

# pHH3 and survivin are co-expressed in high-risk endometrial cancer and are prognostic relevant

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**BACKGROUND:** Phosphohistone-H3 (pHH3) is a promising reliable mitotic count biomarker. Our purpose was to study the relationship between the novel proliferation marker pHH3 and the established anti-apoptotic marker survivin and consider their prognostic relevance in endometrial cancer.

**METHODS:** A total of 106 patients with endometrial cancer (type I/endometrioid,  $n = 81$ ; type II carcinomas,  $n = 18$ ) and simple hyperplasia without atypia ( $n = 7$ ) were investigated. pHH3 and survivin expression were assessed using immunohistochemistry from paraffin-embedded tissue blocks.

**RESULTS:** A strong positive correlation was observed between pHH3 and survivin expression ( $P < 0.0001$ ). Patients with high-grade tumours and patients with type II carcinomas expressed significantly more pHH3 and survivin than low grade and endometrioid tumours ( $P < 0.0001$ ,  $P < 0.0001$ ,  $P < 0.0001$ , and  $P < 0.0001$ , respectively). In univariate survival analysis, overexpression of pHH3 and survivin were associated with increased recurrence and mortality ( $P < 0.0001$ ,  $P < 0.0001$ ,  $P < 0.0001$ , and  $P < 0.0001$ , respectively), in the multivariable Cox regression analyses both pHH3 and survivin could be identified as independent parameters for overall survival ( $P = 0.004$ , and  $P = 0.023$ , respectively).

**CONCLUSION:** In endometrial cancer, pHH3 and survivin were strongly positive correlated and were both associated with type II and high-grade tumours. Increasing expression levels of pHH3 and survivin were associated with adverse prognostic factors.

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In endometrial cancer proliferative activity has been positively correlated to tumour progression (Pirog and Czerwinski, 1992; Tornos *et al*, 1992). The mitotic index (MI), which is defined as the rate of mitotic figures per 10 consecutive high-power fields (HPF) in the area of the highest mitotic activity, has been shown to correlate significantly with tumour grade and prognosis (Christian *et al*, 1993). Pirog and Czerwinski (1992) showed that a MI of  $> 5$  mitosis/10 HPF had a significantly higher 5-year mortality rate; in another study  $\geq 8$  mitosis/10 HPF was identified as an adverse prognostic factor (Tornos *et al*, 1992). In a recently published study concerning the inter-observer agreement, MI on standard H&E-stained slides showed a relatively low reproducibility in comparison with International Federation of Gynecology and Obstetrics 2009 (FIGO) grade, nuclear atypia and growth patterns (Gemert *et al*, 2009). The selection bias is due to the subjective determination of areas of highest mitotic activity, the heterogeneity of mitotic activity in different areas of the tumour, and the difficulty in distinguishing between chromatin changes in cells in mitosis and cells undergoing apoptosis or pycnosis.

In recent years additional diagnostic markers, which indicate tumour cell proliferation have been tested to assess tumour

biology with noteworthy results. In endometrial cancer it was shown that biomarkers like Ki-67/MIB-1 can indicate a more malignant phenotype and overexpression is associated with poor prognosis (Markova *et al*, 2010).

The present study was designed to contrast an established and a novel immunohistochemical proliferative marker. Proliferative activity was determined with antibodies against survivin, a member of the inhibitor-of-apoptosis protein (IAP) family, which promotes survival of tumour cells (Altieri, 2010) and against core histone protein H3 (pHH3), a relatively new mitotic count biomarker, which provides reliable distinct nuclear positivity in cells with mitotic morphology. It has been shown that phosphorylation of histone H3 occurs at Ser 10 almost exclusively during late G2 phase, prophase, and M phase of the cell cycle in order to help coordinate chromatin decondensation and cytokinesis (Hendzel *et al*, 1997). Anti-pHH3 immunohistochemistry has already been applied on several tumour cell lines along with neoplasms, such as breast cancer (Skaland *et al*, 2007), astrocytoma (Habberstad *et al*, 2011), meningioma (Kim *et al*, 2007), melanoma (Casper *et al*, 2010), and uterine smooth muscle tumour (Veras *et al*, 2009), with the result that pHH3 is useful to assess mitotic activity. Moreover, it has been used to support the grading of some tumours and to predict prognosis (Kim *et al*, 2007). Of note, in a series of lymph node-negative breast cancer patients, pHH3 was the strongest prognostic variable (Skaland *et al*, 2007).

