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Differential expression of cyclin-dependent kinase inhibitors and apoptosis-related proteins in endocervical lesions

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ABSTRACT

The development of neoplasia is associated with abnormalities of cell cycle control and apoptosis. In this study, a panel of cyclin-dependent kinase inhibitors (CDKIs) and apoptosis-related proteins (p16, p21, p53, Bcl2 and hsp27) was analysed by immunohistochemistry in 91 glandular cervical lesions. A significant increase in p21 and p53 expression occurred from normal cervix ($n = 11$) through endometriosis/tubo-endometrioid metaplasia (TEM) ($n = 19$) and cervical glandular intraepithelial neoplasia (CGIN)/adenocarcinoma in situ (AIS) ($n = 33$) to invasive adenocarcinoma ($n = 28$). p16 showed diffuse strong expression in CGIN/AIS and invasive adenocarcinoma compared with focal expression in some TEM/endometriosis lesions and no expression in normal cervix. Bcl2 was highly expressed in TEM/endometriosis compared with CGIN/AIS and adenocarcinoma. p16 immunostaining discriminated accurately between neoplastic and non-neoplastic cervical lesions, provided that diffuse strong positivity was present. Similarly, diffuse expression of Bcl2 distinguished endometriosis/TEM from CGIN/AIS. These data demonstrate that analysis of CDKIs and apoptosis-related proteins provides useful information in the diagnostic assessment of glandular lesions of the cervix.

1. Introduction

Invasive cervical adenocarcinoma represents 15–25% of all cervical carcinomas[1] and has increased in frequency in recent years, particularly in young women.[2,3] Like squamous cell carcinoma, invasive adenocarcinoma of the cervix is associated with high-risk human papillomavirus (HPV) infection and arises from non-invasive precursors, namely cervical glandular intraepithelial neoplasia/adenocarcinoma in situ (CGIN/AIS).[4] Glandular lesions account for many problematic issues in diagnostic cervical pathology, since diverse benign glandular lesions of the endocervix raise the possibility of cervical neoplasia.[5] In particular, distinguishing tubo-endometrioid metaplasia (TEM)/endometriosis from CGIN/AIS and invasive cervical adenocarcinoma can be problematic, and there is a need for reliable markers in this situation.

Cyclin-dependent kinase inhibitors (CDKIs) play an important role in regulation of the cell cycle.[6] CDKIs compete with cyclin-dependent kinases (CDK) at their binding sites with cyclins in the G1 phase of the cell cycle. This blocks the kinase activity of CDKs and subsequently prevents phosphorylation of the Rb family of proteins and transition to S phase.[7] Inactivation of

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CDKIs is implicated in the aetiology of various malignant tumours, e.g. breast, pancreatic and bladder carcinomas.[8] Two groups of CDKIs have been identified: the Cip/Kip and Ink4 families.[9] p21 binds and inhibits CDK activity, in particular CDK2. In addition, p21 binds to the proliferating cell nuclear antigen (PCNA) to inhibit DNA replication.[10] Unlike p21, the p16 protein family specifically targets CDK4 and CDK6, preventing them from complexing with D-type cyclins.[11,12]

Bcl2 is considered to be an important protein in the development of cervical cancer. The anti-apoptotic Bcl2 protein, and the pro-apoptotic bax protein, are thought to function by forming homo- and heterodimers that then control progression to apoptosis. p53 is also involved as a down-regulator of Bcl2 and a promoter of bax.[13] Although the E6 proteins of high-risk HPVs bind to and inactivate p53,[14] it has been suggested that the immunodetection of both p53 and Bcl2 proteins in squamous cell carcinoma of the uterine cervix could be used as independent diagnostic markers for cervical cancer associated with human papillomavirus (HPV) infection.[15]

Increased levels of heat shock protein 27 (hsp27), relative to its level in nontransformed cells, have been detected in a number of cancers, such as breast cancer, endometrial cancer, and leukaemia.[16,17] In a previous study, we have shown increased levels of hsp27 expression in both metaplastic and neoplastic lesions of the endocervix.[18]

