Expression of tight and adherens junction proteins in cervical neoplasia

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ABSTRACT

Adherens junctions (AJ) and tight junctions (TJ) play a key role in maintaining the apical-basolateral polarity and cohesive structure of epithelial cells. These epithelial junctions are maintained by the interaction of several key proteins including E-cadherin, claudins, occludin and zona-occludens. The zinc finger protein, Snail, has previously been identified as a possible regulator of several of these AJ and TJ proteins. Expression levels of ZO-1, occludin, claudins, E-cadherin, human Scribble protein and Snail were determined in HeLa and CaSki cervical carcinoma cell lines by imuunohistochemistry (IHC) and Western blotting. In tandem, tissue microarrays were utilised in the IHC-based detection of E-cadherin in 26 cases of non-cancerous cervical tissue, 15 cases of cervical intraepithelial neoplasia 1 (CIN1), 11 cases of CIN2, 12 cases of CIN3, 50 cases of squamous cell carcinoma and two cases of adenocarcinoma. This study found aberrant E-cadherin expression in CIN lesions and cases of squamous cell carcinoma compared to normal epithelium. HeLa cells were E-cadherin-negative while CaSki cells were positive, with HeLa cells showing a high level of Snail expression. Occludin and ZO-1 expression was detected in both cell lines. No expression was observed for claudin 1 or claudin 5 tight junction proteins in HeLa cells or CaSki cells. Loss of expression of claudin 1, claudin 5 and E-cadherin, with concomitant increase in Snail may be associated with loss of epithelial cell polarity and alterations in intercellular adhesion.

KEY WORDS: Adherens junctions.
Cadherins.
Tight junctions.
Uterine cervical neoplasms.

Introduction

Loss of polarity and disruption of cell junctions are common features of cancer cells, and such defects play a role in the pathology of cancer.[1] Cell-to-cell adhesiveness may be reduced in cancers, allowing tumour cells to disseminate, invade neighbouring structures and form metastases.[2] Changes in expression of cytokeratins, epithelial cell adhesion molecules and several other adhesion molecules, including cadherins, have been observed in cervical intraepithelial neoplasia (CIN) lesions and in invasive squamous cell carcinomas.[3]

Epithelial cells demonstrate an organised polarity and the Scribble protein is a regulator of cell shape and polarity, functioning as a scaffold protein using protein-binding motifs.[4] A single mutation in Scribble can induce loss of apical-basal polarity and massive hyperproliferation, demonstrating a role for Scribble in the regulation of polarity and cell proliferation.[5] Scribble deregulation has previously been observed in prostate cancer.[6] In addition, Scribble is targeted for ubiquitin-mediated degradation by high-risk human papillomavirus (HPV) E6 proteins, suggesting that Scribble degradation contributes to the development of HPV-induced cervical carcinoma.[5]

Occludin is an integral membrane protein closely associated with the tight junctions of epithelial and endothelial cells.[7] A decrease in occludin expression is observed in CIN as well as in *in situ* carcinoma. The tight junction protein ZO-1 has been shown to interact with adherens junction proteins.[8] The association of ZO-1 with E-cadherin and Scribble is altered in HPV infection and this leads to loss of epithelial cell polarity. Such defects may contribute to the disease process by deregulating normal proliferation, and thus contribute to immune evasion.

Claudins play a role in the structural establishment of tight junctions, with a role in determining epithelial cell polarity and intercellular permeability.[9] The claudin family consists of at least 24 members, with distribution varying greatly depending on the type of tissue. Claudins consist of four transmembrane domains, two extracellular loops and a short carboxyl intracellular tail.[3] Abnormal claudin expression has been found in breast[10] and ovarian cancer.[11]

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Epithelial cadherin (E-cadherin) is one of the main components of adherens junctions and plays a key role in the maintenance and stability of epithelial cohesion. Several studies have shown that loss of E-cadherin correlates with tumour invasiveness.[12] Studies have also found some epithelial tumour cell lines, in addition to losing E-cadherin expression, have upregulated neural cadherin (N-cadherin), suggesting a change from E-cadherin to N-cadherin may be important in the pathogenesis of epithelial malignancies.[13]

Snail, a zinc fingered transcriptional repressor protein, is also an important regulator of AJ and TJ stability. Active Snail protein binds to e-box motifs in the E-cadherin and claudin-1 promoters, preventing their transcription, thus regulating AJ and TJ stability and epithelial adhesion.[14]

This study aims to explore the expression of TJ and AJ proteins in two cervical cancer cell lines and in tissue microarrays containing non-cancerous tissues, CIN and cervical cancer.

