<tr>
<td>MSH6</td>
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<td>Mob429</td>
<td>Diagnostic BioSystem, Pleasanton, CA</td>
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<td>PMS2</td>
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<td>1:25</td>
<td>MSK064</td>
<td>Diagnostics, Toulouse, France</td>
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<tr>
<td>Ki-67</td>
<td>Mouse MoAb Clone MI81</td>
<td>1:100</td>
<td>M7240</td>
<td>Dakopatt, Glostrup, Denmark</td>
</tr>
<tr>
<td>Estrogen Receptor</td>
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<td>35184</td>
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<td>Progesterone Receptor</td>
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<td>35150</td>
<td>Novocastra Newcastle, UK</td>
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</table>

MoAb: Monoclonal antibody
groups 1 and 2. In group 1, EGFR levels were correlated with those of a) c-Met ($r^*=0.27$, $P=0.05$), b) p-mTOR ($r^*=0.38$, $P<0.005$), c) p-RPS6 ($r^*=0.35$, $P<0.01$), d) global activity for the p-AKT/p-mTOR/p-RPS6 pathway ($r^*=0.40$, $P<0.003$). The relationship between EGFR and p-AKT levels were not quite significant ($P=0.09$) and there was clearly no correlation between EGFR and PTEN levels. In this group, c-Met levels were correlated only with EGFR levels, as reported above. In group 2, EGFR levels were correlated only with the overall activity of the p-AKT/p-mTOR/p-RPS6 pathway ($r^*=0.65$, $P<0.0001$). By contrast with the findings for group 1, c-Met was correlated with PTEN ($r=0.59$, $P<0.0003$) but not with EGFR.

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**Fig. 1.** Histological distribution profiles of immunostaining in endometrial tumors as a function of the number of immunopositive cells per tumor section (A and C) and of intensity (B and D), for EGFR and c-Met, (A and B) and PTEN, p-AKT, p-mTOR, p-RPS6 (C and D). Classes 0 to 4 of immunostaining intensity are defined in the Materials and Methods.
Fig. 2. Examples of immunostaining in endometrial tumours; (A, B detail), EGFR was expressed on cell membranes and sometimes in the cytoplasm. C. Representative pattern of c-Met immuno-reactivity in the cytoplasm of tumour cells. D. Nuclear and cytoplasmic PTEN signal in one tumour; inset: PTEN-negative tumour. (E, F), p-AKT immunoreactivity. The signal was observed in nuclei (E) but also in the cytoplasm and membranes (F). G. p-mTOR was expressed in the cytoplasm, some tumour cells (arrows) showed intense signal. H. p-RPS6 was found in the cytoplasm. Bar: A, 100 µm; B, C, E-H, 25 µm; D, 50 µm.
Analysis of PIK3CA and K-RAS mutations (Table 4)

Eight mutations of PIK3CA (18.5%) were found. For exon 9, DNA was successfully amplified from 42 tumours and two known pathogenic mutations were found in group 1 tumours with an invasive pattern. For exon 20, 43 DNA were amplified. There were three known pathogenic mutations (two in grade 3 endometrioid tumours, one in a group 1 tumour), one stop gained variant, one synonymous variant without protein modification and one new mutation already described in ovarian and gastric cancers. There was no evident correlation between PIK3CA mutations and the loss of PTEN expression. For K-RAS, 40 DNA were amplified. We identified six mutations (15%) known to be pathogenic in exon 2 in endometrioid tumours, four of which belonged to group 1. We also identified in tumours of group 1 two variants of unknown importance.

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**Fig. 3. Survival curves.**

**A.** The presence of numerous tumour cells expressing PTEN tended to be associated with higher rates of patient survival but this was not quite statistically significant. **B.** Survival rates were higher for group 1, corresponding to the less aggressive grade 1 and 2 endometrioid adenocarcinomas, than for group 2, corresponding to grade 3 endometrioid carcinoma and type II (clear cell and serous) carcinomas (P<0.03). **C.** Influence of progesterone receptors (PR), the higher level of expression, the longer the survival.
which have never before been described in endometrial cancer. One corresponded to a synonymous variant with a mutation of the same base c.45C>A described in colon carcinoma. Another mutation was found for the first time targeting a highly conserved alanine (A18V) with p.A18T and p.A18D mutations being reported in certain cancers, among them lung and haematopoietic cancers. One patient with a grade 3 endometrioid cancer displayed both K-RAS and PIK3CA mutations. Genomic DNA was amplified from only two cases of type II carcinoma and no mutation was found.

