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Expression of cell cycle regulators and ki67 in patients with recurrence of early cervical cancer

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Summary

Purpose of Investigation: Recurrence rates in patients with early cervical cancer are generally low. So far, no biomarker has been found to be associated with the risk of recurrence. In other carcinomas, expression of cell cycle regulators and proliferation markers like ki67 corresponded with tumour stage. The expression of those markers were examined in relapsed early cervical cancer after radical vaginal trachelectomy (RVT) to verify if they are suitable for risk stratification. *Materials and Methods:* We examined the expression rates of p16, p21, pRb, and cyclin D1 and ki67. Between March 1995 and March 2013, 310 patients with early cervical cancer underwent RVT as fertility-sparing surgery for early cervical cancer. Until now, ten patients suffered from recurrence. The results of relapsing and non-relapsing tumours were compared. *Results:* p21 was expressed heterogeneously. Neither of the relapsed adenocarcinomas showed an expression of pRb, whereas two of the recurrent squamous cell carcinomas showed positivity of pRb. Adenocarcinomas who relapsed showed a significant lower level of nuclear cyclin D1-expression ($p = 0.021$) than squamous cell carcinomas. Ki67 showed an expression-spectrum from 15-95% but level of expression was not significantly associated to recurrence. *Conclusion:* Nuclear expression of cyclin D1 was found more often in adenocarcinomas in contrast to squamous cell carcinomas. Relapsing adenocarcinomas showed higher expression of cyclin D1. Neither the examination of cell cycle regulators nor ki67 can be transferred in a clinical setting yet. Further studies based on a greater number of relapsed tumours are needed.

Key words: Cervical cancer; Vaginal trachelectomy; Recurrence; Cell cycle regulators; ki67.

Introduction

Early cervical cancer is among the most frequent cancers diagnosed in women throughout their reproductive phase [1, 2]. At the time of diagnosis, up to half of the patients with cervical cancer are between 20-44 years old [3-5]. Patients with stage I cervical cancer have a favourable prognosis with five-year survival rates over 80% [6]. Nevertheless, even in this subgroup relapses occur and the existing knowledge of prognostic factors is limited yet. Therefore, this study aims to improve insight in the molecular patterns of those tumours.

It is most challenging in the treatment of these patients not to compromise the oncologic safety in favour of fertility preservation. Both aspects can be met using radical vaginal trachelectomy (RVT) first carried out by Daniel Dargent in 1994 [7]. The preservation of the uterus while achieving oncologic safety becomes more and more important to informed patients. RVT is a complex and technically challenging procedure described previously by our present group [8, 9]. Patients treated by RVT have to meet strictly the inclusion criteria (Table 1) to reach disease free survival (DFS) and overall survival (OS) as after radical hysterectomy [4]. Oncologic safety of RVT was verified by many retrospective and prospective studies [8, 10, 11]. Pregnancy rates after RVT are similar to that of the general population [12].

Table 1. — *Inclusion criteria for RVT.*

- Desire to have children
- FIGO Stages IA1 L1, IA2 or IB1< 2 cm
- Clear endocervical margins ≥ 0.5 cm
- pN0 (parametran/pelvic), V0
- No neuroendocrine differentiation

Even if the patients fulfil inclusion criteria, cancer relapses occur in a low percentage of patients (4%) [4]. In a recent study comprising 300 women with early cervical cancer treated by RVT, no marker for risk of recurrence could be identified, based on histopathologic findings. [9, 13]. Therefore, other theories must be pursued. Dysregulation of the cell cycle initiates the multistep process of tumorigenesis [14]. Former studies showed that different patterns of expression of cell cycle regulators could be indicators of outcome in various tumours [15-17].

