# p16 is consistently expressed in endometrial tubal metaplasia

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**Abstract.** *Background:* Cell cycle proteins and HIF-$1\alpha$ with downstream factors are often abberrantly expressed in (pre)neoplastic tissue. *Methods:* Paraffin-embedded specimens of inactive endometrium with TM ($n = 15$), ovarian inclusion cysts ($n = 6$), cervix with TM (tubal metaplasia) ($n = 3$), Fallopian tubes ($n = 7$), cycling endometrium ($n = 9$) and a ciliated cell tumor of the ovary were stained for p16 and LhS28. 39 Endometrioid endometrial carcinomas and 5 serous endometrial carcinomas were stained for p16. Additionally, inactive endometrium ($n = 15$) was immunohistochemically stained for p21, p27, p53, cyclin A, cyclin D1, cyclin E, HIF-$1\alpha$, CAIX, Glut-1 and MIB-1. *Results:* A mosaic pattern of expression of p16 was seen throughout in all cases of endometrial TM (15/15), in 2/6 of the ovarian inclusion cysts with TM, in all (3/3) cervical TM and focal in 5/7 of Fallopian tube cases. Mosaic expression was also seen in a ciliated cell tumor of the ovary and in 18/39 of endometrioid endometrial carcinomas, and diffuse p16 expression was seen in 5/5 serous carcinomas. In comparison with normal endometrium, TM areas in the endometrium showed significantly increased expression of HIF-$1\alpha$, cyclin E, p21 and cyclin A, and decreased expression of p27. Membranous expression of CAIX and Glut-1 was only seen in TM areas, pointing to functional HIF-$1\alpha$. *Conclusion:* As p16 is consistently expressed in TM, less and only patchy expressed in the normal Fallopian tube, is paralleled by aberrant expression of cell cycle proteins, HIF-$1\alpha$, CAIX and Glut-1 and resembles the pattern of p16 expression frequently seen in endometrial carcinomas, we propose endometrial TM to be a potential premalignant endometrial lesion.

Keywords: Tubal metaplasia, immunohistochemistry, p16, endometrium, cervix, ovary, carcinogenesis, hypoxia

## 1. Introduction

The tumor suppressor gene p16, also known as INK4A, or MTS1, is located on chromosome 9p21. It encodes the cell cycle regulatory protein p16, a cyclin-dependent kinase inhibitor, which binds to cyclin D1-cyclin-dependent kinase (cdk) 4 and 6 complexes to control the cell cycle at the G1-S transition. p16[INK4A] is considered to be a tumor suppressor gene as it inhibits cell proliferation by regulating cdk4 activity to prevent pRb phosphorylation, leading to G1 arrest. Therefore, inactivation of the p16 [INK-4A] gene could lead to uncontrolled cell growth.

Alterations of p16[INK4A] gene have been detected in various human tumors, but the role of this gene as a tumor suppressor *in vivo* appears to be dependent on tumor type. Loss of p16 function by point mutation, homozygous deletion or hypermethylation of the pro-

moter region of the gene has been implicated in carcinogenesis of several human malignancies. p16 loss per se is not sufficient to induce tumorigenesis, but rather cooperates with exogenous stimuli (e.g. carcinogens) or other spontaneous mutations to facilitate malignant transformation [42]. On the other hand, overexpression of p16 has been described as well, for example in cervical intraepithelial neoplasias associated with oncogenic human papilloma virus (HPV) infections. In clinical practice, p16 is therefore used as a marker of dysplastic cervical cells [11,21,46,51].

The ciliated cell is a normal constituent of the human endometrium, increasing in number during age [15]. However, since the function of these cells within the endometrium is not clear, one could argue that these cells have actually undergone metaplastic differentiation to resemble those of the Fallopian tube showing a mosaic pattern of secretory and ciliated type cells, usually referred to as tubal metaplasia (TM) when it does not concern just scattered individual cells but glands or a stretch of surface epithelium completely composed of ciliated cells. There are more ciliated cells, tubal type

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secretory cells and reserve or intercalary cells than are normally present [35]. Endometrial TM may be extensive, may almost line the surface of the endometrium, especially in peri- and postmenopausal women. Ciliated (tubal) change is seen in normal endometrium, hyperplasia and carcinomas of the endometrium [19,26]. All these changes are thought to reflect a mild degree of estrogenic stimulation [15,35].

This type of differentiation is frequently found in the gynaecological tract. In the cervix, TM refers to endocervical glands that are lined by a Müllerian-type epithelium that closely resembles that of the Fallopian tube. In the ovary, there are often epithelial inclusion cysts, that are traditionally thought to arise from cortical invaginations of the ovarian surface epithelium that have lost their connection with the surface. Recently, it has however been proposed that tubal cells may seed to the ovarian surface, are uptaken and form cysts. These cysts are typically lined by a single layer of columnar cells that are often ciliated, mimicking tubal epithelium [31–33]. Ovarian inclusion cysts are likely the site of origin for most common epithelial tumors of the ovary. Several studies have found that patients with ovarian carcinoma have an increased number of inclusion cysts in the contralateral ovary compared to controls [25,36]. Others could not confirm that, but noticed as well more cortical invaginations in ovaries from women with contralateral ovarian cancer compared to controls [38,45]. In patients at high hereditary risk of ovarian cancer undergoing prophylactic adnectomy, a high frequency of TM inclusion cysts showing aberrant expression of bcl-2, the progesterone receptor, Ki67 and p53 was found [33,38].

Interestingly, several authors have written about 'scattered patterns' or 'focal expression' of p16 positive cells in tubal or tubo-endometrial tissue of the cervix [6,20,27,29,34,46]. They describe a pattern which is not seen in normal tissue, and is different from p16 immunohistochemically negative tissue and diffuse positivity, as is seen in high-risk HPV positive cervical intraepithelial lesions. Inoue considered in a review metaplasia in general to be a precursor of the variant types of endometrial carcinomas, based on the p53 accumulation and PCNA staining, and the fact that endometrial carcinomas are often accompanied by adjacent metaplastic epithelium [18]. However it is, for now, not a generally accepted view that endometrial metaplasias are a precursor of endometrial cancer.