Microsatellite instability in endometrial cancers

We first analysed MSI by molecular biology approaches. Sixteen carcinomas displayed MSI affecting three microsatellite markers and one case affected two markers, corresponding to 40.4% (17/42) of the DNA samples analysed. These 17 tumours were tested with antibodies against four different effectors of mismatch repair. MSH2 was strongly expressed in all tumours and did not seem to play a role in endometrial cancers with MSI. By contrast, MLH1 was absent from 11/17 (65%), PSM2 from 10/17 (59%) and MSH6 from 4/17 (23%) tumours. Two tumours expressed all four markers, but with only a small number of MLH1 labelled cells and eight tumours (47%) expressed neither MLH1 nor PMS2. Fifteen tumours with MSI belonged to group 1, the other two being grade 3 endometrioid adenocarcinomas (group 2). One tumour of group 1 and one of group 2 were deficient in MLH1, MSH6 and PMS2. No MSI was detected in the two available type II tumours. MSI was of no prognostic value.

Expression of hormonal receptors and the Ki67 protein

Of the 69 tumours, 14 (20%) were strictly negative or showed less than 10% of positive cells for both receptors. For ER, 40 tumours were considered as negative whereas 12 showed more than 50% of positive cells, among them 9 low grade endometrioid tumours of group 1. For PR, 20 tumours were negative, whereas 33 showed more than 50% of positive cells. In group 2, of poor prognosis, 9/16 cases remained strictly negative for both receptors, 3 expressed ER and 4 expressed PR. If we considered carcinomas of type II alone, only one case was positive for ER and none for PR. In our series, ER had no prognostic value on the survival of women contrarily to PR. Indeed, the higher the expression level of PR was, the longer the survival was (Fig. 3C). The Ki67 proliferation index was <10% in 16 endometrioid adenocarcinomas. In these cases, histological grade 2 and FIGO staging I predominated. By contrast, 28 tumours had a Ki67 index > to 50%. Twenty-four of these endometrioid carcinomas had grade 2 or 3 associated for 11 of them with FIGO staging III or IV. Nevertheless, cell proliferation did not prove to influence survival.

Table 3. Immunohistochemical scores in endometrial tumours.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Group 1 n=53</th>
<th>Group 2 n=16</th>
<th>P (U test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFR</td>
<td>116.7±16.3</td>
<td>42.2±12.2</td>
<td>=0.01</td>
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<tr>
<td>C-Met</td>
<td>115.7±12.8</td>
<td>166.2±16.1</td>
<td>&lt;0.03</td>
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<tr>
<td>PTEN</td>
<td>60.5±11.9</td>
<td>37.5±14.9</td>
<td>NS</td>
</tr>
<tr>
<td>p-AKT</td>
<td>38.0±3.1</td>
<td>28.6±14.7</td>
<td>NS</td>
</tr>
<tr>
<td>p-mTOR</td>
<td>66.4±11.8</td>
<td>36.3±17.6</td>
<td>=0.05</td>
</tr>
<tr>
<td>p-RPS6</td>
<td>68.6±12.9</td>
<td>60.6±16.8</td>
<td>NS</td>
</tr>
<tr>
<td>m-TOR pathway</td>
<td>58.0±8.4</td>
<td>41.8±13.0</td>
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</table>

Mean value ± 1 SEM. NS, not significant. Group 1 corresponds to endometrioid carcinomas of grade 1 and 2. Group 2 corresponds to endometrioid carcinomas of grade 3 and type II (clear cell and serous carcinomas). Values of mTOR pathway correspond to the individual mean values of p-AKT + p-mTOR + p-RPS6. Results were similar if the three patients with the double localisation of cancer were excluded (i.e. group 1 = 50 patients). * In group 2, c-Met score was clearly higher in type II (202±23, n=5) than in group 1, P<0.05. ** In group 2, PTEN score was still lower in type II (22±14) than in grade 3 endometrioid (46±19).

Table 4. Molecular biology results.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Exons</th>
<th>Amplified DNAs (a)</th>
<th>No. mutations</th>
<th>Nucleotide</th>
<th>Codon</th>
<th>Protein</th>
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<tbody>
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<td>PIK3CA</td>
<td>9</td>
<td>42</td>
<td>2</td>
<td>c.1624G&gt;A</td>
<td>542</td>
<td>p.E542K</td>
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<tr>
<td></td>
<td>20</td>
<td>43</td>
<td>1</td>
<td>c.3062A&gt;G</td>
<td>1021</td>
<td>p.Y1021C</td>
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<td></td>
<td></td>
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<td>c.3075C&gt;T</td>
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<td>p.T1025T</td>
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<td></td>
<td>1</td>
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<td>1037</td>
<td>p.E1037*</td>
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<td>1</td>
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<td>K-RAS</td>
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<td>40</td>
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<td>p.V9I</td>
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<td>4</td>
<td>c.35G&gt;A</td>
<td>12</td>
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<td>13</td>
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<td>p.G15G</td>
<td>Synonymous variant (e)</td>
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<td>1</td>
<td>c.53C&gt;T</td>
<td>18</td>
<td>p.A18V</td>
<td>Missense variant (f)</td>
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</table>