The cell cycle (Figure 1) is a sequence of events leading to cell replication. It is driven by stimulating cyclin/cdk-

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Table 2. — *Antibodies.*

Antibody	Dilution	Isotype	Clone
CINtec p16 histology	1:2		
Monoclonal mouse anti-human p21$^{Waf1/Cip1}$	1:25	IgG1	SX118
pRb lyophilized mouse monoclonal antibody	1:100	IgG1	84-B3-1
Cyclin D1 concentrated rabbit monoclonal antibody	1:200	IgG	EP12
Monoclonal mouse anti-human ki-67 antigen	1:100	IgG1	MIB-1

Table 3. — *Comparison of ki67, cyclinD1, p21, and pRb expression between cases and matched controls.*

	In due course relapsing tumours n=5	Non-relapsing tumours n=21	*p*-value
ki67, mean (SD)	42 (23)	47 (22)	0.706 [a]
Cyclin D1_n			
0	2	3	0.114 [b]
1-3	3	13	
4-6	-	5	
Cyclin D1_C			
0	5	17	0.995 [c]
1-3	-	4	
p21_IRS			
0	1	2	0.833 [b]
1-3	2	14	
4-6	2	5	
pRB_IRS			
0	3	17	0.305 [c]
1-6	2	4	

[a] *Based on linear mixed models.* [b] *Based on ordinal mixed models.* [c] *Based on binary logistic mixed models.*

Table 4. — *Expression of cyclin D1 nuclear (IRS) in all patients (unmatched).*

Cyclin D1 nuclear (IRS)	In due course relapsed tumours (n=5)	Non-relapsing tumours (n=56)	*p*-value[a]
Adenocarcinomas			0.037
0	2	2	
1-3	-	14	
4-6	-	2	
Total	2	18	
Squamous cell carcinomas			1.000
0		5	
1-3	3	32	
4-6	-	1	
Total	3	38	

[a] *p-values for linear trend test calculated using either exact methods or Monte Carlo sampling.*

Table 5. — *Data of patients with recurrence after RVT, who could be examined.*

Recurrence (n)	Recurrence/ *death (m)	Stage	Site	Treatment
2	25/*29	Squam pT1b1pN0G2L0V1	Cervix	Rad HE, RCT
3	12	Adeno pT1b1pN0G2L1V0	Cervix	Rad HE
4	7/*22	Squam pT1b1pN0G2L0V0	Cervix/corpus	Exenteration, RCT
6	3	Adeno pT1b1pN0G2L0V0	Cervix	HE, RCT
10	24/*30	Squam pT1b1pN0G3L1V0	Cervix and distant	Chemotherapy

complexes and inhibited by cdk-inhibitors. The cyclin/cdk complexes exert their stimulating function by phosphorylation of the retinoblastoma gene pRb [18]. Cdk-inhibitors can be divided in two groups: the Cip/Kip-family (p21, p27, and p57) and the INK4 family (p15, p16, p18, and p19). As their function is tumour suppression, missing cell cycle inhibitors could lead to malignant transformation and tumour growth [18, 19].

Table 6. — *Distribution of patient characteristics after matching.*

	Patients with relapse (n=5) (cases)	Patients without relapse (n=21) (controls)
Age at diagnosis in years, mean (SD)	32 (4)	30 (4)
Adenocarcinoma	2	9
Squamous cell carcinoma	3	12
Lymphangio-invasion	3	8
Grading 2	4	17
Grading 3	1	4

Matching was done as propensity score matching according to age, type of tumour, age at surgery, adenocarcinoma or squamous cell carcinoma, grading, and lymphangio-invasion with 1:4 and in one case 1:5 ratio between cases and controls.

Ki67-protein is expressed nuclearly throughout proliferation phases of the cell cycle [20]. It is widely used as gauge for proliferation in different tumours.

The role of cyclin D1 is still controversial. Cyclin D1 overexpression was associated with poor prognosis in squamous cell carcinoma at different sites [21, 22]. However, former studies revealed that downregulation of cyclin D1 is