To our knowledge, this observation has never been compared to staining for LhS28, a ciliated cell marker [8]. Furthermore, p16 expression in TM has never

been placed in a broader context and the expression of p16 has never been studied extensively in TM in the endometrium or in inclusion cysts of the ovary. Interestingly, TM is not merely a process of terminal differentiation that should conceptually be absent in malignancy, as the uncommon endometrial ciliated cell tumour even shows exceptionally high tubal differentiation and may be malignant [14,22]. Ciliated cell tumors of the ovary have also been described [10]. HIF1-$\alpha$, the key regulator of the hypoxia response, has been shown to be expressed in endometrial carcinomas [44], was seen to be expressed in TM areas in our study on the hypoxia response during endometrial carcinogenesis [16].

This study was therefore undertaken to investigate p16 expression in TM in the endometrium, and to compare it to TM in the cervix and ovary, and to the normal Fallopian tube. It is generally thought that endometrial TM is a benign disease, although molecular studies are scarce. Aberrant expression of the proliferation marker MIB-1 (Ki-67) and proteins that are often involved in carcinogenesis such as cell cycle proteins and increased expression of hypoxia inducible factor (HIF)-1 alpha (the key regulator of the carcinogenetic hypoxic response) and its target genes Glut-1 and CAIX in endometrial TM, might necessitate to change the common view that endometrial TM is a purely benign lesion.

## 2. Materials and methods

### 2.1. Patients and tissues

Paraffin-embedded clinical specimens from inactive endometrium ($n = 15$), all showing areas of tubal metaplasia, normal Fallopian tubes ($n = 7$), ovaries with TM in inclusion cysts ($n = 6$), cervix ($n = 3$) with TM, secretory phase endometrium ($n = 3$), interval phase ($n = 1$), proliferative phase endometrium ($n = 5$), and serous ($n = 5$) and endometrioid carcinoma of the endometrium ($n = 39$), and one borderline ciliated cell tumor of the ovary were collected from the archives of the Department of Pathology of the University Medical Center, Utrecht, The Netherlands.

These tissues were derived from patients operated between 1992 and 2005. Haematoxylin and eosin-stained sections were revised by 2 experienced gynecopathologists (PvD, DSG). Anonymous use of redundant tissue for research purposes is part of the standard treatment agreement with patients in our hospital [9].

## 2.2. Immunohistochemistry

All slides were immunohistochemically stained for p16 and LhS28. Additionally, slides with inactive endometrium were further analysed for p21, p27, p53, cyclin A, cyclin D1, cyclin E, HIF-1$\alpha$, Glut-1, CAIX, and MIB-1 (Ki-67) expression.

Immunohistochemistry was performed on 5 $\mu$m thick paraffin slides. Table 1 presents all antibodies, dilutions, incubation times and antigen-retrieval methods used. Slides were deparaffinized with xylene and rehydrated by serial ethanol dilutions.

For HIF-1$\alpha$, endogenous peroxidase was blocked by hydrogen peroxide (Dako CSA kit), after which antigen retrieval followed. A cooling off period of 30 minutes preceded blocking of the avidin by biotin block (Dako; 10 min) and protein block (Dako; 5 min). Then, the primary antibody was applied followed by the catalyzed signal amplification system (Dako CSA kit). Slides were washed in Tris buffer in between.

For all other stainings, endogenous peroxidase activity was blocked for 30 minutes, followed by antigen retrieval. Additionally, slides were incubated with the primary antibody, followed by the secondary antibody after washing slides in between in PBS. Finally, for HIF-1$\alpha$ and other stainings, peroxidase activity was developed with DAB and counterstained with hematoxylin.

Positive controls were used throughout, see table for types of tissue. Negative controls were obtained by omission of the primary antibodies from the staining procedure.

## 2.3. Scoring of staining

Two authors (PvD, NH) scored all slides blinded to clinicopathologic data and results of other stainings. For p16, cytoplasmic and nuclear staining were both considered positive. Each slide was examined for staining pattern, which was classified as negative (no positive cells), focal (some positive cells, though much less than "mosaic staining"), a "mosaic staining" pattern of secretory and ciliated cells, or diffuse staining. A mosaic staining was defined as a typical pattern of positive staining of 50–80% of cells, in a checkerboard pattern of positive and negative cells.

LhS28, a ciliated cell marker, only stained the apical side of the cell, where the basal bodies and cilia are situated; this type of staining was considered positive [8]. LhS28 staining was compared to p16 for colocalization in all slides.

For Glut-1 and CAIX membranous staining was considered positive staining. For all other proteins, the percentage of dark, homogenously stained nuclei was estimated as before [50], ignoring cytoplasmic staining.

## 2.4. Statistics

Slides with parts of normal inactive endometrium and parts with TM were scored (% of positive nuclei) separately, and differences in expression were analyzed with non-parametric paired testing (Wilcoxon Signed Ranks Test). Two sided $p$-values $< 0.05$ were considered significant. All statistical analysis were performed with SPSS for Windows version 12.0.1, 2003 (SPSS Inc., Chicago, IL).

## 3. Results

### 3.1. p16

All (15/15) cases of endometrial TM showed a mosaic p16 expression pattern in the TM areas (Fig. 1B). In the normal Fallopian tube, some focal p16 expression was seen in 5/7 cases (Fig. 1D), 2 cases were negative. 3/6 cases of TM in ovarian inclusion cysts were p16 negative, while 1 case showed some focal positivity and 2 showed a mosaic pattern of p16 expression. One of the positive ovaries harboured 4 inclusion cysts (Fig. 1E, F), one being p16 negative not showing much TM, but the other 3 smaller cysts were strongly p16 positive in a mosaic pattern and showed extensive TM. In the cervix all (3/3) cases with TM showed a mosaic expression of p16 expression (Fig. 1H).