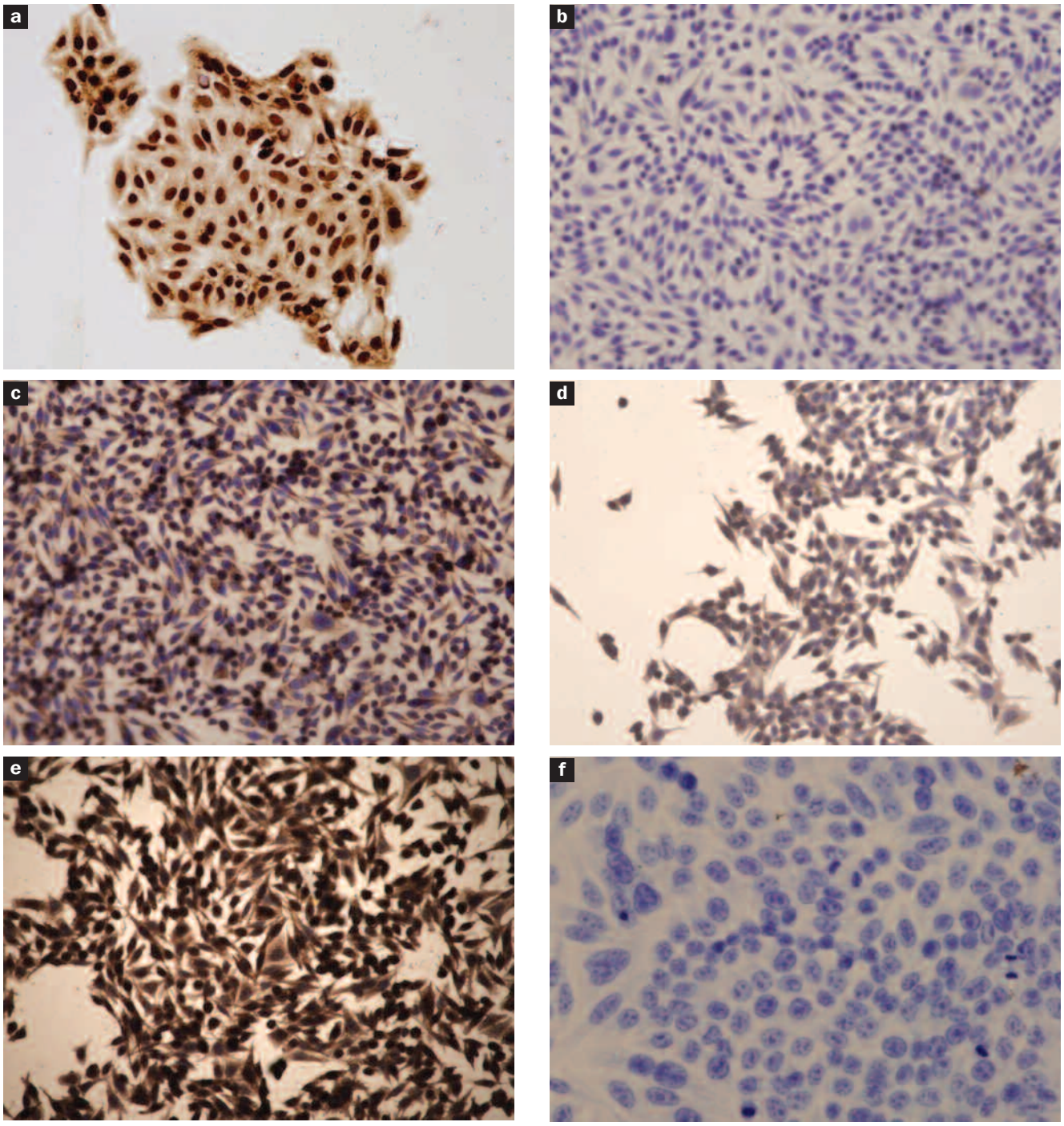

Fig. 1. Expression in HeLa cells: **a)** Snail; **b)** claudin-5; **c)** N-cadherin; **d)** Scribble; **e)** ZO-1; and **f)** negative control.