The aim of this work was to perform a detailed immunohistochemical study of normal cervix, TEM/endometriosis and neoplastic cervical glandular lesions in order to determine the potential usefulness of the expression of p16, p21, p53 and Bcl2 in differentiating these groups of lesions. The findings were also compared to our previous study of hsp27 expression.[18]

2. Materials and methods

2.1. Cervical tissues

Cases were identified from the archives of the Department of Pathology at the Royal Liverpool University Hospital between 1989 and 2001. Normal endocervical tissues from hysterectomies for various non-neoplastic conditions were employed as controls ($n = 11$). This study was performed in accordance with local ethical guidelines. Included as cases were unselected examples of (i) tubo-endometrioid metaplasia (TEM) and cervical endometriosis ($n = 19$); (ii) high-grade cervical glandular intra-epithelial neoplasia/adenocarcinoma in situ (CGIN/AIS, $n = 33$); and (iii) invasive cervical adenocarcinoma of endocervical type ($n = 28$, 14 well differentiated, 11 moderately differentiated and 3 poor differentiated carcinomas).

The haematoxylin and eosin slides were reviewed by two investigators (AAE and CSH) and the diagnoses agreed by consensus. Where more than one histological component was identified, the lesions were classified according to the highest grade present. Only the latter was then analysed for immunohistochemical expression.

2.2. Immunohistochemistry

Immunostaining was performed on 5 μm sections from paraffin wax blocks. Briefly, sections were dewaxed with xylene, rehydrated through graded ethanols followed by blocking of endogenous peroxidase activity in H_2O_2/methanol for 12 min. Antibody-binding epitopes were retrieved by pressure-cooking the tissue sections for 2.5 min in 10 mM EDTA, pH 7.0 and identified with mouse monoclonal antibodies listed in Table 1. Sections were incubated with primary antibodies for 40 min at room temperature. After washing twice with Tris-buffered saline (TBS; 50 mM Tris–HCl, 150 mM NaCl, pH 7.4), slides were incubated with antimouse immunoglobulin (Envision, Dako, UK) for 30 min. Sections were immersed in diaminobenzidine (DAB) for 10 min. All incubations were performed at room temperature. Washes with TBS were performed between each step. Antibodies were diluted in Tris buffered saline containing 5% (w/v) bovine serum albumin. Nuclei were counterstained with Meyers haemalum before mounting the slides in DPX.

Table 1 – Primary antibodies used for immunostaining

Antibody	Clone	Positive control	Dilution
p16	E6H4	Cervical adenocarcinoma of known positivity	1:100
p21	4D10	Ultraviolet irradiated skin	1:100
p53	DO-7	Colonic cancer	1:100
Hsp27	2B4	Breast carcinoma	1:20
Bcl2	124	Lymphocytes from tonsil	1:20

Negative controls in which the primary antibody was omitted and positive controls (Table 1) for each antibody were included in each batch of immunohistochemistry.

2.3. Assessment of immunostaining

Only the glandular epithelial component of the cervix was analysed in all tissues. For each case, the number of positively stained epithelial cells was estimated visually by scanning the entire tissue at low power using conventional light microscopy. The percentage of positively stained cells was counted in each focus and then averaged to give a mean percentage per case. The staining intensity was recorded as follows: [0 = no staining, 1 = weak, 2 = moderate and 3 = strong].[19,20] Analysis of immunostaining was performed by two investigators (AAE and AMS).

2.4. Statistical analysis

Data were analysed by the non-parametric, two-sided Mann–Whitney test and Spearman's rank correlation coefficient, using the Statistical Package for Social Sciences (SPSS® package, version 10). For all calculations, the median values and the inter-quartile ranges (IQ) of marker expression were used. P values of $\leqslant 0.05$ defined statistical significance. Receiver operator characteristic (ROC) curve was drawn to identify the best marker, among a group of markers, for differentiating benign (normal endocervical glands and TEM/endometriosis) and neoplastic (CGIN/AIS and adenocarcinoma) lesions.

3. Results

Patients' ages ranged from 24 to 92 years with a mean of 43(±12.53) years. The mean age of patients with a normal

cervix was 42.6 ± 7.9, with TEM/endometriosis it was 39 ± 6.4, with CGIN/AIS it was 41.3 ± 10.1 and with invasive adenocarcinoma it was 47.9 ± 17.6. Spearman rank correlation coefficient showed no correlation between patient age and CDKI or apoptosis-related protein expression in the four different groups.