All normal parts of the proliferative endometrium ($n = 5$) stained negative for p16, the parts with ciliated cell change ($n = 5$) showed p16 positivity in a mosaic pattern. Interval phase endometrium ($n = 1$) was p16 negative. In the 3 slides with secretory endometrium 2 showed parts with TM, both of these showed p16 in a mosaic pattern in the TM, the normal parts were p16 negative ($n = 1$) or focally p16 positive ($n = 1$); one slide showed only normal secretory endometrium, which was p16 negative.

Serous carcinoma of the endometrium showed a diffuse pattern of p16 expression in 5/5 cases. Of 39 endometrioid endometrial carcinomas 1 was p16 negative, 7 were focally positive, 18 showed a mosaic pattern of expression, and 13 were diffusely positive.
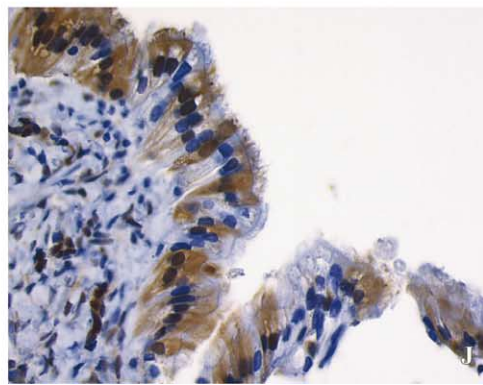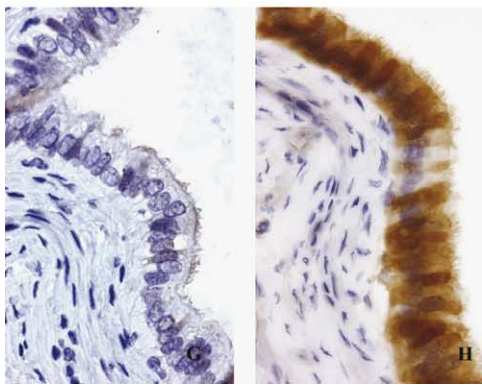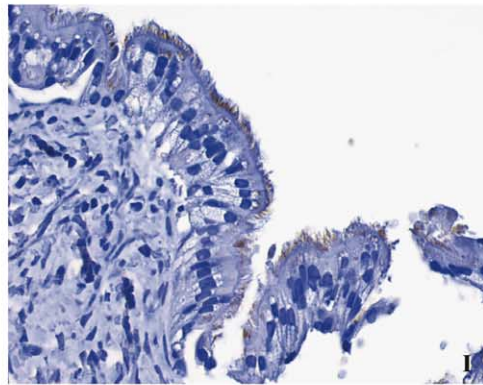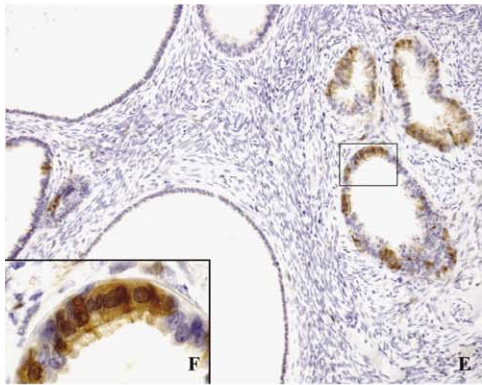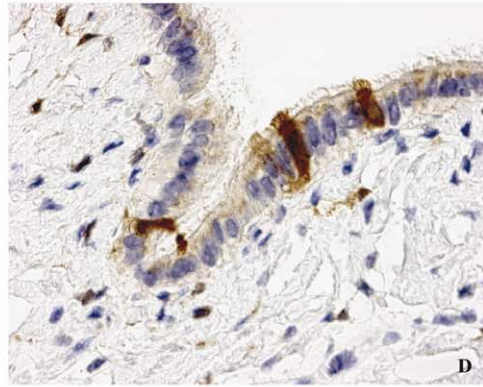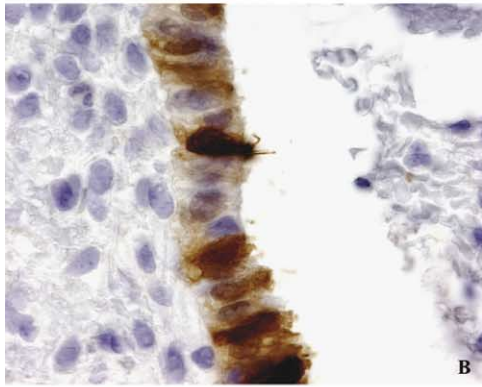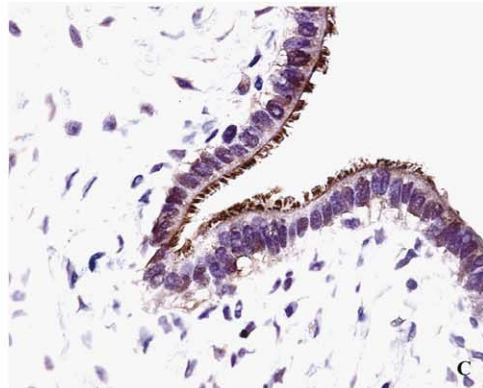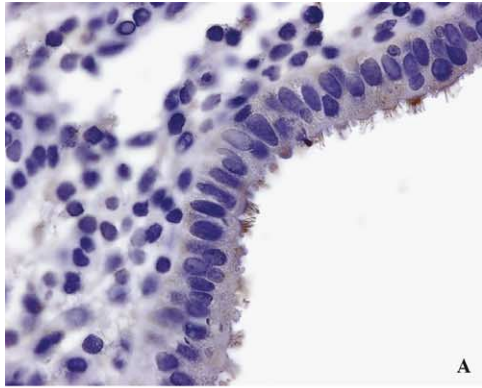
Table 1
Overview of the antibodies used and tissue processing details