Materials and methods

Coverslips were sterilised by submerging them in 100% ethanol and drying them in a flame for 5 sec, then placed in a sterile six-well plate. Both chamber slides and coverslips were then seeded with either HeLa or CaSki cells diluted in RPMI-1640 medium with 10% fetal bovine serum. Coverslips and chamber slides were then incubated at 37˚C until the cells reached 60–70% confluence. Samples were then fixed using a 50% methanol/50% acetone solution which was applied to each section for 15 min. Hydrogen peroxide (3%) in methanol was then applied to the sections for 5 min.

The sections were washed three times in PBS for 5 min before being stained using the Vectastain Elite ABC kit (Vector Laboratories). Normal horse serum was applied to sections for 5 min, before application of the primary antibody for 1 h at room temperature. Antibodies used were E-cadherin (1 in 50; Dako), ZO-1 (1 in 100; Invitrogen), claudin-1 (1 in 200; Invitrogen), claudin-5 (1 in 100; Invitrogen), occludin (1 in 200; Invitrogen), N-cadherin (1 in 125; Epitomics), Snail (1 in 500; Abcam) and Scribble (1 in 50; Abcam).

A biotinylated secondary antibody was then applied for 15 min, followed by three washes in PBS for 5 min. The avidin-

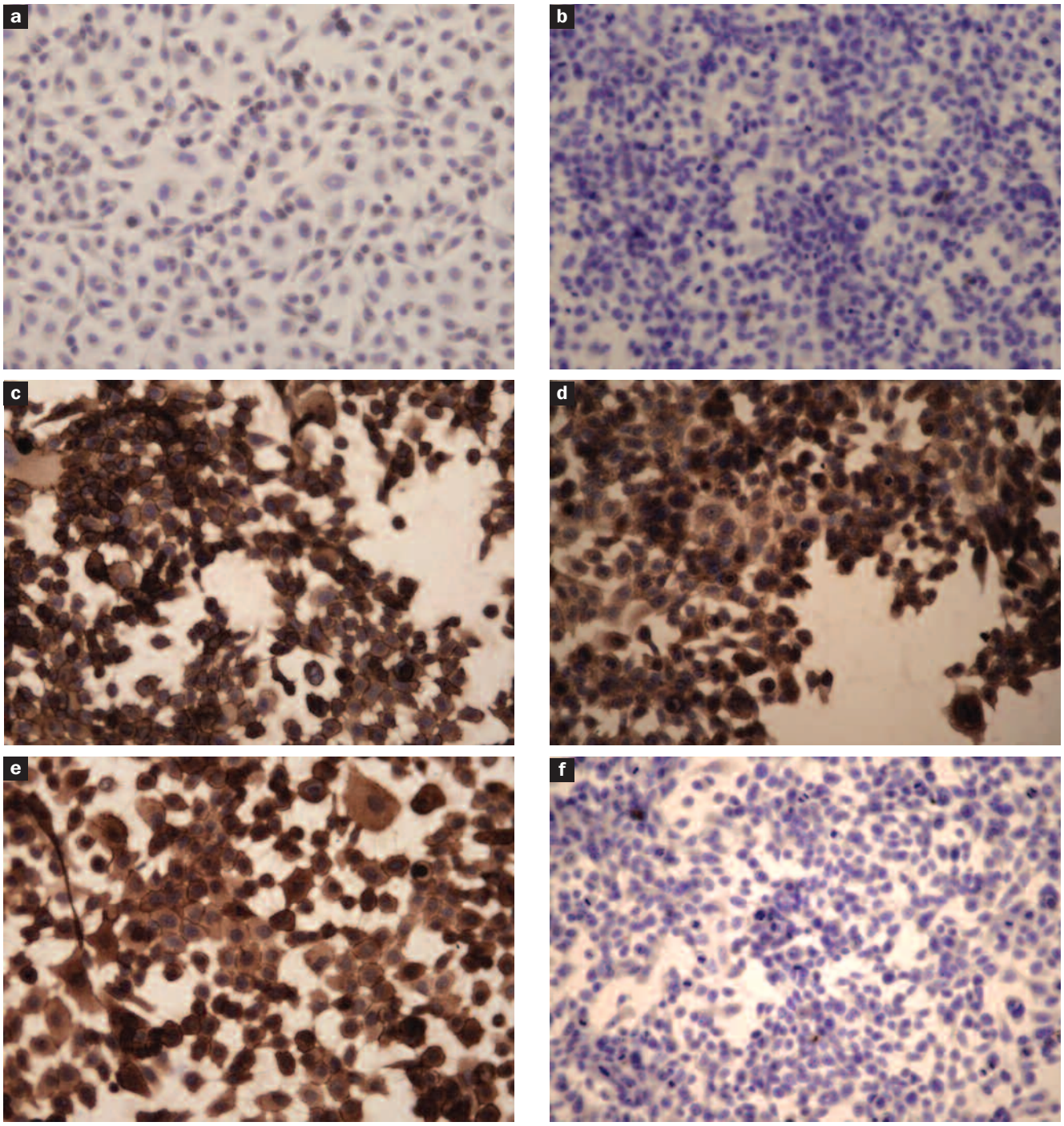

Fig. 2. Expression in CaSki cells: **a)** claudin-1; **b)** claudin-5; **c)** N-cadherin; **d)** ZO-1; **e)** Scribble; and **f)** negative control.

biotin enzyme complex (ABC) was applied to sections for 15 min before the sections were washed again in PBS for 5 min. Peroxidase labelling was visualised using 0.02% diaminobenzidine (DAB) and 0.03% hydrogen peroxide applied to sections for 8 min. Sections were washed in PBS for 5 min and then counterstained in Mayer's haematoxylin. Finally, samples were dehydrated in graded alcohol, cleared in xylene and mounted in DPX.

Tissue microarrays (TMAs) were acquired from Biomax US. Samples were collected with full informed donor consent and according to IRB- and HIPPA-approved protocols. Additionally, samples were assessed and their disease status graded by pathologists working for Biomax US.

Samples examined for E-cadherin expression included 16 cases of non-neoplastic cervical epithelium, 10 cases of CIN1, 10 cases of CIN2, 13 cases of CIN3, 57 cases of squamous cell carcinoma and two cases of adenocarcinoma. Sections were dewaxed in xylene and rehydrated through graded alcohol before IHC using the Vectastain Elite ABC kit. Normal cervical epithelium treated with PBS instead of primary antibody was used as a negative control. Sections were graded according to the level of positive staining, the range being 3+ (intense staining), 2+ (moderate staining), 1+