3.1. *Cyclin-dependent kinase inhibitors (CDKIs)*

Staining was mainly nuclear for both p16 and p21; however, cytoplasmic staining for p16 was observed in some cases of CGIN/AIS and invasive adenocarcinoma.

3.1.1. *Control normal epithelium, TEM and endometriosis*

There was no p16 expression in the majority (7 of 11) of normal cervices (median 0%, IQ: 0–1%). Where p16 expression was present in normal endocervical glands, it was confined to scattered columnar epithelial cells, with at most 15% positive cells (Table 2). Foci of TEM/endometriosis expressed moderate levels of this marker within epithelial cells (median 35%, IQ: 8–45%). Most of these lesions showed moderate staining intensity in a focal rather than diffuse pattern. No staining for p21 was seen in normal cervical glands and TEM/endometriosis (median 0%, IQ: 0–0%, median 0%, IQ: 0–3%, respectively) (Table 2).

3.1.2. *CGIN/AIS and invasive cervical adenocarcinoma*

Expression of p16 was seen in all cases of CGIN/AIS and invasive adenocarcinoma. The median expression of p16 in CGIN/AIS was 95% (IQ: 75–100%) (Fig. 1a). No further increase in expression was noted in invasive adenocarcinoma (median 90%, IQ: 80–95%) (Fig. 1b). Diffuse moderate and strong expression was seen in lesions of both categories; occasional lesions showed only focal expression. Minimal staining for p21 was identified in CGIN/AIS (median 1.5%, IQ: 0–5%) (Fig. 1c) but an increase in the level of p21 positivity was observed in adenocarcinoma (median 9%, IQ: 2.25–15%) (Table 2, Fig. 2a). In invasive adenocarcinoma, p16 and p21 showed no correlation with tumour grade, lymph node involvement or lymphovascular space invasion.

Using a cutoff value of 50% for p16 expression, the neoplastic lesions (CGIN/AIS and adenocarcinoma) could be discriminated accurately from the benign glandular epithelia (normal and TEM/endometriosis) at a sensitivity of 90% and specificity of 87%.

3.2. *Apoptosis-related proteins*

The expression of both hsp27 and Bcl2 was predominantly cytoplasmic with occasional membranous staining, particularly with hsp27. However, p53 staining was mainly nuclear. The data for hsp27 expression have been reported previously[18] but are included here for comparison.

3.2.1. *Normal epithelium, TEM and endometriosis*

Minimal expression of hsp27 was identified in normal cervical glands (median 10%, IQ: 5–15%). The majority of normal cervices were unstained for p53 (median 0%, IQ: 0–0%) and Bcl2 (median 0%, IQ: 0–0%), respectively.

Foci of TEM/endometriosis expressed moderate levels of hsp27 within epithelial cells (median 35%, IQ: 15–80%). Although some cases (5 of 19) exhibited nuclear staining for p53, the overall median expression in this group was zero (IQ: 0–1). Moderate to strong staining for Bcl2 was seen in 11 of the 19 cases (median 45%, IQ: 0–70%) (Table 2, Fig. 1d).

Table 2 – Summary of CDKI and apoptosis-related protein expression

Histology	p16		p21		p53		Bcl2		hsp27[m]	
	Median, % (IQ)	Range (%)	Median, % (IQ)	Range (%)	Median, % (IQ)	Range (%)	Median, % (IQ)	Range (%)	Median, % (IQ)	Range (%)
Normal cervix (n = 11)	0 (0–1)	0–15	0 (0–0)	0–0	0 (0–0)	0–1	0 (0–0)	0–0	10 (5–15)	0–60
TEM/endometriosis (n = 19)	35 (8–45)[a]	0–90	0 (0–3)	0–15	0 (0–1)	0–35	45 (0–70)[g]	0–100	35 (15–80)[j]	1–100
CGIN/AIS (n = 33)	95 (75–100)[b]	5–100	1.5 (0–5)[d]	0–20	0 (0–3)	0–70	0 (0–1)[h]	0–60	60 (32–80)[k]	2–90
Adenocarcinoma (n = 28)	90 (80–95)[c]	25–100	9 (2.25–15)[e]	0–70	0.75 (0–7.25)[f]	0–95	0 (0–5)[i]	0–90	40 (25–80)[l]	1–95