The purpose of this study was to evaluate the role of pHH3 and survivin in a series of patients with endometrioid and type II

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endometrial carcinomas, compare the staining results with clinicopathologic variables, and assess their prognostic value.

## MATERIALS AND METHODS

In this retrospective study a total of 106 patients with endometrial cancer (type I/endometrioid,  $n=81$ ; type II carcinomas,  $n=18$ ) and simple hyperplasia without atypia ( $n=7$ ), who were treated at the Department of Obstetrics and Gynaecology, Thermenklinikum Moedling/Vienna, Austria, were enrolled. Hysterectomy, bilateral salpingo-oophorectomy, and cytological examination of peritoneal fluid were performed. Lymphadenectomy was conducted, except for stage IA with histological grades 1 and 2 and endometrioid histology. Sections were obtained from curettage specimens. Histological and clinical staging were performed according to the staging system of the FIGO 2009 (Mutch, 2009). Subsequent to the definitive treatment, all patients were followed up in 6-month intervals. Patient charts were reviewed to obtain clinical data about age, FIGO tumour stage, tumour grade, histopathological tumour type, time of recurrence, and death or date of last follow-up.

### Immunohistochemistry

The histological classification of endometrial carcinomas was done in accordance with the current WHO classification (Nucci and Oliva, 2009) by an experienced pathologist (HB). Grading was done according to the FIGO grading system. A standard immunohistochemical technique was performed using Ventana BenchMark XT immunostainer (Ventana Medical Systems, Illkirch, Cedex, France) with a prediluted rabbit polyclonal antibody to phosphohistone-H3 (pHH3, Cell Marque, Rocklin, CA, USA) and a prediluted rabbit anti-human monoclonal antibody to survivin (clone EP2880Y, Biogenex, San Ramon, CA, USA). Heat epitope retrieval as provided by the immunostainer was done in a TRIS-based buffer supplied by the manufacturer (CC1 cell-conditioning solution, Ventana Medical Systems, Tucson, AZ, USA) at pH 7.5 for 60 min. Both antibodies were incubated at 37°C for 60 min. The enzymatic reactivity was visualised with Ultra View RED for pHH3 and 3–3 diaminobenzidine for survivin (Ventana Medical Systems). A case of skin with squamous cell carcinoma and adjacent normal epidermis served as an external positive control. For negative controls serial sections of the same specimens were used, omitting the primary antibody from the staining protocol and substituting it by commercially available mouse non-immune IgG serum (DAKO, Carpinteria, CA, USA).

### Evaluation of staining

The staining of pHH3 was recorded by counting positive cells in prophase, metaphase, anaphase, and telophase in selected areas with most positive nuclei, that is, 'hot spots' (Nasr and El-Zammar, 2008). The total number of mitoses per 10 HPF (using  $\times 400$  magnification) was determined.

Survivin staining was evaluated as described previously, with slight modifications (Brustmann *et al*, 2011). Immunoreactivity was exclusively observed in nuclear patterns and was scored semiquantitatively. The percentage of immunoreactive cells was evaluated throughout the whole slide. Only moderate and strong staining intensities were considered eligible for evaluation. Strong intensity corresponded to the intensity of the positive controls. Sections were scored as follows: (–) 0% immunoreactive cells; (+) 1–25% immunoreactive cells; (++) 26–50% immunoreactive cells; (+++) 51–75% immunoreactive cells; (++++) 76–100% immunoreactive cells. Finally, to define a cutoff level cases with scores 0, + and ++ were considered negative (group 0), those with scores +++ and ++++ were considered positive for overexpression (group1).

### Statistical analysis

Correlation of pHH3 and survivin was assessed by Kendall's tau correlation coefficient for ordinal data. For further evaluation, patients were categorised into group 0 (score negative, + and ++) and group 1 (scores +++ and ++++) for survivin expression and into group 0 (pHH3 $\leq$ median) and group 1 (pHH3>median) for pHH3 staining. The proportion of patients in group 1 was compared between subgroups defined by clinical characteristics by the chi-squared test and Fisher's exact test. For statistical purpose, patients were classified into a risk stratification schema based upon the estimated risk of disease recurrence as follows: low risk (endometrioid tumours IA and  $\leq$ G2), intermediate risk (endometrioid tumours IB and  $\leq$ G2), and high risk (endometrioid tumours  $\geq$ IA and G3, >II, or type II) (Creasman *et al*, 2006).