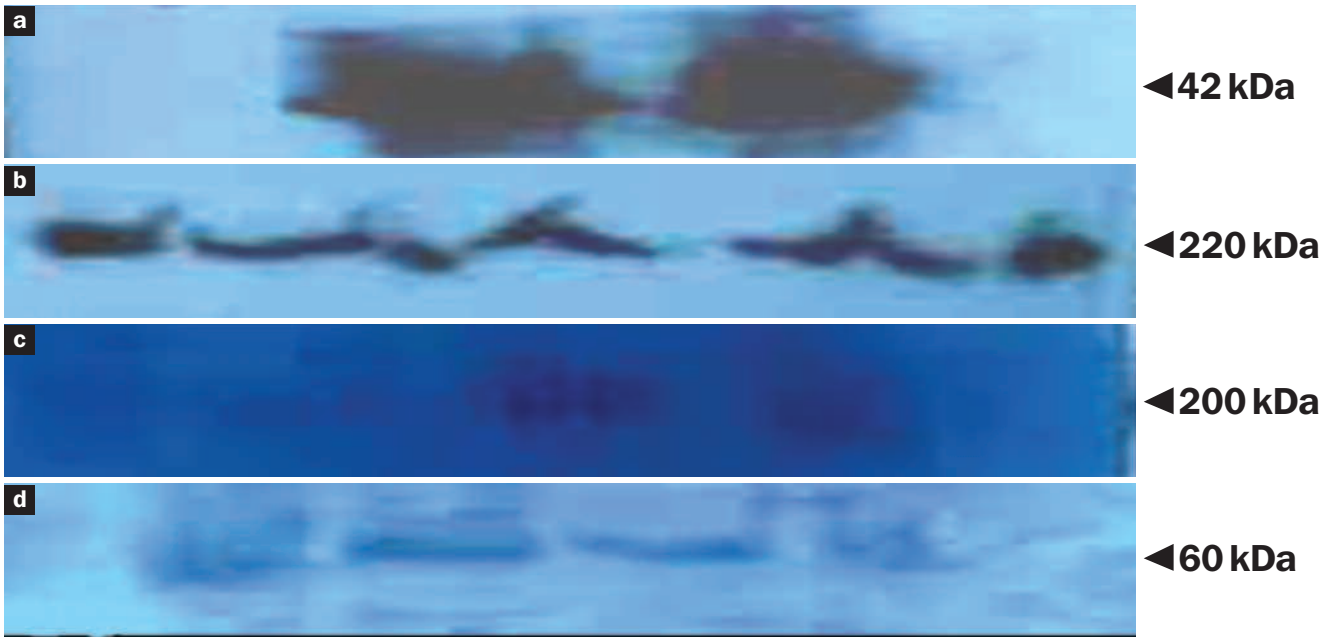

Fig. 3. Western blot detection in HeLa cells: **a)** β-actin; **b)** ZO-1; **c)** Scribble; and **d)** occludin.

(weak staining) and 0 (no staining). Additionally, a record was made of whether the positive staining was membranous or cytoplasmic, or both. Absence of staining (grade 0) or a non-exclusive membranous staining pattern was deemed to show aberrant expression.

Western blot analysis of tight junction expression of HeLa and CaSki cells was performed as described by Campbell *et al*.[8] Dilutions of primary antibodies were as follows: anti-occludin (1 in 200), anti-Scribble (1 in 200), anti-ZO-1 (1 in 200) and anti-β-actin (1 in 100).

Results

This study assessed the expression profile of cell adhesion and polarity proteins in two cervical carcinoma cell lines (i.e., HeLa and CaSki). Initially, it examined the expression of E-cadherin, Snail, claudin-1, claudin-5, N-cadherin, Scribble and ZO-1 in HeLa and CaSki cells using IHC. The results demonstrate high expression of Snail, N-cadherin, Scribble and ZO-1 in HeLa cells (Figs. 1a, 1c, 1d and 1e), but no expression of claudin-5 (Fig. 1b). Scribble appears mislocalised and present in the cytosol, in contrast to the membrane localisation of Scribble associated with normal epithelial cells. CaSki cells showed high expression of N-cadherin, ZO-1 and Scribble (Figs. 2c, 2d and 2e), with no expression of claudin-1 or claudin-5 (Figs. 2a and 2b).