TEM, tubo-endometrioid metaplasia; IQ, InterQuartile range; CGIN, cervical glandular intraepithelial neoplasia; AIS, adenocarcinoma in situ.
a Higher than normal cervix ($P < 0.001$).
b Higher than TEM/End. ($P < 0.001$), higher than normal cervix ($P < 0.001$).
c Higher than TEM/End. ($P < 0.001$), higher than normal cervix ($P < 0.001$).
d Higher than normal cervix ($P = 0.003$).
e Higher than CGIN ($P < 0.001$), higher than TEM/End. ($P < 0.001$), higher than normal cervix ($P < 0.001$).
f Higher than TEM/end. ($P = 0.037$), higher than normal cervix ($P = 0.005$).
g Higher than normal cervix ($P = 0.004$).
h Higher than normal cervix ($P = 0.038$), lower than TEM/end. ($P = 0.004$).
i Higher than normal cervix ($P = 0.037$), lower than TEM/end. ($P = 0.008$).
j Higher than normal cervix ($P = 0.007$).
k Higher than normal cervix ($P < 0.001$).
l Higher than normal cervix ($P = 0.001$).
m Date from previous study.[18]

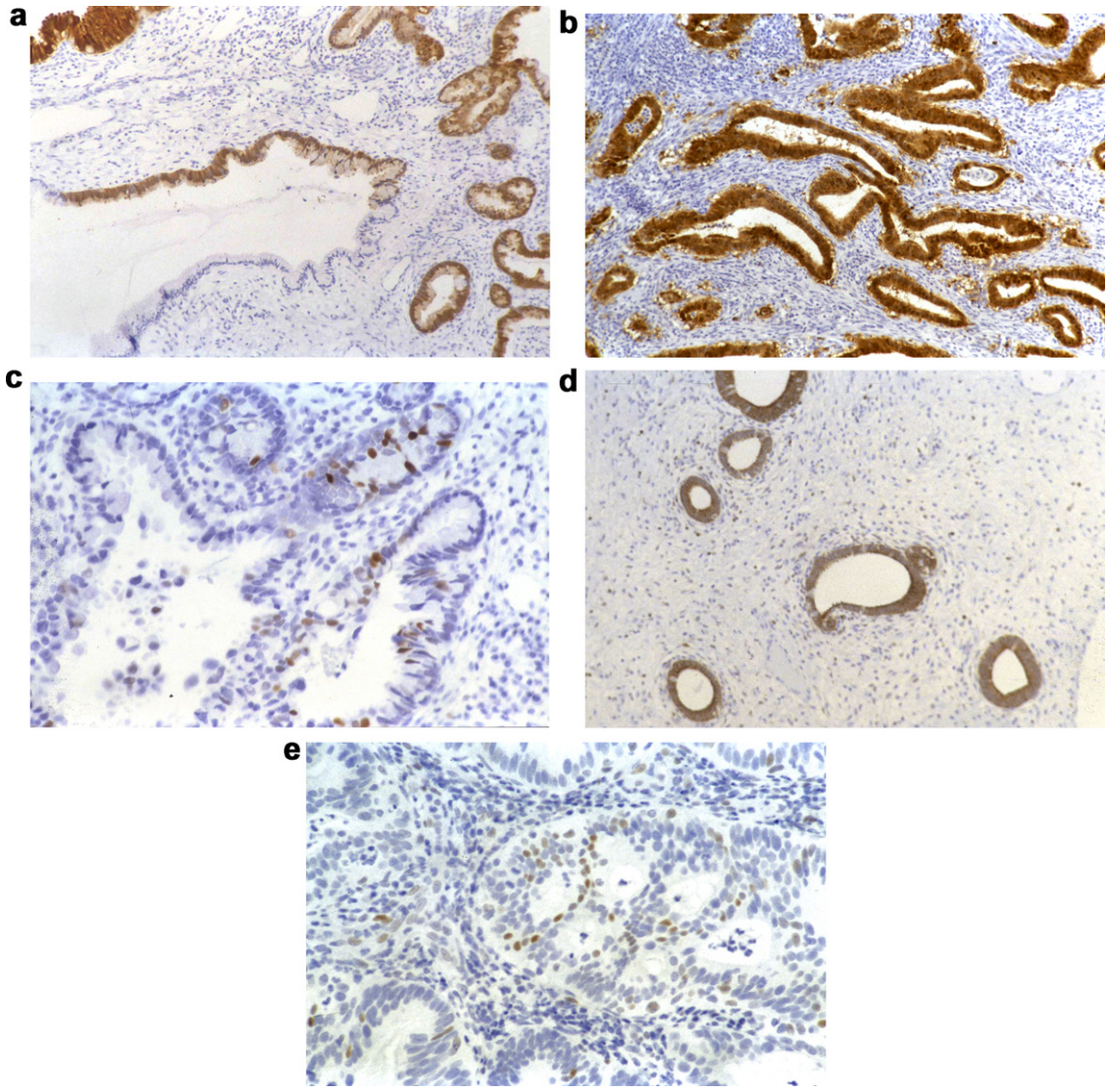

Fig. 1 – Immunohistochemical staining in cervical glandular lesions. (a) CGIN/AIS stained for p16. There is abrupt transition between neoplastic cells (strongly expressing the protein) and normal (negative) cells (×100). (b) p16 expression in well-differentiated invasive cervical adenocarcinoma. There is strong expression in almost all malignant cells (×100). (c) p21 expression in CGIN/AIS showing scattered positive nuclear staining (×250). (d) A focus of endometriosis showing strong Bcl2 expression in the majority of epithelial cells (×100). (e) Invasive cervical adenocarcinoma showing nuclear expression of p53 in a minority of malignant cells (×250).