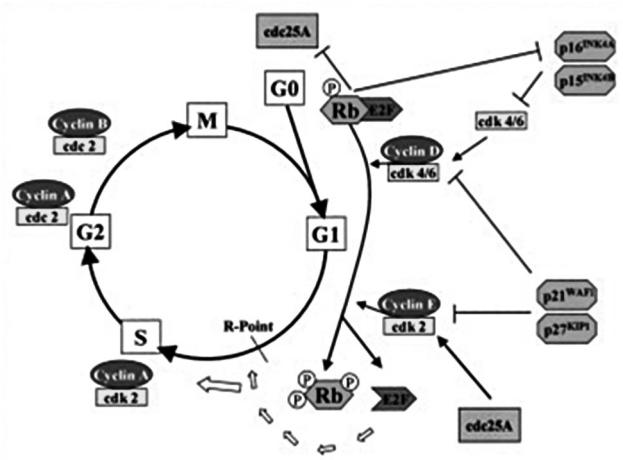

Figure 1. — Cell cycle regulation [34] (with permission from the author).

seen more often in malignant lesions of cervical neoplasia [23]. A recent study described absent cyclin D1 as well as focal positivity in neoplastic endocervical lesions. The authors hypothesized a role of cyclin D1 in tumour progression rather than in tumorigenesis [24].

p21 is detected in proliferating cells and missing in quiescent cells [25]. High expression levels of p21 can induce growth arrest and prevent apoptosis. p21 was deregulated in cervical cancer and found an independent predictor of poor prognosis in patients with adenocarcinoma of the cervix [26, 27].

p16 is an element of the key regulatory p16/cyclinD1-cdk4/6/Rb-pathway. Disruption of this pathway is an essential part of tumorigenesis [25]. p16 can also prevent the phosphorylation of the retinoblastoma protein (Rb) and control the S-phase progression [20]. Normally Rb is phosphorylated and thereby activated by p16 resulting in entering from G1 in S-phase [28].

In applying this knowledge to tumour tissue of the present cohort of patients, we attempted to establish a new categorization of tumour characteristics with regards to the risk of incidence of recurrence.

Materials and Methods

This retrospective study was approved by the Ethics Commission of the Charité – Universitätsmedizin Berlin. Informed consent to collect data was given by all patients. The authors examined a subgroup consisting of 65 patients of 310 patients with cervical cancer who underwent RVT between March 1995 and March 2013 at the university hospitals of Jena, Berlin, Munich, and the clinic Köln-Hohelind. Data was recorded retro- and prospectively. All patients met the inclusion criteria for RVT as established before [4, 8].

Standardised automatic immunohistochemistry (IHC) with an autostainer was performed. BenchMark ultra IHC/in situ hybridizationstaining (ISH) module formalin fixed, paraffin embedded material was also used. The antibodies included are shown in Table 2. Positive controls were included in all staining series, using tonsil tissue for all antibodies.

Interpretation of IHC staining of the tested markers was performed in two different approaches. First, the numbers of stained cells were established semiquantitatively in all slides. The whole slide was screened for stained areas and the ratio of the tumour area was estimated. If this ratio was not completely obvious, numbers of stained nuclei were counted. In a second step staining intensity was registered for p21, pRb, and cyclin D1 using immunoreactive score (IRS). Focal staining patterns and the presence of cytoplasmatic staining were registered when present. To achieve consensus of intra- and interobserver variations, samples in question were reviewed using a two-headed microscope. Data were pseudonymously entered into a database, and evaluated using the statistical program SPSS 22.

Patients with relapse were compared to those 56 patients without relapse. Additionally, matched cases and controls were analysed to control for confounding. For the matching of patients with relapse and without relapse, the authors used the following matching criteria: type of carcinoma (adenocarcinoma and squamous cell carcinoma; adenosquamous carcinomas were excluded), age at surgery, status of lymphangio-invasion (yes/no), and grading (1, 2 or 3). Matching was performed using the propensity score for relapse (probability for relapse calculated by binary logistic regression with the co-variables for age, histologic type, lymphangio-invasion and grading). The matching procedure aimed to match cases and controls in a relation of 1:4. Expression