Western blot analysis of HeLa extracts showed a positive band at 60 kDa (Fig. 3d), corresponding to occludin protein, and a band at 200 kDa (Fig. 3c), corresponding to Scribble protein. HeLa cells were found to express ZO-1 tight junction protein, as a band corresponding to a molecular weight of 220 kDa was detected (Fig. 3b).

Analysis of TMAs comprising 16 cases of non-neoplastic cervical epithelium, 10 cases of CIN1, 10 cases of CIN2, 13 cases of CIN3, 57 cases of squamous cell carcinoma and two cases of adenocarcinoma demonstrated aberrant E-cadherin expression in CIN lesions and cases of squamous cell carcinoma compared to normal epithelium. This aberrant expression had two distinct patterns. The first was found

Table 1. E-cadherin staining of cervical TMAs, detailing mean staining intensity and percentage of samples with aberrant expression for each disease category.

Disease status	Number of samples	Mean staining intensity	Samples with aberrant expression (%)
Normal	16	1.4	0
CIN1	10	2.0	70
CIN2	10	2.3	50
CIN3	13	1.77	92
Carcinoma	59	1.00	89

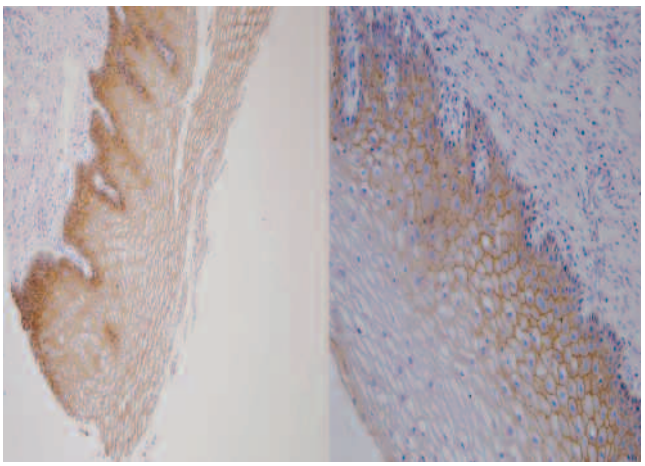

Fig. 4. Normal cervical tissue from TMAs stained for E-cadherin demonstrating membranous staining of cells in the cervical epithelium.