3.2.2. *CGIN/AIS and invasive cervical adenocarcinoma*

In cases of CGIN/AIS, moderate to strong staining for hsp27 was detected in 29 of the 33 cases. The median expression of hsp27 was 60% (IQ: 32–80%). Positive staining for p53, of weak to moderate intensity, was present in 15 of the 33 cases. The median expression of p53 was 0% (IQ: 0–3%). Bcl2 was not detected in the majority of CGIN/AIS cases; however, weak staining was found in 7 patients (median 0, IQ: 0–1%) (Fig. 2b).

All cases of invasive adenocarcinoma exhibited positive staining for hsp27 (median 40%, IQ: 25–80%) (Table 2). Focal staining for p53 was present in 18 of the 28 cases (median 0.75%, IQ: 0–7.25%). Weak intensity for p53 was observed in 12 of the 18 positive cases (42.9%, Fig. 1e); however, 3 cases (10.7%) exhibited strong staining intensity. Bcl2 stained occasional malignant glandular cells in 13 of the 28 cases of invasive adenocarcinoma (median 0; IQ: 0–5%). No significant differences in apoptosis-related protein expression were found between different grades of adenocarcinoma.

In adenocarcinoma, hsp27, p53 and Bcl2 positivity showed no correlation with tumour grade, lymph node involvement or lymphovascular space invasion.

3.3. *Relationship between marker expression and histological diagnosis*

No correlation was found between p16 and p21 expression in non-neoplastic lesions (normal cervix and TEM/Endometriosis). A positive correlation was found between both markers in CGIN/AIS ($r = 0.424$, $P = 0.016$). This positive correlation was not maintained in adenocarcinoma ($r = 0.153$, $P = 0.447$).