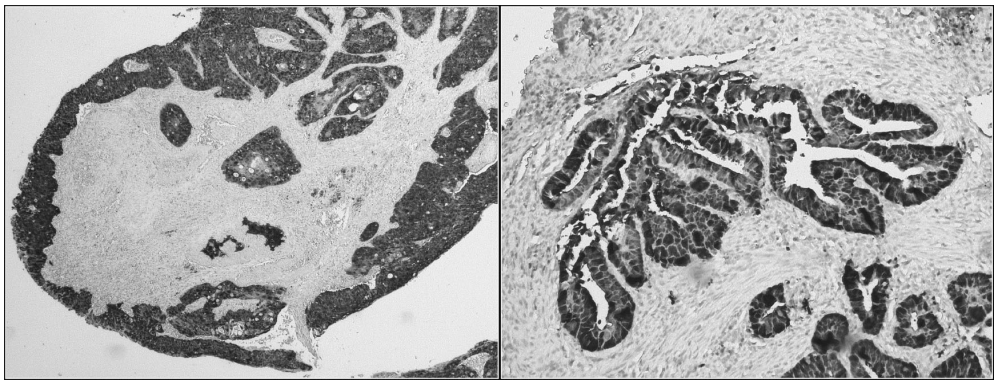

Figure 2. — In due course relapsing squamous cell carcinoma, 100% p16 staining intensity (left). In due course relapsing adenocarcinoma, 100% p16 staining intensity (right).

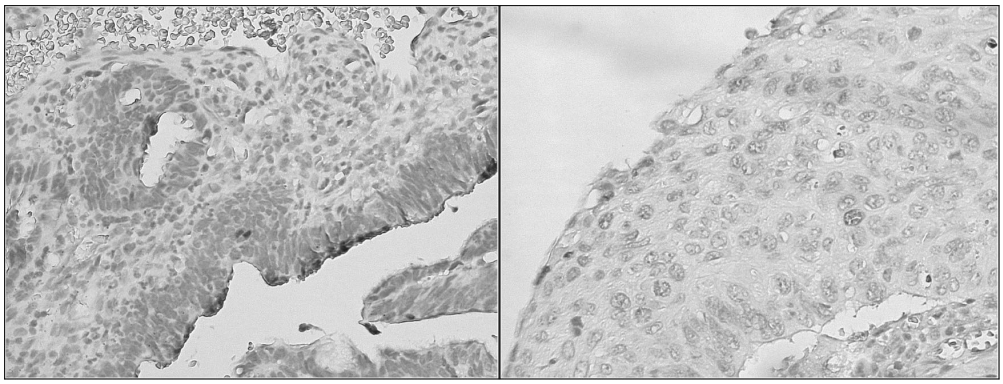

Figure 3. — In due course relapsing adenocarcinoma, p21 IRS 1-3 (left). In due course relapsing squamous cell carcinoma, p21 IRS 1-3 (right).

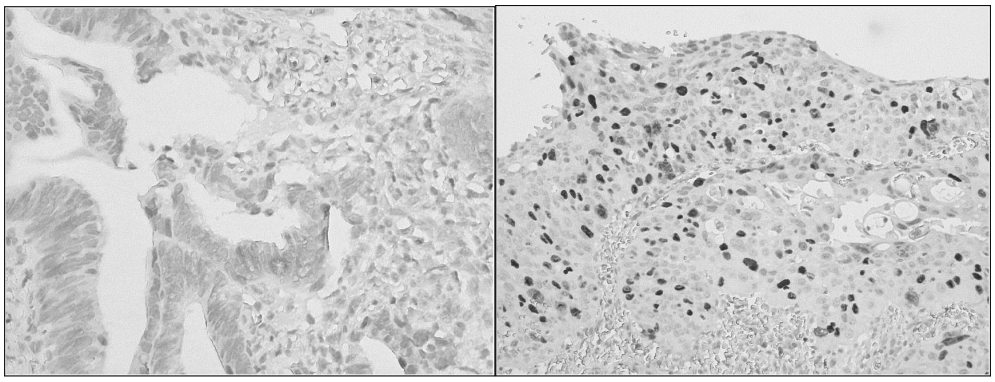

Figure 4. — In due course relapsing adenocarcinoma, pRb IRS 0 (left). In due course relapsing squamous cell carcinoma, pRb IRS 4-6 (right).