| Primary Antibody* | Company** | Dilution | Antigen Retrieval† | Second step‡ | Positive control | Incubation time/temp (primary antibody) | Procedure |
|---|---|---|---|---|---|---|---|
| LhS28 | Abcam | 1:500 | Citrate pH 6.0 | Strep AB(2) | Cilliated cell Fallopian tube | 60 minutes/ room temp | By hand |
| p16 | Neomarkers | 1:160 | EDTA pH 9.0 | PV | Cervical Carcinoma room temp | 60 minutes/ | Automatic staining device |
| p21 | Dako | 1:25 | EDTA pH 9.0 | PV | Colon | 60 minutes/ room temp | By hand |
| p27 | Transduction | 1:500 | Citrate pH 6.0 | PV | Skin | o/n 4C | By hand |
| p53 | Dako | 1:400 | Citrate pH 6.0 | Strep AB(2) | Serous endometrial carcinoma | o/n 4C | By hand |
| cyclin A | Novocastra | 1:100 | Citrate pH 6.0 | Strep AB(2) | Tonsil | o/n 4C | By hand |
| cyclin D1 | Novocastra | 1:20 | EDTA pH 9.0 | Strep AB(3) | Mantle Cell Lymphoma | 60 minutes/ room temp | Automatic staining device |
| cyclin E | Novocastra | 1:50 | Citrate pH 6.0 | Strep AB(2) | Placenta | o/n 4C | By hand |
| HIF-1$\alpha$ | Pharmingen | 1:50 | TRS, DAKO, 45 min, 97°C | CSA | Breast Carcinoma | 60 minutes/ room temp | By hand |
| Glut-1 | Dako | 1:200 | Citrate pH 6.0 | G-aR IgG + Strep AB(1) | Red blood cells in slide | 60 minutes/ room temp | Automatic staining device |
| CAIX | Novus Biologicals | 1:1000 | Citrate pH 6.0 | PV | Grawitz tumor | 60 minutes/ room temp | By hand |
| MIB-1 (Ki-67) | Immunotech | 1:100 | Citrate pH 6.0 | Strep AB (3) | Tonsil | 60 minutes/ room temp | Automatic staining device |

*All primary antibodies used are monoclonal antibodies, except for Glut-1 and CAIX. HIF-1$\alpha$ = hypoxia-inducible factor -1$\alpha$.
**Neomarkers, Fremont, USA; Abcam, Cambridge, UK; Pharmingen, BD Biosciences, BD Pharmingen, San Diego, CA, USA; Dako, Dako-Cytomation, Glostrup, Denmark; Novus Biologicals, Littleton, CO, USA; Transduction, BD Biosciences, BD Transduction Laboratories, San Diego, CA, USA; Novocastra, Newcastle upon Tyne, UK.
†TRS = Target Retrieval Solution, DAKO S1700.
‡CSA = catalyzed amplification kit, Dako; G-aR IgG = biotinylated Goat-anti Rabbit IgG (BA-1000, Vector laboratories, CA, diluted 1:500) + Strep AB (1) = Streptavidin peroxidase labeling (Streptavidin HRP, IM0309, Beckman Coulter, diluted 1:1000); Strep AB (2) = biotinylated rabbit-anti-mouse, diluted 1:500 in PBS, Dako, followed by streptavidin-biotin complex, diluted 1:200 in PBS, Dako; Strep AB(3) = biotinylated horse-anti-mouse, diluted 1:500 in PBS, Vector BA-2000, followed by streptavidin-biotin complex, diluted 1:1000 in PBS, Immunotech; PV = Powervision ready to use (Poly-HRP-anti Ms/Rb/RtIgG biotin free, ImmunoLogic, ImmunoVision Technologies, Brisbane, CA, USA).

In 2 endometrioid carcinomas, expression was particularly pronounced in the squamous parts. One cilliated cell tumor of the ovary showed the typical mosaic p16 expression pattern (Fig. 1J).

LhS28 expression colocalized with p16 mosaic pattern in TM in endometrium (Fig. 1A, B), cervix (Fig. 1G, H), ovary and Fallopian tubes (Fig. 1C and D) and in ciliated cell tumor of the ovary (Fig. 1I, J).

Fig. 1. Ciliated cell change in various parts of the gynecological tract. (A) Endometrium with tubal metaplasia stained by LhS28, a ciliated cell marker, and in (B) for p16. (C) Normal Fallopian tube showing LhS28 staining, and (D) focal p16 expression. (E) Subcortical ovarian inclusion cysts showing varying degrees of tubal metaplasia, with a mosaic pattern of p16 expression in the cysts with the most pronounced ciliated cell change (F). (G) Cervix with tubal metaplasia showing positivity for LhS28 and H) p16 in a mosaic pattern. (I) Endometrial ciliated cell tumor of the ovary showing positivity for LhS28 and (J) p16 in a mosaic pattern.

Table 2

Expression of cell cycle proteins (% of positive cells) in normal inactive endometrium and tubal metaplasia (TM) of the endometrium.

| | Normal inactive endometrium ($n = 15$) | | | TM endometrium ($n = 15$) | | | Test for difference between groups: Wilcoxon Signed Ranks test |
|---|---|---|---|---|---|---|---|
| | Mean | Median | Range | Mean | Median | Range | $p$-value |
| cyclin A | 0.6 | 0 | 0–2 | 2.73 | 2.0 | 0–5 | 0.001 |
| cyclin D1 | 0 | 0 | 0 | 0.47 | 0 | 0–2 | 0.083 |
| cyclin E | 2.67 | 0 | 0–35 | 7.07 | 0 | 0–35 | 0.039 |
| P21 | 1.07 | 1.0 | 0–5 | 6.87 | 5.0 | 0–35 | 0.005 |
| P27 | 59.67 | 65.0 | 5–100 | 42.00 | 35.0 | 5–100 | 0.007 |
| P53 | 0.07 | 0 | 0–1 | 0.07 | 0 | 0–1 | 1.0 |
| HIF-1$\alpha$ | 0 | 0 | 0 | 18.33 | 5.0 | 0–90 | 0.005 |
| MIB1 | 2.80 | 2.0 | 0–20 | 3.53 | 2.0 | 0–10 | 0.344 |