Overall survival was defined as time between diagnosis and death. Patients without recurrence at their last follow-up visit were censored. Deaths without recurrence were considered as competing events, and the cumulative incidence of recurrence was compared between groups by Gray's test (Gray, 1988). Patients alive at their last follow-up visit were censored. Survival probabilities were computed by the Kaplan–Meier method (Kaplan and Meier, 1958). Crude (unadjusted) and adjusted hazard ratios (HR) were computed by univariate and multivariable Cox regression analyses, respectively (Cox, 1972). As the number of events was low for overall survival (22 events), we could not estimate a multivariable model, adjusting the effects of pHH3 and survivin for the potential confounders FIGO tumour stage, histological grade, histological type, age, and estimated risk of recurrence simultaneously. Instead, we extracted the first two principal components of these confounders, which captured 98.2% of the total variance of all confounders (Harrell *et al*, 1996). We fitted two small-sample-bias-corrected Cox regression models including pHH3 or survivin, adjusting for these principal components (Cox, 1972; Heinze and Schemper, 2001). Two-sided  $P$ -values  $\leq 0.05$  were considered as indicating statistical significance. The statistical software R (R Development Core Team, Vienna, 2008, www.r-project.org) was used for statistical analysis.

### Institutional Review Board

Approval for this study was obtained by the Institutional Review Board.

## RESULTS

Patients' characteristics are given in Table 1. Histologically, 81 tumours were classified as endometrioid, 18 tumours were classified as type II carcinomas (serous carcinoma ( $n=15$ ), clear-cell carcinoma ( $n=3$ )). Of the total of 99 patients we found 25 patients with high-grade tumour (G3). Lymph node status was available in 30 patients. Lymph node involvement was noted in five patients. The median (IQR) follow-up in our study was 72 (51, 99) months. A positive staining reaction of pHH3 and survivin is shown in Figures 1 and 2.

A strong positive correlation was observed between pHH3 and survivin staining (Kendall's correlation coefficient tau-b = 0.75,  $P < 0.0001$ ).

In simple endometrial hyperplasia without atypia ( $n=7$ ) the rate of pHH3-positive cells ranged from 51 to 73 (median 61) per 10 HPF. In the cohort of endometrioid carcinomas ( $n=81$ ) the median rate of pHH3-positive cells was 31 (6–117), and was 94 (41–173) in type II carcinomas ( $n=18$ ) ( $P < 0.0001$ ). Figure 3 shows that pHH3 expression increased from endometrioid carcinoma to hyperplasia and was manifestly highest in the group of type II carcinomas. The rate of pHH3-positive cells was significantly associated with histological type ( $P < 0.0001$ ),

**Table 1** Characteristics of patients with primary endometrial cancer

Characteristic	Measure
Number of patients with endometrial cancer	99
Age at diagnosis (years)	66.5 (60.6–74.6) <sup>a</sup>
Abnormal uterine bleeding (n)	85 (84.8%)
Endometrial thickness measured by transvaginal ultrasound (mm)	15.8 (± 8.7) <sup>b</sup>
<i>Tumour stage (FIGO) (n)</i>	
IA	65 (65.7%)
IB	15 (15.1%)
II–IV	19 (19.2%)
<i>Histological grading</i>	
G1	49 (49.4%)
G2	25 (24.8%)
G3	25 (24.8%)
<i>Histopathological tumour type (n)</i>	
Endometrioid histology (type I carcinoma)	81 (81.8%)
Non-endometrioid histology (type II carcinoma)	18 (18.2%)
Serous carcinoma	15 (15.1%)
Clear-cell carcinoma	3 (3%)
Involvement of vascular space (n)	19 (19.2%)
<i>Management after surgery (n)</i>	
Radiotherapy	28 (28.3%)
Chemotherapy (carboplatin/paclitaxel)	4 (4.1%)
<i>Recurrence status (n)</i>	
No. of patients with recurrent disease	15 (15.2%)
Time to recurrent disease (months)	14.9 (2.1–27.8) <sup>a</sup>
<i>Survival rate (n)</i>	
Disease-free survival	77 (77.7%)
Specific-tumour survival	84 (84.8%)
Tumour related death	15 (15.2%)
Death as a result of other causes	7 (7.1%)