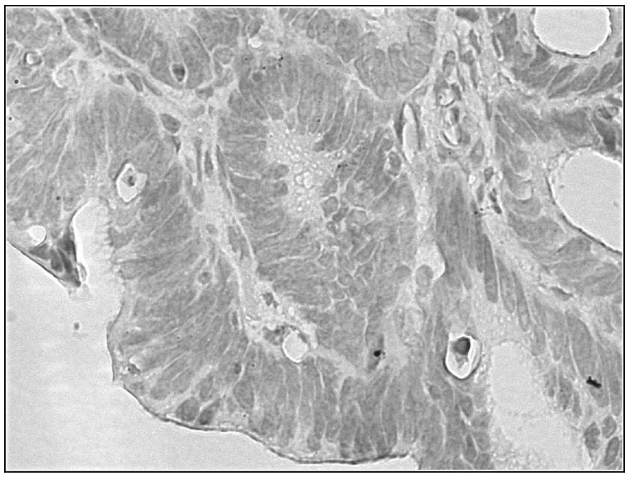

Figure 5. — In due course relapsing adenocarcinoma, cyclin D1 nuclear staining IRS 0.

rates of p16, p21, pRb and cyclin D1, and ki67 were analysed using linear mixed models to account for the matching (Table 3). In case of skewed distribution (|skewness |>1) the authors used log-transformed data for the regression. Unmatched data were analysed with linear trend tests (Table 4). A two-sided significance level of α=0.05 was considered. No adjustment for multiple testing was applied.

Results

Ten patients from the patients who were treated by RVT for early cervical cancer in our clinic suffered from relapse. One patient had to be excluded because final pathology showed adenosquamous cervical cancer. Nine patients were included in this study of which four suffered from adenocarcinoma and five suffered from squamous-cell-carcinoma (Table 5). Due to difficulties in cooperation with other clinics and institutes, we could only manage to re-

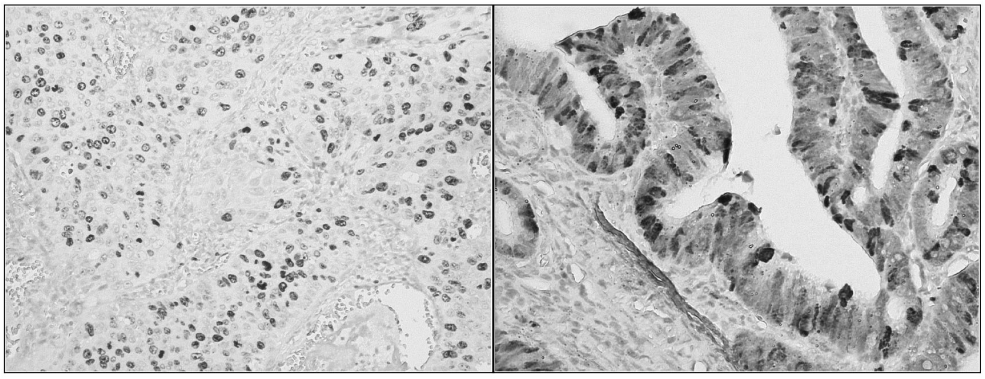

Figure 6. — In due course relapsing squamous cell carcinoma, ki67 (left). In due course relapsing adenocarcinoma, ki67 (right).

ceive material of the primary tumour of five patients. Additionally, we obtained material of relapsed tumours of four patients. These tumours were compared with tissue from 21 matched patients with early stage cervical carcinomas from the collective of RVT-patients who did not suffer from recurrence so far. Since recurrences in this setting occurred in some cases after 26 months, matching tumours were chosen from 1998–2012 to obtain an adequate follow-up interval. Median follow-up for patients without relapse was 45 months (IQR: 10-54 months). The median age at first diagnosis was 30 years (IQR: 24-38 years), and median age at relapse was 32 years (IQR: 25-40 years). A detailed description of patient characteristics is given in Tables 5 and 6.

The matching procedure resulted in 21 controls matched to the five cases with relapse with similar distribution in age, type of cancer, status of lymphangio-invasion, and grading (Table 6).