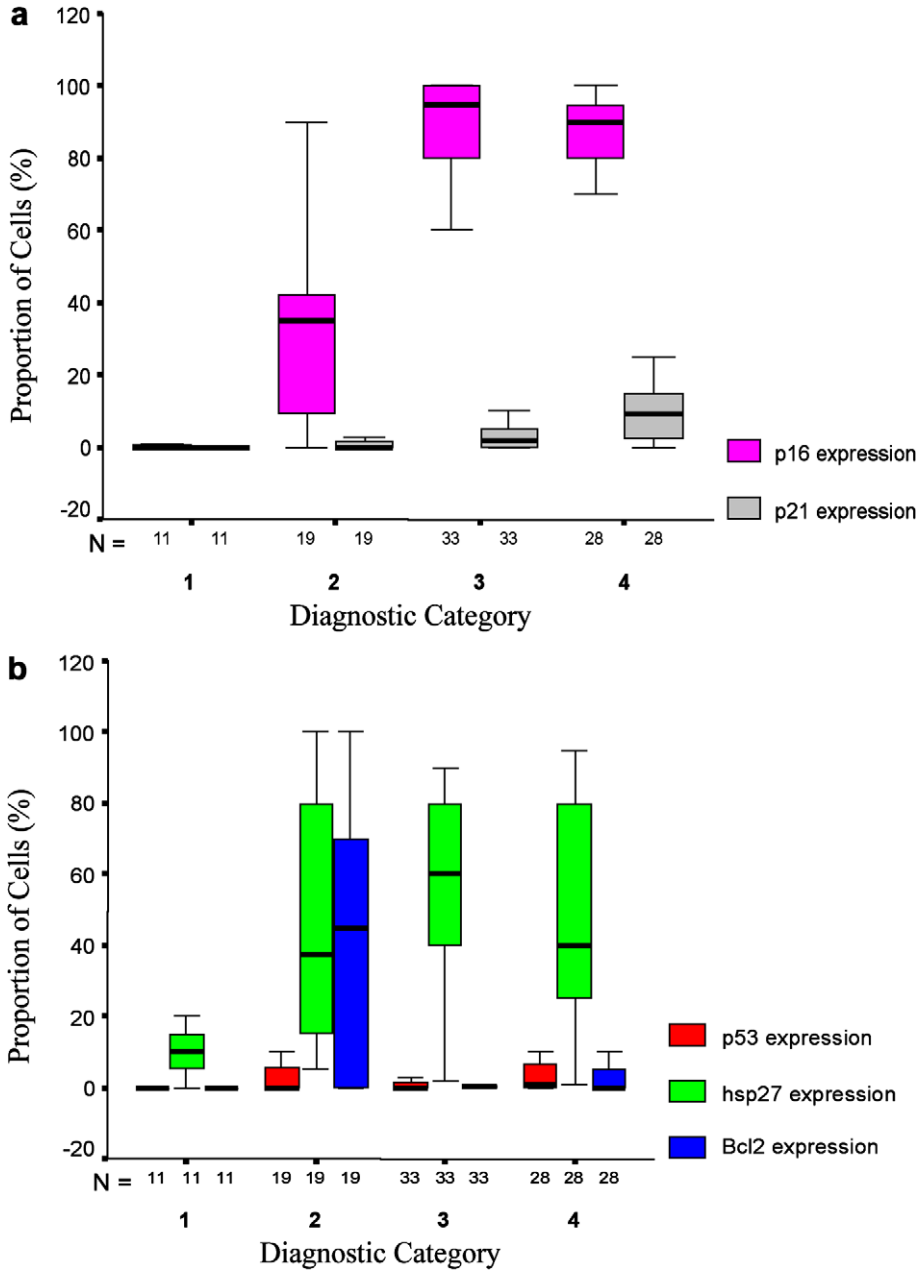

Fig. 2 – Boxplot graphs plotted for p16 and p21 (a) and p53, hsp27 and Bcl2 (b) showing percentage expression in normal cervix (1), TEM/endometriosis (2), CGIN/AIS (3) and invasive cervical adenocarcinoma (4). The boxes contain the values between the 25th and 75th percentiles, the lines across the boxes represent the medians, the whiskers extend to the highest and lowest values excluding outliers, open circles and asterisks identify outliers and extreme values.

No correlation was found between hsp27, p53 and Bcl2 expression in the four studied groups.

Overall, no significant correlation was found between all markers in normal endocervical glands and TEM/endometriosis. A trend towards positive correlation was only noted between p53 and p21 ($r = 0.353$, $P = 0.052$) in cases of CGIN/AIS.

The ability of CDKIs and apoptosis-related proteins to correctly separate neoplastic from benign lesions was tested using ROC curves. This revealed that p16 followed by p21 and hsp27 could significantly discriminate between both groups. The area under the curve was 0.951 for p16, 0.789 for p21 and 0.649 for hsp27. Conversely, p53 and Bcl2 could not significantly classify these two groups (area under the curve 0.628, $P = 0.065$ and 0.438, $P = 0.372$, respectively) (Table 3, Fig. 3).