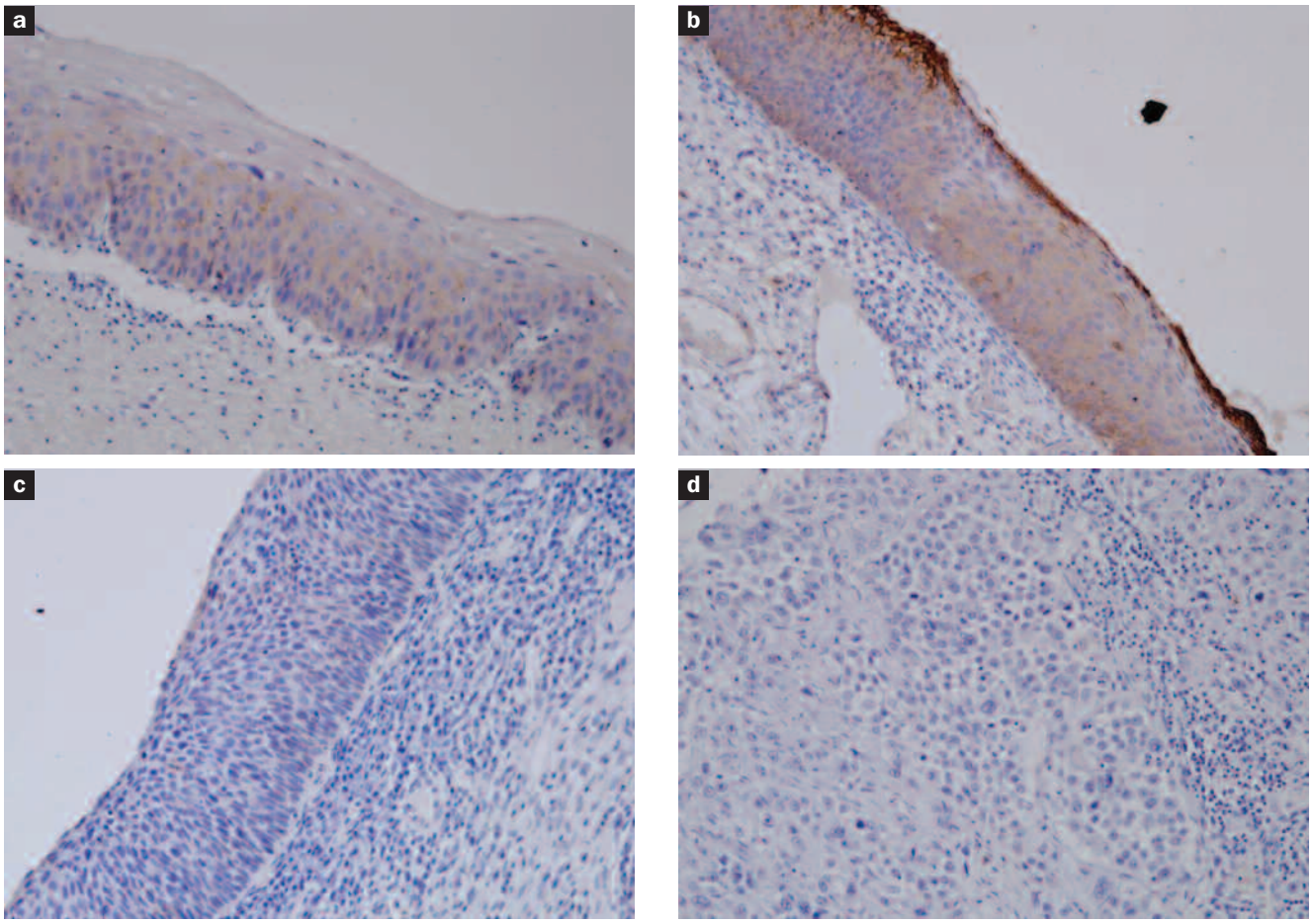

Fig. 5. Reduced expression of E-cadherin compared to non-neoplastic epithelium: **a)** CIN1; **b)** CIN2; **c)** CIN3; and **d)** squamous cell carcinoma.