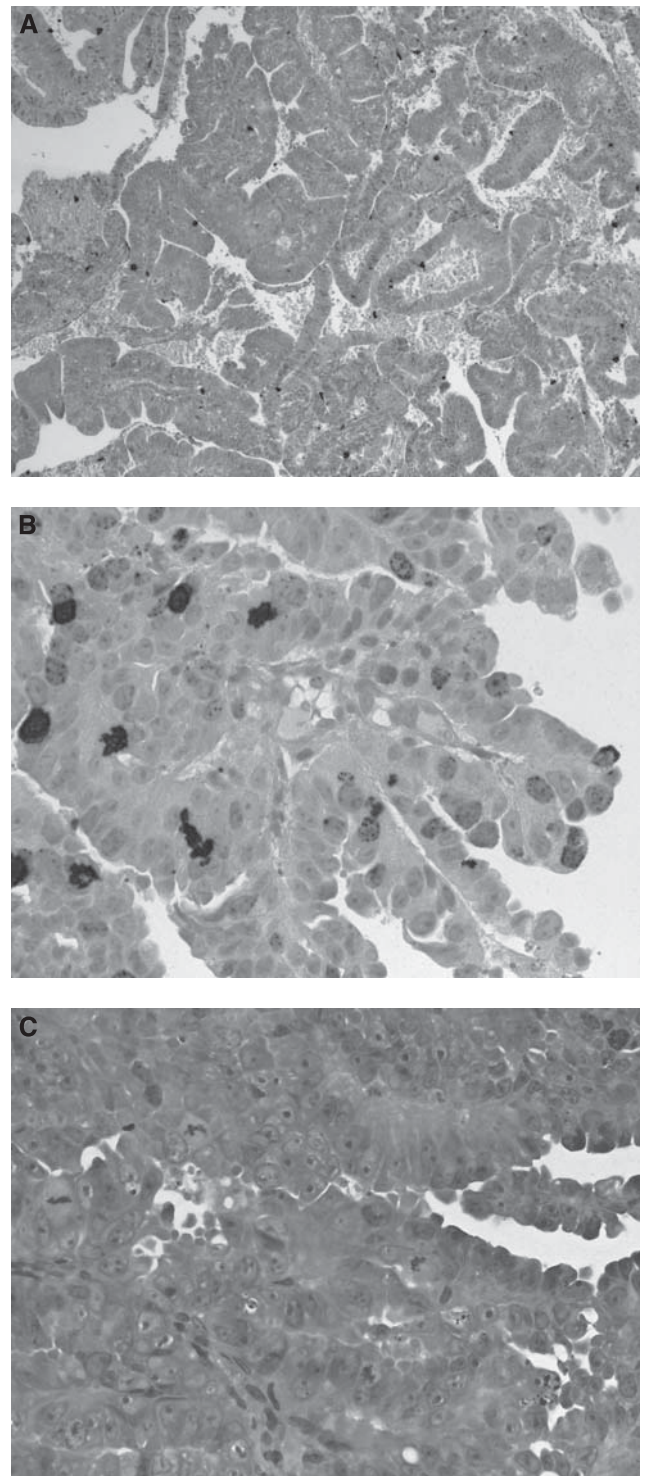
Abbreviations: FIGO=International Federation of Gynecology and Obstetrics according to classification 2009; IA=tumour invades <50% of the myometrial wall; IB=tumour invades ≥50% of the myometrial wall. <sup>a</sup>Median (IQR). <sup>b</sup>Mean (s.d.).

histological grade ( $P<0.0001$ ), risk stratification ( $P<0.0001$ ), and vascular space involvement ( $P=0.018$ ), but not with advanced tumour stage ( $P=0.3$ ) (Table 2).

We found no overexpression of survivin in any of the hyperplasia lesions. Survivin overexpression was present in 30/81 patients with endometrioid carcinoma and 18/18 in type II carcinoma ( $P<0.0001$ ). Patients with high-grade tumours (G3) and patients with an estimated high risk of disease recurrence expressed significantly more survivin than low-grade and low-risk tumours ( $P<0.0001$  and  $P<0.0001$ , respectively). We found no significant correlation between immunostaining of survivin and FIGO stage ( $P=0.06$ ) or vascular space involvement ( $P=0.063$ ) (Table 2).