### 3.2. HIF-1α, Glut-1, CAIX, p21, p27, cyclin A, cyclin D1, cyclin E

The TM areas in the endometrial cases were further analyzed for aberrant expression of cell cycle proteins (other than p16) and HIF-1$\alpha$ (Table 2). In comparison with normal parts of the endometrium, TM areas in the endometrium showed increased expression of HIF-1$\alpha$ ($p = 0.005$). The HIF-1$\alpha$ downstream genes Glut-1 and CAIX showed no expression in inactive endometrium, whereas TM areas showed positive cell membranes for Glut-1 in 4/15 cases and for CAIX in 2/15 cases, which colocalized with the HIF-1 $\alpha$ positive areas. TM areas also showed increased expression of cyclin E ($p = 0.039$), cyclin A ($p = 0.001$) and p21 ($p = 0.005$), and decreased expression of p27 ($p = 0.007$) compared to normal parts of the endometrium. For MIB-1 (Ki-67) no difference in staining between inactive endometrium and tubal metaplasia ($p = 0.344$) was noticed.

## 4. Discussion

The objective of the study was to assess p16 expression in TM in the endometrium in comparison with normal endometrium, normal Fallopian tube and TM in ovarian inclusion cysts and cervix; and to verify whether cell cycle proteins, HIF-1$\alpha$, Glut-1 and CAIX were expressed equally in TM and normal parts of the endometrium.

On the basis of the data presented in this study, the comparison with the ciliated cell marker LhS28 and the published literature, we conclude that mosaic expression of p16 is a consistent phenomenon of TM in the female genital tract [6,20,27,29,34,46], especially in the endometrium. We also noticed, as others did before [15,26], that ciliated cells are more present during the proliferative phase compared to secretory endometrium. p16 expression has been observed before in cyclic endometrium, more in the proliferative than the secretory phase, but has not been studied and linked incessantly to TM as we did [4,27,29,34,43,47]. The typical expression of p16 which we observed in TM was not seen that extensively in the normal Fallopian tube. In the ovary, p16 expression paralleled the degree of tubal metaplasia. A ciliated cell tumor of the ovary showed the typical mosaic pattern of p16 expression we observed in TM. We propose that TM of the endometrium could be a potentially premalignant lesion, based on the following arguments.

Firstly, aberrant expression of p16 is regarded as a carcinogenetic event in many tumors, including those in the gynecological tract. p16 is very frequently aberrantly expressed in cervical dysplasia and carcinoma [1,6,20,24] where it may however be a bystander effect of HPV E6/E7 cell cycle activation without an inherent contribution to carcinogenesis, but in other malignancies the carcinogenetic role of p16 alterations are much better defined [13,30]. Further, Umezaki [48] suggested that tubal metaplasia should be considered a neoplastic entity of uterine cervical glandular lesions that may have the potential to undergo malignant transformation, although this is up to now not a widespread view. Secondly, in the present study, p16 is also aberrantly expressed in TM in some ovarian inclusion cysts. These cysts have been proposed to be precursors of ovarian cancer [38]. In ovarian cancer, especially the serous type, p16 is often aberrantly expressed [2]. Furthermore, in endometrial carcinoma of

the endometrium, p16 positivity rates vary from 30-94% [1,4,28,37,39,42,43,47], in endometrioid carcinoma especially in the squamous areas [20], and aberrant p16 expression has also been found in endometrial hyperplasia [4,47]. In serous and clear cell endometrial cancers, p16 positivity is also frequent [1,2,37,39], although the number of cases studied so far is low.

Additionally, we found that the TM areas in the endometrium showed aberrant expression of many cell cycle proteins, HIF-1$\alpha$, Glut-1 and CAIX compared to normal tissue. The cell cycle consists of four phases: G1, S, G2, M. Cyclins form a complex with cyclin-dependent kinases and by doing this they make transition to the next phase of cell cycle possible. This will induce cell growth, unless inhibited by tumor suppressor gene products such as p53 or cdk-inhibitors (cdki's), such as p16, p21 and p27. The transition of G1/S is partly controlled by cyclin E and cyclin D1; p27 and p21 inhibit cyclin E; p16 and p21 inhibit cyclin D1. In the transition of S/G2 cyclin A plays a role, with the cdki's p27 and p21. Overexpression of cell cycle stimulating factors such as the cdk's and cyclins, and underexpression of inhibiting factors such as cdki's are frequently found in tumors. In general, aberrant expression of cell cycle regulators correlated with a more malignant subtype, a higher proliferation rate, recurrence and a worse survival in different tumors. We found a significantly higher expression of cyclin A, cyclin E and p21, and a lower expression of p27 in TM parts with TM compared to the normal endometrium, which points to disturbance of the G1/S, S/G2 and G2/M transitions in endometrial TM, consistent with potential premalignant change. Cyclin A and MIB-1 are markers for proliferation, the first being more expressed in TM, but this was not apparent for MIB-1. The changes in cell cycle regulators are however not accompanied with obvious morphological changes like in other dysplastic lesions. It is therefore not in this stage possible to morphologically discriminate potentially premalignant from harmless TM. As obvious dysplastic changes are lacking, it is also difficult to indicate to what kind of cancer TM could progress. In view of the p16 expression patterns seen in endometrioid and serous carcinomas, we suggest that both these cancers but especially serous cancer could be at the far end of the progression spectrum of TM.

It is unclear why HIF-1$\alpha$ is overexpressed in TM. HIF-1$\alpha$ is the key regulator of the hypoxia response, and has been implicated in carcinogenesis in many different epithelia including the female epithelia of the endometrium [16,44] and breast [5]. In the absence of necrosis, it is unlikely that hypoxia causes HIF-1$\alpha$ overexpression. Therefore, the observed HIF-1$\alpha$ overexpression is possibly caused by aberrant expression of oncogenes and tumor suppressor genes that are known to be able to upregulate HIF-1$\alpha$ [41]. This deserves to be further studied.