(a) There were initially 43 available DNA tumour samples. (b) Mutation reported in ovarian and gastric cancers but not in endometrial cancer (COSMIC 33700). (c) COSMIC 1562184. (d) COSMIC 535. (e) A mutation in c.45C>A was reported in intestinal cancer (COSMIC1360879). (f) Not reported in endometrial cancer but p.A18T and p.A18D are mentioned in lung and haematopoietic cancers (COSMIC 541, 542).
Discussion

Conflicting results have been obtained in the punctual and fragmentary studies of different cohorts of patients, reflecting the complexity of the endometrial carcinogenesis process. This study is the first to investigate in a single series of endometrial cancers from a cohort of consecutive women, the relationships between TKR, the AKT/mTOR signalling pathway and PTEN expression, \(PIK3CA\) and \(K-RAS\) mutations, the presence of MSI and patient survival. The choice to classify tumours into two histological groups was justified by i) WHO histological criteria, in particular the suggestion that grade 3 endometrioid cancers with bizarre nuclear atypia may also correspond to type II serious differentiation; ii) survival rates, which differed significantly between these two groups; iii) the immunohistochemical scores for the various factors studied and the correlations observed between them highlighting the markedly different behaviour of these two groups, likely corresponding to different entities of potential value for prognosis and therapy.

Concerning TKR, EGFR levels were clearly lower in group 2 and each of its subgroups than in group 1. This finding is close to other results (Konecny et al., 2009) showing significantly lower levels of EGFR expression in type II than in endometrioid carcinomas. This pattern contrasts with that observed in the neoplastic anogenital area, in which EGFR expression increased with lesion severity (Walker et al., 2009). Only a few studies have evaluated c-Met expression in endometrial cancers (Wagatsuma et al., 1998; Bishop et al., 2011). All the tumours studied here expressed c-Met, which was present at significantly higher levels in group 2, particularly type II, than in group 1. Similarly, the expression of c-Met has been reported to increase with lesion severity in anogenital lesions (Walker et al., 2003, 2009). TKR and their ligands have been identified as promising candidate molecular targets for cancer treatment. Based on our data, anti-EGFR antibodies (cetuximab) and/or EGFR inhibitors such as erlotinib (Oza et al., 2008) may be useful for the treatment of group 1 endometrial cancers. Blockade of the HGF/c-Met pathway with a monoclonal antibody against HGF (Giordano, 2009) or c-Met (Spiegel et al., 2012) may be particularly useful for the treatment of group 2 endometrial cancers.

PTEN and the PI3K/AKT pathway are involved in the development and/or progression of endometrial carcinoma. Loss or decrease in PTEN protein expression (mostly due to gene deletion, silencing or mutation) was observed in 52% of our tumours, consistent with the literature, 50-65% (An et al., 2002; Kanamori et al., 2002; Uegaki et al., 2005). Mean PTEN score was lower in group 2 than in group 1 for which the prognosis was better. By contrast, in a series comparable to ours, An et al. (2002) found a PTEN expression in most type II serous carcinomas (4/5 cases) and a loss or decrease in PTEN expression in endometrioid carcinomas. The potential prognostic value of PTEN expression in endometrial carcinoma also remains a matter of debate. Thus, PTEN and p-AKT levels would be inversely related (Kanamori et al., 2002; Uegaki et al., 2005), although no such relationship was observed here. Patients with PTEN-positive and p-AKT-negative tumours clearly had higher survival rates than other patients (Uegaki et al., 2005). Like others (Kanamori et al., 2002), we found that patients with PTEN-positive tumours survived for longer than those with PTEN-negative tumours. In addition, we found no relationship between PTEN negative tumours and \(PIK3CA\) alterations. PI3K activation leads to the phosphorylation of AKT, mTOR and RPS6. The levels of activation of these molecules have never before been studied simultaneously in endometrial cancers and few data are available concerning the phosphorylation of mTOR and RPS6. Our study is the first report on the status of these three PI3K effectors in the same series of tumours. The number of tumours expressing these factors increased progressively with the phosphorylation cascade. This may reflect the dysregulation of other signalling pathways, such as the RAS/MAPK pathway responsible for the activation of mTOR and p70S6K proteins. More than 92% of the group 1 tumours expressed p-mTOR. This factor may be predictive of the response to treatment with rapamycin derivatives in that group (Fairf et al., 2006), as recently reported for metastatic endometrial cancers (Gadducci et al., 2008). By contrast, in group 2 and particularly in the type II subgroup, immunohistochemical scores for p-AKT and p-mTOR were constantly low, a result replicated in type II cancers microarrays. Our results are, therefore, consistent with those of others (Choi et al., 2010) who found a decrease of p-mTOR in severe cancers. This suggests that other signalling pathways may be implicated in these endometrial carcinomas of poorer prognosis. Only one other previous study involved immunohistochemical analysis of p70S6K expression in endometrial carcinomas (Lu et al., 2008). In our series, we found that scores for p-RPS6, the activation product of p70S6K, were roughly similar in endometrioid cancers of all grades but were clearly lower in type II carcinomas.