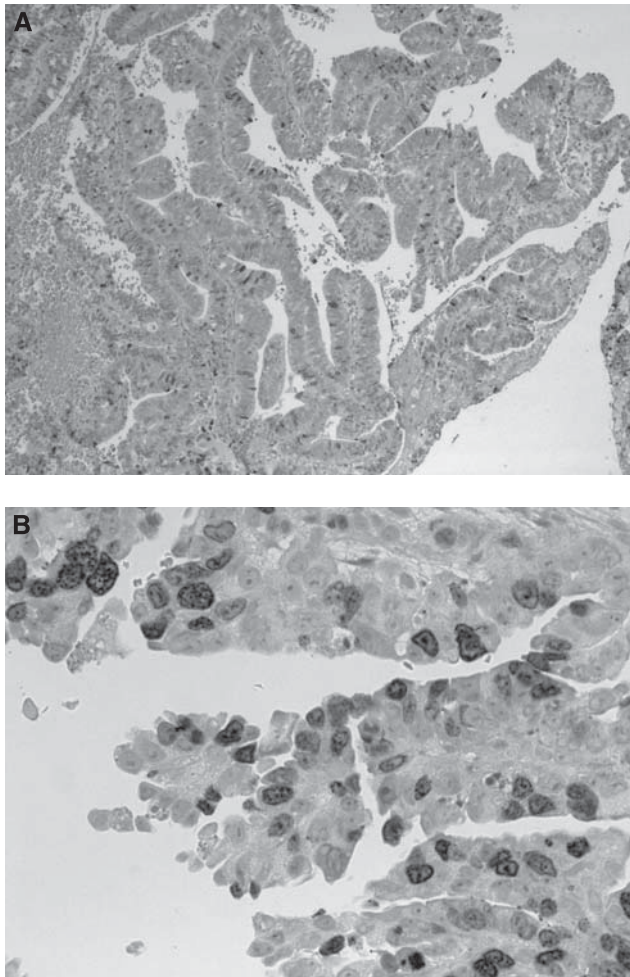
### pHH3 and survivin expression correlate with prognosis

We further analysed the prognostic significance by comparing carcinomas with high mitotic rate measured by pHH3 staining and survivin overexpression to those with low mitotic rate and less survivin expression in the whole cancer group. High rate of pHH3-positive cells (>median) and overexpression of survivin ( $\geq+++$ ) were associated with increased mortality (5-year overall survival rates 63.1% vs 100% ( $P<0.0001$ ), 63.3% vs 100% ( $P<0.0001$ ), respectively). A Kaplan–Meier curve regarding the association between pHH3 rate, survivin overexpression and overall survival is shown in Figure 4. High rate of pHH3-positive cells (>median) and overexpression of survivin ( $\geq+++$ ) were



**Figure 1** Immunohistochemical staining of phosphohistone-H3 (pHH3) in endometrial carcinoma. pHH3-labelled mitotic figures are easily detectable at (A) low power (magnification  $\times 100$ ) in a case of type I endometrioid carcinoma; (B) strong immunohistochemical staining in a case of type II endometrioid carcinoma (magnification  $\times 400$ ); (C) corresponding H&E staining of the tumour shown in B (magnification  $\times 400$ ).

associated with increased recurrence (5-year cumulative incidence of recurrence 29.6% vs 0% ( $P<0.0001$ )] 30% vs 0% ( $P<0.0001$ ), respectively) (Figure 4). Comparing survival and cumulative recurrence between positive and negative pHH3 and survivin



**Figure 2** Immunohistochemical localisation of survivin in nuclear pattern. Representative tissue sections of (A) low frequency (magnification  $\times 100$ ) in endometrioid adenocarcinoma; (B) high frequency (magnification  $\times 400$ ) in type II carcinoma.