Although HIF-1$\alpha$ is well-known as the key regulator for survival of hypoxic tumor cells, another direct effect of HIF-1$\alpha$ in hypoxia is proposed to be promotion of cell cycle arrest, for example by influencing p21 or p27 [7,12], however, this has not been studied in endometrial cells. Therefore, whether the increase in expression of p21 in TM in this study is HIF-1$\alpha$-driven remains to be proven. The decrease of p27 in TM is inexplicable in relation to HIF-1$\alpha$.

Glut-1 and CAIX are well characterized downstream targets of HIF-1$\alpha$, which facilitate survival of cells in acid and low glucose circumstances. Carbonic anhydrase IX (CAIX) is a membrane-associated carbonic anhydrase, that plays a role in pH regulation [49]. A role for this enzyme in the adaptation of tumor cells to hypoxic conditions and in tumor cell progression is suggested by a significant overlap between CAIX expression and regions of hypoxia in solid tumors [23,53]. In endometrial hyperplasia, expression of Glut-1, a glucose transporter upregulated by HIF-1$\alpha$, appeared to be associated with (pre)neoplastic stages of the endometrium and is therefore proposed to be a useful indicator of high risk for development of endometrial carcinoma [3,17,52]. On the whole, these studies reveal an indication to preneoplastic progress in tissue expressing CAIX and Glut-1 in the membranes. This is remarkable as we noticed this type of expression in parts of TM.

In conclusion, ciliated cells are regarded by some to be normal constituents of the endometrium [23]. We show here that endometrial TM, the far morphological end of tubal differentiation, shows aberrant expression of several cell cycle regulators, HIF-1$\alpha$, the key regulator of the hypoxia response, Glut-1 and CAIX. This implies that TM might not be as benign as generally accepted, and may in fact be a potential premalignant lesion. This warrants further molecular studies on genetic aberrations in TM to better found these preliminary results. In view of the consistent expression of p16 in a characteristic mosaic pattern in TM, p16 immunohistochemistry may help to identify TM areas in the gynecological tract as an alternative for the ciliated cell marker LhS28.

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## References

[1] M.A. Ansari-Lari, A. Staebler, R.J. Zaino, K.V. Shah and B.M. Ronnett, Distinction of endocervical and endometrial adenocarcinomas: immunohistochemical p16 expression correlated with human papillomavirus (HPV) DNA detection, *Am. J. Surg. Pathol.* **28** (2004), 160–167.

[2] J.E. Armes, R. Lourie, M. de Silva, G. Stamaratis, A. Boyd, B. Kumar, G. Price, S. Hyde, D. Allen, P. Grant and D.J. Venter, Abnormalities of the RB1 pathway in ovarian serous papillary carcinoma as determined by overexpression of the p16(INK4A) protein, *Int. J. Gynecol. Pathol.* **24** (2005), 363–368.

[3] A. Ashton-Sager, A.F. Paulino and A.M. Afify, GLUT-1 is preferentially expressed in atypical endometrial hyperplasia and endometrial adenocarcinoma, *Appl. Immunohistochem. Mol. Morphol.* **14** (2006), 187–192.

[4] A.M. Bamberger, L. Riethdorf, K. Milde-Langosch, C.M. Bamberger, I. Thuneke, I. Erdmann, H.M. Schulte and T. Loning, P16/MTS1 and pRB expression in endometrial carcinomas, *Virchows Arch.* **434** (1999), 23–28.

[5] R. Bos, H. Zhong, C.F. Hanrahan, E.C. Mommers, G.L. Semenza, H.M. Pinedo, M.D. Abeloff, J.W. Simons, P.J. van Diest and E. van der Wall, Levels of hypoxia-inducible factor-1 alpha during breast carcinogenesis, *J. Natl. Cancer Inst.* **93** (2001), 309–314.

[6] R.I. Cameron, P. Maxwell, D. Jenkins and W.G. McCluggage, Immunohistochemical staining with MIB1, bcl2 and p16 assists in the distinction of cervical glandular intraepithelial neoplasia from tubo-endometrial metaplasia, endometriosis and microglandular hyperplasia, *Histopathology* **41** (2002), 313–321.

[7] P. Carmeliet, Y. Dor, J.M. Herbert, D. Fukumura, K. Brusselmans, M. Dewerchin, M. Neeman, F. Bono, R. Abramovitch, P. Maxwell, C.J. Koch, P. Ratcliffe, L. Moons, R.K. Jain, D. Collen and E. Keshert, Role of HIF-1alpha in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis, *Nature* **394** (1998), 485–490.

[8] M.T. Comer, A.C. Andrew, H.J. Leese, L.K. Trejdosiewicz and J. Southgate, Application of a marker of ciliated epithelial cells to gynaecological pathology, *J. Clin. Pathol.* **52** (1999), 355–357.

[9] P.J. van Diest, No consent should be needed for using leftover body material for scientific purposes, *BMJ* **325** (2002), 648–651.

[10] J.H. Eichhorn and R.E. Scully, Endometrioid ciliated-cell tumors of the ovary: a report of five cases, *Int. J. Gynecol. Pathol.* **15** (1996), 248–256.

[11] T. Ekalaksananan, C. Pientong, S. Sriamporn, B. Kongyingyoes, P. Pengsa, P. Kleebkaow, O. Kritpetcharat and D.M. Parkin, Usefulness of combining testing for p16 protein and human papillomavirus (HPV) in cervical carcinoma screening, *Gynecol. Oncol.* 2006.

[12] N. Goda, H.E. Ryan, B. Khadivi, W. McNulty, R.C. Rickert and R.S. Johnson, Hypoxia-inducible factor 1alpha is essential for cell cycle arrest during hypoxia, *Mol. Cell. Biol.* **23** (2003), 359–369.