predominantly in cases of squamous cell carcinoma and involved the reduction or absence of E-cadherin expression (Fig. 5). The second pattern was observed predominantly in CIN lesions, and involved over-expression of E-cadherin, but with cytoplasmic positivity (Fig. 6) as opposed to the strict membranous pattern observed in normal epithelium (Fig. 4). On average, CIN lesions showed the highest levels of E-cadherin expression, but in the vast majority of cases this expression was mislocalised to the cytosol. Cases of squamous cell carcinoma showed proportionally the lowest expression of E-cadherin; lower than CIN lesions or normal cervical epithelium (Table 1).

Discussion

Cell-to-cell and cell-to-extracellular matrix interactions are important in tumour progression, and development of some cancers may be associated with epithelial cells unable to form tight junctions, leading to loss of apical-basolateral polarity, with adherens junctions and tight junctions playing a key role in maintaining the apical-basolateral polarity and cohesive structure of epithelial cells.[15] Adherens and tight junctions are maintained by the interaction of several key proteins including E-cadherin, occludin, zona-occludens and claudins.[16]

The present study examined tight junction expression in HeLa cells and CaSki cells. The HeLa cell line is isolated from a human uterine tumour and contains an integrated human papillomavirus (HPV) DNA type 18. This study found expression of occludin, ZO-1, Scribble and E-cadherin in HeLa cells, with Scribble mislocalised and present in the cytosol, in contrast to the membrane association of Scribble seen in normal epithelial cells. Vaira *et al*.[17] recently described aberrant localisation of Scribble in several tumour cell lines.

This study found no expression of claudin-1 and claudin-7 tight junction proteins in HeLa cells; a finding consistent with that of Daugherty *et al*.,[18] who also showed that HeLa cells do not express claudins. Loss of expression of claudins 1 and 5 may be associated with loss of epithelial cell polarity and alterations in intercellular adhesion. The lack of expression of AJ and TJ proteins may correlate with the high Snail expression, as Snail has been shown to be a potent repressor of E-cadherin and claudin-1 in other cell lines.[14]

Interestingly, this study found that the CaSki cell line does not express claudins, but does express occludin and Scribble. The CaSki cell line, isolated from a human uterine tumour, contains integrated human papillomavirus DNA type 16. Scribble is a potential tumour suppressor and has been identified as a substrate of ubiquitin-mediated degradation by high-risk HPV E6 and the E6AP ubiquitin-protein ligase. Deficiency of Scribble impairs many aspects of cell polarity and cell movement, and the PDZ domain of Scribble binds directly to the HPV E6 protein. The association of ZO-1 with E-cadherin and Scribble may be important in HPV infection