\(PIK3CA\) alterations, mostly in exons 9 and 20 but sometimes detected in exons 1, 4 and 7 (Konopka et al., 2011), seem to be correlated with a poor prognosis, with exon 20 mutation frequently observed in high-grade deeply invasive carcinoma (Catasus et al., 2009). In our study, \(PIK3CA\) mutations and \(K-RAS\) mutations occurred in 18.5% and 15% of the available cases, respectively, consistent with the frequency of 13-16% recently reported (Garcia-Dios et al., 2012; Peterson et al., 2012). Some authors have suggested that \(PIK3CA\) and \(K-RAS\) mutations are mutually exclusive in endometrial carcinomas (Velasco et al., 2006; Kang et al., 2008). We and others (Oda et al., 2008; Konopka et al., 2011), have shown the coexistence of these two types of mutations in the same endometrial tumour. As in colorectal cancer, \(K-RAS\) activation may invalidate an anti-EGFR treatment.

Sporadic endometrioid carcinomas display MSI in 20-45% of cases, a percentage different from that for
PTEN, mTOR pathway in endometrial cancer

non-endometrioid carcinomas (Okuda et al., 2010; Samarnthai et al. 2010; Matias-Guiu and Prat, 2013). The frequency of 40.4% MSI found here agrees with these findings. Based on the literature a) inactivation of the mismatch repair gene MLH1 by methylation of its promoter seems to be the most frequent cause of MSI in sporadic endometrioid carcinoma and the loss of its expression the main phenomenon identified in tumours with high-MSI (those positive for at least four microsatellite markers) (Stefansson et al., 2002; Okuda et al., 2010; Samarnthai et al., 2010); b) the loss of MSH2 and MSH6 is more frequent in tumours displaying modifications for 2-3 microsatellite markers (Stefansson et al., 2002). Like others (Peterson et al., 2012), we found that the absence of MLH1 and PMS2 expression was the major phenomenon, but in tumours with only 2-3 microsatellite markers. The expression of MSI protein markers does not affect patient survival (us, Stefansson et al., 2002).

Concerning hormonal receptors, we agree with the literature that high grade tumours lacked ER and PR expression (Zhu et al., 2009; Okuda et al., 2010). According to Llaurado et al., 2012, estrogen-dependent carcinomas have a good prognosis. However, in the current study, PR were more often present than ER and, alone, their presence correlated with better survival. As expected, Ki67 index was weak in low grade and low FIGO staging and increased with the severity of tumour, fitting with other data (Kounelis et al., 2000; Zhu et al., 2009).

In summary, we provide evidence for the existence of two different profiles in group 1 (low grade) and group 2 (high grade) adenocarcinomas. EGFR and PTEN expression and the activity of AKT/mTOR/RS6 pathway were higher in group 1 than in group 2, whereas c-Met expression was higher in group 2. These molecular alterations may have an impact on future treatment strategies. Thus, these two groups of patients may benefit from different selected targeted therapies. Our findings support the notion that grade 3 endometrioid cancers are more similar to type II cancers than to low-grade endometrioid tumours. Interestingly, while our work was in progress, Voss et al.(2012) investigating HER2 and hormonal receptors expression in endometrial cancers considered that grade 3 endometrioid cancer is better characterised as type II cancer.

References


Kanamori Y., Kigawa J., Itamochi H., Sultana H., Suzuki M., Ohwada M., Kamura T., Sugiyama T., Kikuchi Y., Kita T.,
PTEN, mTOR pathway in endometrial cancer


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