[13] R. Gonzalez-Quevedo, C. Garcia-Aranda, A. Moran, C. De Juan, A. Sanchez-Pernaute, A. Torres, E. Diaz-Rubio, J.L. Balibrea, M. Benito and P. Iniesta, Differential impact of p16 inactivation by promoter methylation in non-small cell lung and colorectal cancer: clinical implications, *Int. J. Oncol.* **24** (2004), 349–355.

[14] M.R. Hendrickson and R.L. Kempson, Ciliated carcinoma–a variant of endometrial adenocarcinoma: a report of 10 cases, *Int. J. Gynecol. Pathol.* **2** (1983), 1–12.

[15] M.R. Hendrickson and R.L. Kempson, Uterus and Fallopian Tubes, in: S.S. Sternberg (Ed.), *Histopathology for Pathologists*. Raven Press, New York, PA, 1992, 797–834.

[16] N. Horree, P.J. van Diest, P. van der Groep, D.M.D.S. Sie-Go and A.P.M. Heintz, Hypoxia and angiogenesis in endometrial carcinogenesis. *Submitted*.

[17] M.T. Idrees, P. Schlosshauer, G. Li and D.E. Burstein, GLUT1 and p63 expression in endometrial intraepithelial and uterine serous papillary carcinoma, *Histopathology* **49** (2006), 75–81.

[18] M. Inoue, Current molecular aspects of the carcinogenesis of the uterine endometrium, *Int. J. Gynecol. Cancer* **11** (2001), 339–348.

[19] T. Kaku, N. Tsukamoto, N. Tsuruchi, K. Sugihara, T. Kamura and H. Nakano, Endometrial metaplasia associated with endometrial carcinoma, *Obstet. Gynecol.* **80** (1992), 812–816.

[20] J.T. Keating, A. Cviko, S. Riethdorf, L. Riethdorf, B.J. Quade, D. Sun, S. Duensing, E.E. Sheets, K. Munger and C.P. Crum, Ki-67, cyclin E, and p16INK4 are complimentary surrogate biomarkers for human papilloma virus-related cervical neoplasia, *Am. J. Surg. Pathol.* **25** (2001), 884–891.

[21] R. Klaes, T. Friedrich, D. Spitkovsky, R. Ridder, W. Rudy, U. Petry, G. Dallenbach-Hellweg, D. Schmidt and M. von Knebel Doeberitz, Overexpression of p16(INK4A) as a specific marker for dysplastic and neoplastic epithelial cells of the cervix uteri, *Int. J. Cancer* **92** (2001), 276–284.

[22] S.E. Low and A. Nicol, Ciliated cell variant of endometrioid adenocarcinoma: a rare tumour, *J. Clin. Pathol.* **57** (2004), 1341–1342.

[23] J.A. Loncaster, A.L. Harris, S.E. Davidson, J.P. Logue, R.D. Hunter, C.C. Wycoff, J. Pastorek, P.J. Ratcliffe, I.J. Stratford and C.M. West, Carbonic anhydrase (CA IX) expression, a potential new intrinsic marker of hypoxia: correlations with tumor oxygen measurements and prognosis in locally advanced carcinoma of the cervix, *Cancer Res.* **61** (2001), 6394–6399.

[24] W.G. McCluggage and D. Jenkins, p16 immunoreactivity may assist in the distinction between endometrial and endocervical adenocarcinoma, *Int. J. Gynecol. Pathol.* **22** (2003), 231–235.

[25] K.R. Mittal, A. Zeleniuch-Jacquotte, J.L. Cooper and R.I. Demopoulos, Contralateral ovary in unilateral ovarian carcinoma: a search for preneoplastic lesions. *Int. J. Gynecol. Pathol.* **12** (1993), 59–63.

[26] S. Moritani, R. Kushima, S. Ichihara, H. Okabe, T. Hattori, T.K. Kobayashi and S.G. Silverberg, Eosinophilic cell change of the endometrium: a possible relationship to mucinous differentiation, *Mod. Pathol.* **18** (2005), 1243–1248.

[27] N. Murphy, C.C. Heffron, B. King, U.G. Ganuguapati, M. Ring, E. McGuinness, O. Sheils and J.J. O'Leary, p16INK4A positivity in benign, premalignant and malignant cervical glandular lesions: a potential diagnostic problem, *Virchows Arch.* **445** (2004), 610–615.

[28] R. Nakashima, M. Fujita, T. Enomoto, T. Haba, K. Yoshino, H. Wada, H. Kurachi, M. Sasaki, K. Wakasa, M. Inoue, G. Buzard and Y. Murata, Alteration of p16 and p15 genes in human uterine tumours, *Br. J. Cancer* **80** (1999), 458–467.

[29] G.P. Nielsen, A.O. Stemmer-Rachamimov, J. Shaw, J.E. Roy, J. Koh and D.N. Louis, Immnohistochemical survey of p16INK4A expression in normal human adult and infant tissues, *Lab. Invest.* **79** (1999), 1137–1143.

[30] D.F. Peng, Y. Kanai, M. Sawada, S. Ushijima, N. Hiraoka, S. Kitazawa and S. Hirohashi, DNA methylation of multiple tumor-related genes in association with overexpression of DNA methyltransferase 1 (DNMT1) during multistage carcinogenesis of the pancreas, *Carcinogenesis* **27** (2006), 1160–1168.

[31] J.M. Piek, P.J. van Diest, R.P. Zweemer, P. Kenemans and R.H. Verheijen, Tubal ligation and risk of ovarian cancer, *Lancet* **358** (2001), 844.

[32] J.M. Piek, R.H. Verheijen, P. Kenemans, L.F. Massuger, H. Bulten and P.J. van Diest, BRCA1/2-related ovarian cancers are of tubal origin: a hypothesis, *Gynecol. Oncol.* **90** (2003), 491.

[33] J.M. Piek, R.H. Verheijen, F.H. Menko, A.P. Jongsma, J. Weegenaar, J.J. Gille, G. Pals, P. Kenemans and P.J. van Diest, Expression of differentiation and proliferation related proteins in epithelium of prophylactically removed ovaries from women with a hereditary female adnexal cancer predisposition, *Histopathol.* **43** (2003), 26–32.