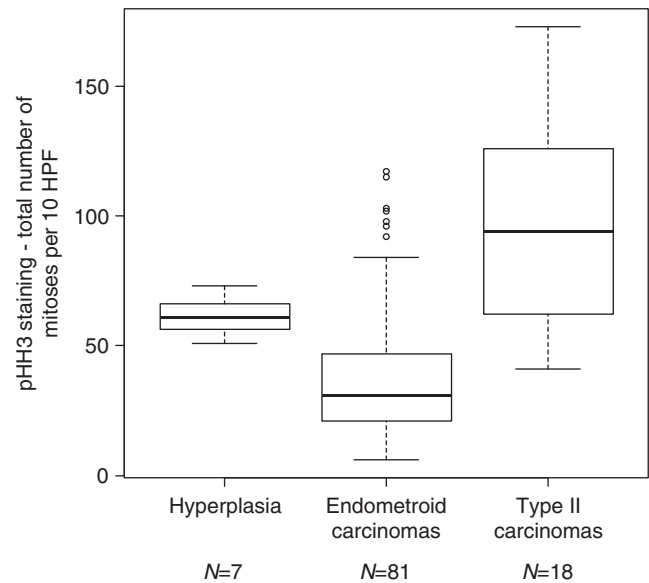
overexpression *vs* no overexpression corresponding to the subgroup of endometrioid carcinoma patients ( $n=81$ ), we found still significant results ( $P=0.007$ ,  $P=0.044$ ,  $P=0.003$ , respectively,  $P=0.003$ ). As all type II carcinomas were positive for pHH3 and survivin overexpression, no statistical comparison was possible in this subgroup.

In the confounder-adjusted multivariable Cox regression analyses both pHH3 and survivin could be identified as independent prognostic parameters for overall survival (pHH3  $>$  median *vs*  $\leq$  median: HR = 5.9, 95% confidence interval: 1.7, 31.4,  $P=0.004$ ; survivin overexpression *vs* low expression: HR = 3.8, 95% confidence interval: 1.2, 15.6,  $P=0.023$ ).

## DISCUSSION

In this immunohistochemical study, we demonstrate the positive correlation between the mitotic marker pHH3 and survivin, a member of the IAP family. Both markers are associated with type II and high-grade tumours, and strong expression indicates a poorer prognosis in survival analysis.

Mitotic activity is essential in the histopathological assessment of endometrial cancer. Mitosis is one of the shortest and least variable phases of the cell cycle and therefore mitotic indices



**Figure 3** pHH3 staining grouped by types of tissues. Boxplots shows median (thick solid line), 25th and 75th percentiles (lower and upper ranges of boxes, respectively), and range of values extending outside the 25th and 75th percentiles (vertical lines). Values  $>$  75th percentiles + 1.5 interquartile ranges are separately depicted as dots. Abbreviation: HPF = high-power fields.

provide a precise estimate of the proliferative rate (Pirog and Czerwinski, 1992). Identification of mitotic figures in H&E-stained slides is time consuming, moreover identification of mitotic figures can be hampered by several factors including squeezed cells from curettage specimens and similarities to chromatin changes that is, in apoptotic cells, or necrosis.