The results of the IHC were analysed descriptively using primary tumours of five patients who relapsed in comparison with 56 specimens of tumours that did not relapse yet.

p16: Four primary tumours showed 100% staining intensity, whereas one tumour showed only marginal staining and was therefore classified as negative. Fifty-two of 56 tumours of the comparative collective showed 100% staining intensity, typical for invasive carcinomas. Three cases showed 50% staining intensity and one tumour only 10%. No significant difference could be identified between relapsing tumours and tumours who did not relapse, neither in adenocarcinomas ($p = 0.10$) nor in squamous cell carcinomas ($p = 1.0$) (Figure 2).

p21 is heterogeneously expressed. No significant difference could be found between relapsed and non-relapsing tumours, neither in adenocarcinomas ($p = 1.0$) nor in squamous cell carcinomas ($p = 0.625$). More of the relapsed tumours showed moderate and high expression of p21, whereas the distribution among non-relapsing tumours was more diverse (Figure 3).

pRb: Two of three relapsing squamous cell carcinomas showed an expression of pRb, whereas no staining could be found in adenocarcinomas. This difference in staining behaviour between squamous cell carcinomas and adenocarcinomas could also be found in the comparative collective. Yet, there was no significant difference between the groups with relapse *vs*. without relapse in adenocarcinomas and squamous cell carcinomas with $p = 0.10$ and $p = 0.052$, respectively (Figure 4).

Cyclin D1 expression was found in the nucleus and in the cytosol. Therefore, results are described as nuclear and cytosolic staining. In squamous cell carcinomas cyclin D1 showed no significant difference in expression between patients with relapse and patients without relapse ($p = 1.000$). In contrast to that, no nuclear cyclin D1–expression was seen in adenocarcinoma relapse in contrast to tumours of the comparative collective without relapse (only 2 of 18 without nuclear cyclin D1-expression), which was a significant finding ($p = 0.037$). Cytosolic expression showed no significant difference (Figure 5 – cytosolic expression not shown).

Ki67: Adenocarcinomas which relapsed had a median of 35% ki67-staining intensity (IQR: 23-46%). Adenocarcinomas without relapse showed a slightly higher ki67-staining intensity of 41% (IQR: 29-73%). Results were similar in squamous cell carcinomas – relapsing tumours showed a median ki67 staining intensity of 44%, whereas non-relapsing tumours showed a median staining intensity of 41% (results not shown).

In addition to this descriptive overview of the results, we compared the relapsed tumours with matched pairs using mixed models. We analysed the staining results of all markers in the matching groups. No significant difference between the matched groups could be found (Table 3 and Figure 6).

Discussion

RVT is an oncologically safe therapeutic approach for patients with early cervical cancer who wish to preserve fertility. Relapse is extremely rare. In this collective of 310 patients treated by RVT, only ten patients relapsed. Although many characteristics of these relapsing tumours have been analysed, no pattern of recurrence could be es-

tablished so far [9]. In accordance to several studies investigating the prognostic impact of cell cycle regulators and proliferation markers in cervical cancer, we examined the expression of some of these markers in the relapsing tumours. The essential weakness of our study – the low number of relapses – is on the other hand an immense strength of the method and must not be lost track on. To our knowledge, up to now no other study evaluated the expression of cell cycle regulators in recurrent early cervical cancer.

By using automatized IHC, we deliberately chose an "easily available method, which is applicable for routine use. Our aim was to find a marker for relapse in cervical cancer which could be examined very easily in practice. All antibodies used were commercially available and were evaluated beforehand. Therefore, no methodically bias was to be expected. To compensate for the heterogeneities in analysis, we chose areas in the specimen with the highest positivity. The background of this approach is the acknowledged principle that the most proliferating area of a tumour reflects the biological behaviour and therefore the prognostic significance of the tumour best [29].

No statistically significant prognostic value of any analysed marker could be established. Even using matched pairs and the propensity score for matching, no difference in expression of the markers in neither group could be observed.

Former studies analysing cell cycle regulators in cervical cancer also found varying expression patterns which could not be sufficiently explained by different collective of patients or methodical approaches [14, 28, 30]. Therefore, by using IHC staining the estimation of cell cycle regulators seems to be very difficult.