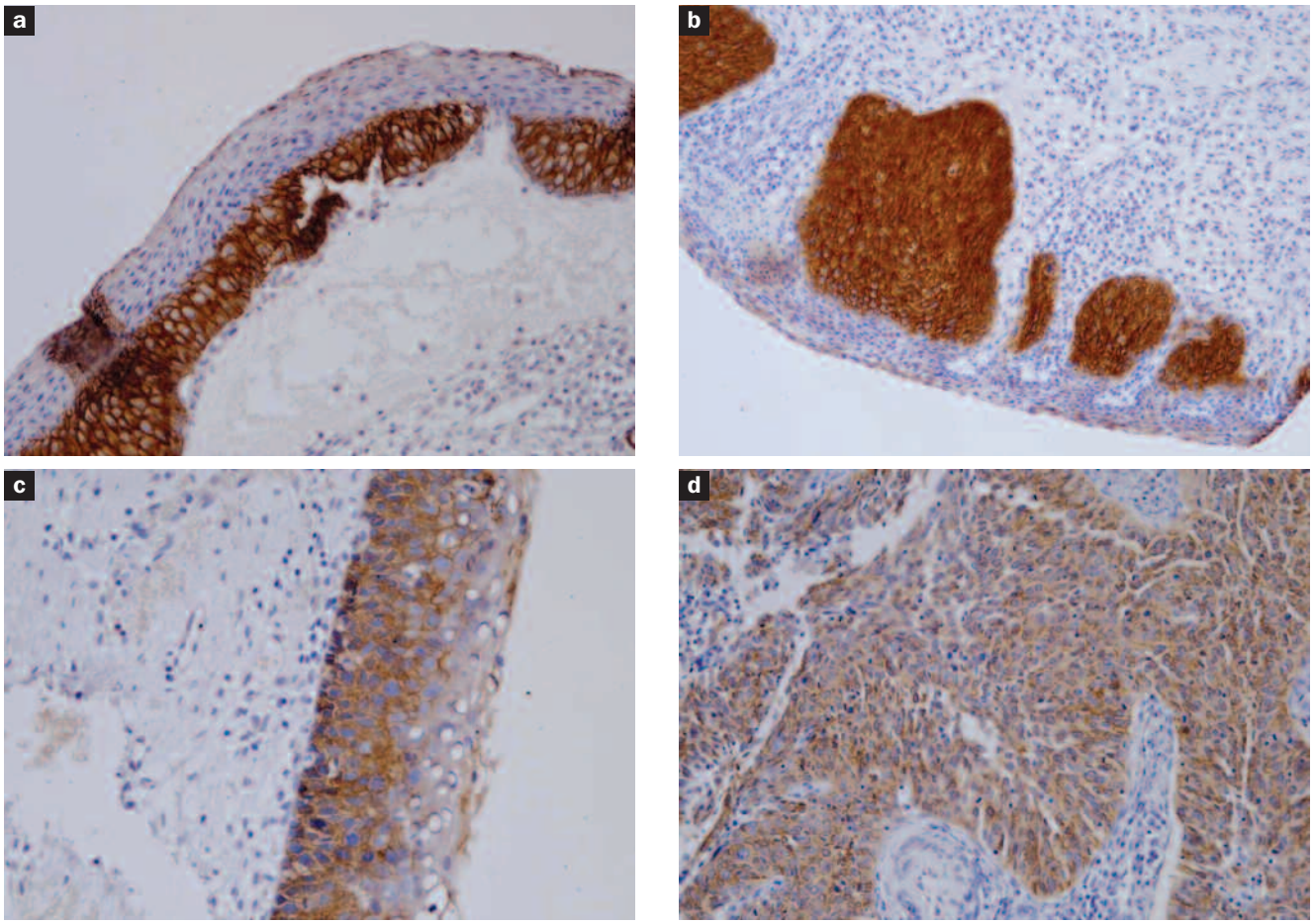

Fig. 6. Aberrant cytoplasmic expression of E-cadherin: **a)** CIN1; **b)** CIN2; **c)** CIN3; and **d)** squamous cell carcinoma.

and there is evidence that Scribble interacts with ZO-2,[19] which may be important for cell polarity.

High expression of N-cadherin was observed in both cell lines. N-cadherin expression is not normally associated with epithelial cells, and its expression may be of significance as other studies have found that N-cadherin helps to facilitate transendothelial migration, which is a crucial mechanism in metastasis.[20]

This study found aberrant E-cadherin expression in cases of cervical neoplasia but not in normal cervical epithelium, and two main patterns of aberrant expression were identified. In CIN lesions, E-cadherin was predominantly mislocalised to the cytosol, while in most squamous cell carcinoma samples expression was totally lacking. Of particular interest was the high proportion of pre-invasive (CIN1, -2 and -3) lesions that showed aberrant E-cadherin expression (Table 1). These results indicate that mislocalisation of E-cadherin may be an early-stage occurrence in the progression of the disease, and may therefore be of use as an early clinical marker/prognostic indicator in the diagnosis of cervical carcinoma, when used in conjunction with traditional histological and cytological methods.

Correlating the IHC results from this study with the HPV status of the cervical samples may have provided further insight into the pathogenesis of the disease, possibly identifying specific HPV genotypes associated with loss of cell polarity and cohesion. This study was unable to determine the HPV status of the cervical samples as the tissue microarrays were obtained from an external company that was unable to provide this information.

Further work will seek to identify whether or not the aberrant E-cadherin expression seen in the cervical tissue samples corresponds with increased Snail and N-cadherin expression and the lack of claudin expression that was observed in the cervical carcinoma cell lines. □

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