4. Discussion

The hypothesis of this study was that the use of cell cycle and apoptosis markers might aid in the distinction between neoplastic and non-neoplastic glandular lesions of the cervix.

In the present study, p16 was generally absent in normal cervical glands but was markedly overexpressed in both CGIN/AIS and invasive adenocarcinoma. Moreover, p16

Table 3 – The significance of different markers in detecting neoplastic cervical glandular lesions

Test result variable(s)	Area under the curve				
	Area under curve	Standard error[a]	Asymptotic significance[b]	Asymptotic 95% confidence interval	
				Lower bound	Upper bound
p16 expression	0.951	0.021	0.000	0.910	0.993
p21 expression	0.789	0.049	0.000	0.693	0.885
hsp27 expression	0.649	0.072	0.032	0.508	0.790
p53 expression	0.628	0.066	0.065	0.498	0.758
Bcl2 expression	0.438	0.078	0.372	0.285	0.590

a Under the nonparametric assumption.
b Null hypothesis true, area = 0.5.

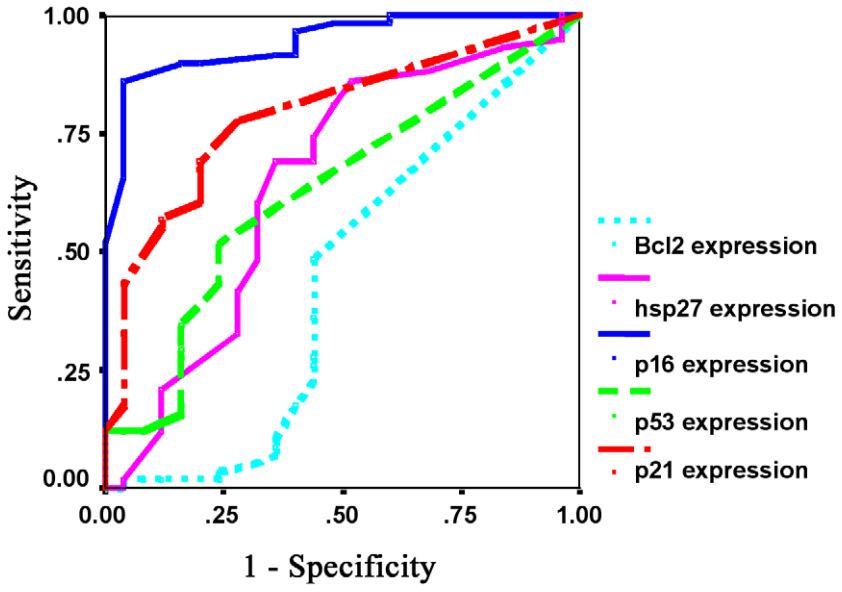

Fig. 3 – Receiver operating characteristic (ROC) curves of different markers for the distinction between non-neoplastic and neoplastic endocervical lesions.

immunostaining distinguished these neoplastic lesions from TEM/endometriosis if diffuse strong expression was present. In HPV-associated neoplasia, the mechanism of overexpression of p16 is thought to relate to abrogation of pRb function by the high-risk HPV E7 protein,[21] and p16 expression has been shown to be diagnostically useful in both squamous[22] and glandular[23] lesions of the cervix. However, the *p*16 gene can be silenced by, for example, methylation in both intraepithelial and invasive cervical neoplasia[24,25] and hence the absence of widespread p16 immunopositivity does not exclude the possibility of a neoplastic cervical lesion. This is consistent with our findings (see Table 2).