One problem posed the fact that p16 and cyclin D1 were expressed nuclearly as well as in the cytosol. Cytoplasmatic staining is very difficult to quantify and could vary from one staining process to another [14]. An exact quantitative staining percentage could therefore not be received. We used a scoring system for calculation of a rough estimation, otherwise we confined to descriptive analysis.

Apart from one relapsing adenocarcinoma, all relapsing tumours showed 100% p16 staining. Due to the fact that also almost all so far non-relapsing tumours from the control group showed high staining levels, we attribute this effect to the known biological background of p16 dysregulation and resulting overexpression in proliferating cells [19, 20]. In contrast to other studies that found low levels of p16 associated with worse outcome, this seems not to apply to our patients with early cervical cancer [18, 31].

In our analysis we could not find pRb-expression in adenocarcinoma, nor in relapsing neither in non-relapsing tumours. Only two of the relapsing squamous cell carcinomas showed expression of pRb. pRb has been examined in cervical intraepithelial lesions before where increasing invasiveness of the lesion was accompanied with decreasing pRb expression [32]. We could not find any association between p16- and pRb-expression that has been described by others before [32]. These inconsistent results could either be attributed to the small number of patients in our study or could be one of the many different effects caused by disruption of the cell cycle pathways.

The hypothesis resulting from biological knowledge of the cell cycle regulators was that cell cycle inhibitors like p21 showed a lower expression in tumours with proliferative potential e.g. relapsing tumours [14]. In one study p21 decrease proved to be an independent predictor of poor prognosis in adenocarcinoma of the cervix [27]. In contrast to that high level, p21 in squamous cell cervical cancer was found in different studies [33, 34]. In our study we only found a slight tendency towards higher expression rates of p21 in non-relapsing tumours. Due to the low number of relapsed tumours, no significance could be established.

Even if the role of cyclin D1 is still not completely clear, it is obvious that the expression of the protein changes during tumour progression [35]. Revealed absence of expression or only focal positivity in a recent study suggested that it could be a good marker in glandular lesions [24]. In our cohort of patients, we found no nuclear expression of cyclin D1 in adenocarcinomas who relapsed in contrast to non-relapsing carcinomas ($p = 0.037$). These results fall in line with results from a study where sparser cyclin D1 expression was seen in HSIL *vs.* normal tissue [36].

The analysis of the nuclear protein ki67 being widely used as proliferation marker showed unrewarding results in our study. Because of its strong expression even in normal tissue, it is not as specific in diagnosis of differences in tumour stages [37]. Even when used as predictive marker in other studies, the results were partly inconsistent or showed a trend to better overall survival [38, 39]. In analysing several studies concerning the expression of cell cycle regulators in cervical cancer, mostly heterogenous results were revealed. Therefore, it seems unlikely that these markers alone could be of potential use as prognostic markers in routine. Only the results of cyclin D1-examination in adenocarcinomas warrants further studies because of their easy routine management and cost effectiveness.

Conclusion

Patients with early cervical cancer can be treated oncologically safe using organ preserving surgery. However, in rare cases these patients also suffer from relapse. So far, no tumour characteristic could have been found that serves as predictor for recurrence. In this study we examined several known markers for tumour proliferation to improve the biological understanding of relapsing early cervical cancer. In conclusion the most important weak spot of the present study lies in the number of analysed samples of recurrent tumours. To pinpoint significant markers for relapse among the examined cell cycle regulators and proliferation mark-

ers, greater studies with more tumour samples must be carried out. Nevertheless, this weakness in numbers is a real strength for the applied operation technique (RVT) and therefore for the patients. One could hypothesize that there is no biological pattern to be found for these patients who suffered from relapse. Perhaps recurrence in these patients is to be accountable by external factors like operation method, certain time of operation, and expertise of the surgeon.

The unrewarding results of expression patterns could also be attributed to the complexity of dysregulation in the different affected pathways. Probably, the signature of each single tumour has to be evaluated to receive further information of its potential for future recurrence.

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