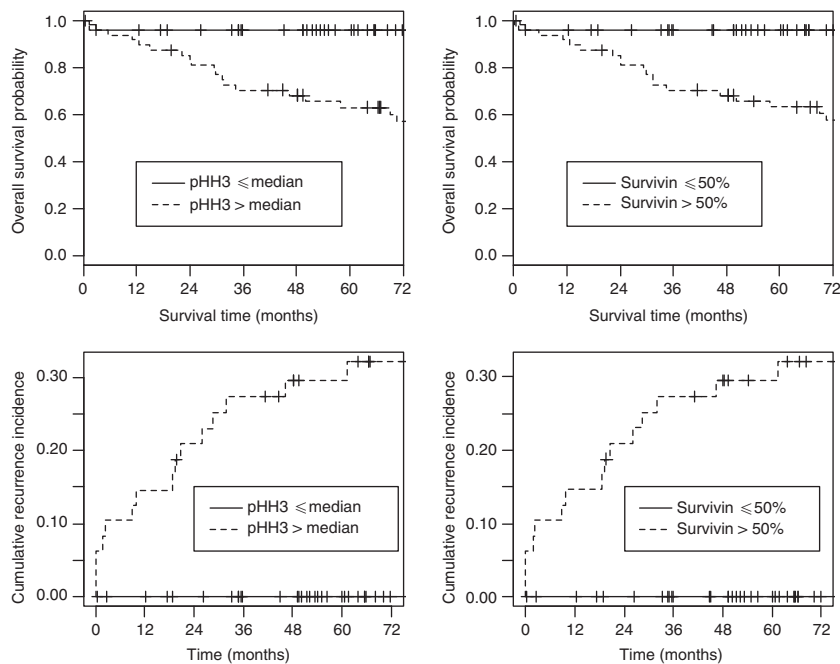
The immunohistochemical labelling of MFs with an antibody against pHH3 seems to be a novel promising method; however, the experience in endometrial tumour pathology is limited. To our knowledge we found only two reports on the topic of pHH3 and endometrial alteration. Brenner *et al* (2003) showed that expression of pHH3 and the mitotic indices were highly correlated in samples across the cycle. In another series of 15 patients, including 7 cases of endometrial carcinoma, the MI calculated from pHH3 immunostaining was zero in all cases of benign specimens, 2.3% in atypical hyperplasia and 4.8% in endometrial carcinomas (2.4% for grade 1, 3% for grade 2, and 7.8% for serous carcinomas) (Shah and Mazur, 2008). Our results are partly discrepant, as we found a higher rate of pHH3 in benign hyperplasia in comparison to endometrial tumours; however, we evaluated the most distinct and strongest expression of pHH3 in less differentiated and type II carcinomas as a sign of tumour progression. Staining of pHH3 facilitates the mitotic counting of cells in all stages of mitosis, including early prophase, when histone phosphorylation is initiated, and thus increases the sensitivity and specificity of this method relative to the conventional H&E procedure. Corresponding results with an increased number of identified mitosis by using pHH3 staining are described in other tumour types (Kim *et al*, 2007; Skaland *et al*, 2007). Of note, we also observed some discrete granular reactivity in cells without any morphological indicators of mitosis, consistent with interphase nuclei, which was reported as a major drawback of the method (Haberstad *et al*, 2011). However, from our point of view, distinct staining and objective determination of mitotic morphology prevent misinterpretation.

The biological implications of survivin are complex. Survivin expression is increased in the G2/M phase of the cell cycle, when pHH3 is active and acts as an inhibitor-of-apoptosis by blocking mitochondrial-induced apoptosis (Beardmore *et al*, 2004).

**Table 2** Correlation between pHH3 and survivin expression and clinicopathologic parameters in patients with endometrial cancer

Characteristic	n	pHH3 > median (36 mitoses/10 HPF) n (%)		P-value <sup>†</sup>	n	Survivin overexpression (+++ and ++++) n (%)		P-value <sup>a</sup>
		n	(%)			n	(%)	
Histological type (endometrioid)	81	30	(37.1)		81	30	(37.1)	
Histological type (type II carcinomas)	18	18	(100)	<0.0001	18	18	(100)	<0.0001
Tumour grade (I and II)	74	24	(32.4)		74	24	(32.4)	
Tumour grade (III)	25	24	(96)	<0.0001	25	24	(96)	<0.0001
Risk stratification (low <sup>b</sup> )	53	13	(24.5)		53	15	(28.3)	
Risk stratification (intermediate <sup>c</sup> and high <sup>d</sup> )	46	33	(76.1)	<0.0001	46	33	(71.7)	<0.0001
Stage (IA)	65	25	(38.5)		65	27	(41.5)	
Stage (IB and II–IV)	34	23	(67.6)	0.3	34	21	(61.7)	0.06
Age (≤70 years)	59	28	(47.5)		59	28	(47.5)	
Age (>70 years)	40	20	(50)	0.9	40	20	(50)	0.8
Vascular space involvement (neg.)	75	30	(40)	0.018	75	31	(41.3)	0.063
Vascular space involvement (pos.)	19	14	(73.7)		19	13	(68.4)	

Abbreviations: HPF = high-power fields; IA = tumour invades <50% of the myometrial wall; IB = tumour invades ≥50% of the myometrial wall. <sup>a</sup>P-value chi-square test. <sup>b</sup>Endometrioid tumours IA and ≤G2. <sup>c</sup>Endometrioid tumours IB and ≤G2. <sup>d</sup>Endometrioid tumours ≥IA and G3; >II; papillary serous or clear-cell uterine tumours.