The presence of p16 expression in some normal endocervical glands is consistent with other studies.[26] This observation has implications for the diagnostic utility of p16 immunostaining but it is of note that the pattern of expression in these normal glands is different from that in neoplastic glandular lesions: in the former only scattered cells are positive whereas, in the latter, p16 expression is widespread. Indeed, if a cut-off value of 50% was used for p16 expression, neoplastic lesions (CGIN/AIS and adenocarcinoma) could be discriminated in our study from benign glandular epithelia (normal glands and TEM/endometriosis) with a sensitivity of 90% and specificity of 87%. It is possible that larger studies, employing image analysis-based assessment of p16 immunostaining, could improve this performance still further. Nevertheless, the presence of p16 expression should be interpreted with caution, taking these considerations into account, and viewed in conjunction with the appearances in routine H&E-stained sections. The mechanism of p16 overexpression in normal glands is not known but p16 expression has been reported in the benign lesion lobular endocervical glandular hyperplasia in the absence of HPV infection, suggesting that it may be overexpressed in this context by an HPV-independent mechanism.[27]

p16 may also play a role in the progression of cervical neoplasia. In one study in which the time interval for disease progression from initial biopsy to CIN3 or invasive cancer was compared with p16 expression, lesions that stained positive for p16 progressed within 64.2 months as compared with 122.3 months for those that did not ($P < 0.01$). These results suggest that p16 may be a valuable marker for prediction of the progression of cervical neoplasia.[28]

Unlike p16, p21 targets a wide variety of CDK/cyclin complexes. It was initially thought that elevated levels of p21 protein in response to extracellular signals mediated cell-cycle arrest, predominantly at the G1 phase of the cell cycle. However, several laboratories have demonstrated that p21 protein does not inhibit cyclin D-CDK; on the contrary, it facilitates the assembly[29] and proper nuclear translocation of the complex.[30,31] Regarding p21 protein, the existing data fail to reach consensus. As cited in an extensive review by Tsihlias and colleagues,[32] the various methodologies used in staining and scoring often make it difficult to compare data from different laboratories. Nevertheless, when p21 expression was evaluated, the present study showed a difference in its expression in CGIN and adenocarcinoma, indicating that increased expression of this protein accompanies neoplastic progression in some lesions. In keeping with our results, p21 and p16 expression were found to be increased in cervical squamous cell carcinoma, with high expression being observed in 20% (44/221) and 43% (94/220) of tumours, respectively.[33]

The current study showed an increased level of Bcl2 in tubo-endometrioid metaplasia and cervical endometriosis. Previous studies have interestingly shown that normal Fallopian tube epithelium and proliferative endometrial glands express Bcl2.[34,35] Our data support the use of diffuse Bcl2 expression as a diagnostic marker in the distinction between TEM/endometriosis and CGIN/AIS, particularly when used in combination with p16: this is consistent with the conclusions reached by Cameron and colleagues.[36]

Although mutations of the *p53* gene are the most common genetic alteration in human tumours, they are relatively rare in cervical carcinomas, largely because oncogenic HPVs code for an oncoprotein that leads to the ubiquitin-mediated destruction of p53.[37,38] In this work, increased expression of p53 was found in a minority of CGIN/AIS and invasive adenocarcinomas. When compared with CGIN, adenocarcinoma did not show significant differences in p53 expression. This is in agreement with another study, which reported the same findings in squamous cervical lesions.[39]

As previously reported,[18] hsp27 expression was increased in both TEM/endometriosis and neoplastic cervical glandular lesions, when compared with normal cervical glands. However, no significant differences were found in the levels of expression between CGIN/AIS and invasive adenocarcinoma.

ROC curve analysis (Fig. 3) suggests that p16, p21, and hsp27 analyses are diagnostically useful in the distinction between neoplastic and non-neoplastic lesions. Further scrutiny of Fig. 2 confirms this for p16 and demonstrates that diffuse positivity is most discriminatory. However, Fig. 2 also demonstrates that, although p21 immunostaining is statistically capable of discriminating between these lesions, the extent of p21 positivity is insufficient for diagnostic use. Similarly, the extent of expression of hsp27 by TEM/endometriosis limits its diagnostic application in this context.

In conclusion, the current study demonstrates diagnostically useful dysregulation of cell-cycle regulatory, and apoptosis-related, proteins in glandular lesions of the cervix uteri. In particular, p16 immunostaining discriminated accurately between neoplastic and non-neoplastic cervical lesions, provided that diffuse strong positivity was present. Similarly, diffuse expression of Bcl2 distinguished endometriosis/TEM from CGIN/AIS. Further larger-scale prospective studies are needed to explore the clinical significance of these findings and the possible prediction of high-risk patients who could benefit from close follow-up and early treatment.

Conflict of interest statement

None declared.

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