[34] L. Riethdorf, S. Riethdorf, K.R. Lee, A. Cviko, T. Loning and C.P. Crum, Human papillomaviruses, expression of p16, and early endocervical glandular neoplasia, *Hum. Pathol.* **33** (2002), 899–904.

[35] B.M. Ronnett and R.J. Kurman, Precursor Lesions of Endometrial Carcinoma, in: R.J. Kurman (Ed.), *Blaustein's pathology of the female genital tract*, Springer-Verlag, New York, PA, 2002, 487–498.

[36] H. Salazar, A.K. Godwin, M.B. Daly, P.B. Laub, W.M. Hogan, N. Rosenblum, M.P. Boente, H.T. Lynch and T.C. Hamilton, Microscopic benign and invasive malignant neoplasms and a cancer-prone phenotype in prophylactic oophorectomies, *J. Natl. Cancer Inst.* **88** (1996), 1810–1820.

[37] H.B. Salvesen, S. Das and L.A. Akslen, Loss of nuclear p16 protein expression is not associated with promoter methylation but defines a subgroup of aggressive endometrial carcinomas with poor prognosis, *Clin. Cancer Res.* **6** (2000), 153–159.

[38] P.W. Schlosshauer, C.J. Cohen, F. Penault-Llorca, C.R. Miranda, Y.J. Bignon, J. Dauplat and L. Deligdisch, Prophylactic oophorectomy: a morphologic and immunohistochemical study, *Cancer* **98** (2003), 2599–2606.

[39] A. Semczuk, C. Boltze, B. Marzec, A. Szczygielska, A. Roessner and R. Schneider-Stock, p16INK4A alterations are accompanied by aberrant protein immunostaining in endometrial carcinomas, *J. Cancer Res. Clin. Oncol.* **129** (2003), 589–596.

[40] A. Semczuk, R. Miturski, D. Skomra and J.A. Jakowicki, Expression of the cell-cycle regulatory proteins (pRb, cyclin D1, p16INK4A and cdk4) in human endometrial cancer: correlation with clinicopathological features, *Arch. Gynecol. Obstet.* **269** (2004), 104–110.

[41] G. Semenza, Signal transduction to hypoxia-inducible factor 1, *Biochem. Pharmacol.* **64** (2002), 993–998.

[42] N.E. Sharpless, INK4a/ARF: a multifunctional tumor suppressor locus, *Mutat. Res.* **576** (2005), 22–38.

[43] T. Shiozawa, T. Nikaido, M. Shimizu, Y. Zhai and S. Fujii, Immunohistochemical analysis of the expression of cdk4 and p16INK4 in human endometrioid-type endometrial carcinoma, *Cancer* **80** (1997), 2250–2256.

[44] E. Sivridis, A. Giatromanolaki, K.C. Gatter, A.L. Harris and M.I. Koukourakis; Tumor and Angiogenesis Research Group, Association of hypoxia-inducible factors 1alpha and 2alpha with activated angiogenic pathways and prognosis in patients with endometrial carcinoma, *Cancer* **95** (2002), 1055–1063.

[45] F. Tresserra, P.J. Grases, R. Labastida and A. Ubeda, Histological features of the contralateral ovary in patients with unilateral ovarian cancer: a case control study, *Gynecol. Oncol.* **71** (1998), 437–441.

[46] B. Tringler, C.J. Gup, M. Singh, S. Groshong, A.L. Shroyer, D.E. Heinz and K.R. Shroyer, Evaluation of p16INK4a and pRb expression in cervical squamous and glandular neoplasia, *Hum. Pathol.* **35** (2004), 689–696.

[47] H. Tsuda, K. Yamamoto, T. Inoue, I. Uchiyama and N. Umesaki, The role of p16-cyclin d/CDK-pRb pathway in the tumorigenesis of endometrioid-type endometrial carcinoma, *Br. J. Cancer* **82** (2000), 675–682.

[48] K. Umezaki, M. Sanezumi, F. Kanemori, T. Yoshimura, H. Ohdate, A. Ikuta and H. Kanzaki, Immunohistochemical demonstration of aberrant glycosylation and epidermal growth factor receptor in tubal metaplasia of the uterine cervix, *Gynecol. Oncol.* **70** (1998), 40–44.

[49] R.D. Vaughan-Jones and K.W. Spitzer, Role of bicarbonate in the regulation of intracellular pH in the mammalian ventricular myocyte, *Biochem. Cell. Biol.* **80** (2002), 579–596.

[50] M.M. Vleugel, A.E. Greijer, A. Shvarts, P. van der Groep, M. van Berkel, Y. Aarbodem, H. van Tinteren, A.L. Harris, P.J. van Diest and E. van der Wall, Differential prognostic impact of hypoxia induced and diffuse HIF-1alpha expression in invasive breast cancer, *J. Clin. Pathol.* **58** (2005), 172–177.

[51] G. Volgareva, L. Zavalishina, Y. Andreeva, G. Frank, E. Krutikova, D. Golovina, A. Bliev, D. Spitkovsky, V. Ermilova and F. Kiseljov, Protein p16 as a marker of dysplastic and neoplastic alterations in cervical epithelial cells, *BMC Cancer* **4** (2004), 58.

[52] B.Y. Wang, T. Kalir, E. Sabo, D.E. Sherman, C. Cohen and D.E. Burstein, Immunohistochemical staining of GLUT1 in benign, hyperplastic, and malignant endometrial epithelia, *Cancer* **88** (2000), 2774–2781.

[53] C.C. Wykoff, N.J. Beasley, P.H. Watson, K.J. Turner, J. Pastorek, A. Sibtain, G.D. Wilson, H. Turley, K.L. Talks, P.H. Maxwell, C.W. Pugh, P.J. Ratcliffe and A.L. Harris, Hypoxia-inducible expression of tumor-associated carbonic anhydrases, *Cancer Res.* **60** (2000), 7075–7083.