**Figure 4** Overall survival and cumulative recurrence incidence of pHH3 and survivin.

However, survivin also regulates mitosis, partly by preserving microtubule stability and other not-conclusively-elucidated signaling pathways (Altieri, 2010). Survivin has a number of splicing variants, which may differ in their subcellular localisation and function, consistent with the regulation of both cell viability and cell division. The nuclear pool of survivin is considered to be involved in proliferation (Li *et al*, 2005). Although the number of type II carcinomas was low in our study ( $n = 18$ ), we could show a strong correlation between nuclear survivin overexpression and histological type. The rate of nuclear survivin overexpression was 37% in endometrioid carcinomas, whereas 100% in type II carcinomas. Studying the literature, we found inconsistent results concerning the nuclear and cytoplasmic pool of survivin, histological type, and prognosis. In a series of endometrioid and type II carcinomas it was stated that cytoplasmic survivin was frequently expressed, but no statistical significant correlation was shown between histological type, grade, stage, overall survival, and mitotic indices (Pallares *et al*, 2005). Likewise, a recent report found similar findings concerning cytoplasmic staining and

lacking association to histological type. However, in that study a significant correlation with clinical stage, histological grade, and survival rate was shown (Lambropoulou *et al*, 2010). Another earlier finding noted strong nuclear staining and some cytoplasmic reaction in endometrial carcinoma patients with similar clinicopathological results unfortunately without specifying the histological type of carcinoma (Takai *et al*, 2002). Concerning only endometrioid type of endometrial cancer it was recently evaluated that combined biomarkers including nuclear survivin, p21, and p53 are prognostically relevant (Steinbakk *et al*, 2009). In sharp contrast, other reports found no correlation between cytoplasmic (Erkanli *et al*, 2007) or nuclear survivin expression (Erkanli *et al*, 2006) and classical prognostic factors or survival in patients with endometrioid carcinomas.

We expanded our study and examined also the immunohistochemical expression of both markers in some patients with simple endometrial hyperplasia without atypia. In general, we found some diffuse nuclear staining but no overexpression of survivin in the hyperplasia cases. This result is different to earlier findings, where

mitotic and anti-apoptotic effects were correlated with cytoplasmic survivin immunoreaction (Erkanli *et al*, 2007). The discrepancy may result because of the use of different antibodies, which in our study stained exclusively nuclear patterns. In contrast pHH3 showed strong immunoreactivity in simple endometrial hyperplasia and was higher in comparison with endometrioid carcinomas. This proliferative phenomenon was also observed in earlier immunohistochemical studies, which revealed that the mean MI in endometrial hyperplasia was not significantly different from that in well-differentiated endometrioid carcinomas (Pirog and Czerwinski, 1992; Shah and Mazur, 2008). It is known that even in benign proliferative endometrium the MI can be threefold more than in endometrioid carcinomas (Ioffe *et al*, 1998). Previous studies with proliferation marker Ki-67 showed labelling of 21.8% in cases of simple hyperplasia and expression patterns were variable in endometrial carcinomas ranging from 15.7% for grade 1 and 25.3% for grade 3 (Morsi *et al*, 2000). The higher rate of pHH3 in benign cases is caused by the influence of elevated oestrogen, which varies through the menstrual cycle or is provided externally to patients suffering from menopausal symptoms (Shah and Mazur, 2008).

A limitation of our study is the over-representation of low-risk patients in our study group. So patients with important prognostic marker like positive lymph node involvement were relatively under-represented and a reliable statistical analysis with focus on

this prognosticator could not be considered. Another drawback is the low number of patients with type I grade 3 endometrioid carcinoma (EC-3) ( $n=7$ ). Thus, we could not perform statistical analysis to assess differences in the immunostaining profile of pHH3 and survivin in EC-3 and type II carcinomas.

In conclusion, our data indicate, that in terms of tumourgenesis, strong expression of pHH3 and survivin occur more often in type II and high-grade carcinomas as a sign of predominate proliferation and a feasible late event in the development of endometrium cancer. In our experience, immunohistochemistry of pHH3 seems to be an applicable and reliable method to determine areas of highest mitotic activity and to assist in rapid and precise differentiation between mitotic figures and apoptotic nuclei. We could show that pHH3 and survivin are positively correlated and are overexpressed in type II and high-grade tumours, which suggests that these proteins might share a common molecular pathway or enhance each other's actions during the cell cycle in the tumour cells. Further studies are warranted to prove the independent prognostic value of both biomarkers.

### Conflict of interest

The authors declare no conflict of interest.

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