The link between integration and expression of human papillomavirus type 16 genomes and cellular changes in the evolution of cervical intraepithelial neoplastic lesions

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We have matched a PCR assay which detects disruptions in the E2 reading frame of human papillomavirus type 16, with RNA *in situ* hybridization patterns and shown that in 15 out of 16 cervical intraepithelial neoplastic (CIN) III lesions and in 19 out of 19 tumours, the E2 gene is disrupted with no detectable E2 transcripts. Varying levels of E6–E7 transcripts are detected in CIN III lesions, with stronger signals in tumours. The cytokeratin profile of most tumours: cytokeratin 10-, 14- and 19-positive and 4-, 13- and 18-negative, is also detected in CIN III lesions. The changes in levels of $\alpha 2$, $\beta 1$ and $\beta 4$ integrins, CD44 and E-cadherin occur during the evolution of high-grade CIN lesions. Increases in the levels of expression of CD44 and E6–E7 transcripts, coupled with changes in the cellular localization of the Notch protein, define the transition from CIN III lesions to tumours.

Cervical tumours evolve very slowly from precursor lesions known as dysplasias or cervical intraepithelial neoplastic (CIN) lesions, which may regress or persist (for review see Howley, 1991). Most human papillomavirus (HPV) type 16-associated cervical tumours have integrated viral DNA. Several studies have used Southern hybridizations to analyse physical state (Dürst *et al.*, 1985; Cullen *et al.*, 1991) and RNA *in situ* hybridization to examine patterns of HPV-16 gene expression. The results suggest that CIN lesions have episomes, and tumours have integrated HPV-16 genomes (for review see zur Hausen, 1991). This is also supported by a study using a PCR assay which reported the presence of episomes in all grades of CIN lesions (Das *et al.*, 1992). This PCR assay is based on detecting disruption of the viral E2 gene, a transcription factor which is believed to function predominantly as a repressor of the upstream regulatory region (URR) and is found to be disrupted in most cervical tumours (for review see McBride *et al.*, 1991). The implication of detectable episomes in precursor lesions is that integration of the virus correlates with, or is the cause of, progression to invasiveness.

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However, on the contrary, some groups have previously used Southern hybridization as a technique to analyse physical state and reported the presence of a fair number of integrated genomes in CIN lesions (Di Luca *et al.*, 1986; Lehn *et al.*, 1988). Using the PCR assay developed by Das and colleagues, we have also previously reported that we were unable to detect episomes in high-grade CIN lesions (Daniel *et al.*, 1995). In this paper, we have matched the PCR analysis with an RNA *in situ* hybridization analysis and the results support our previous assertion that the integration event is associated with the development of high-grade CIN lesions. We have also analysed changes in cytokeratins, markers of invasiveness and the Notch signal transduction pathway during the evolution of cervical lesions.

The absence of amplification with primers for the E2 gene in the presence of an amplification with primers corresponding to the URR and E6 sequences correlated very well with the presence of integrated HPV-16 DNA (Das *et al.*, 1992). In this study, we undertook a PCR analysis along with RNA *in situ* hybridization, using standard protocols, with fragments spanning two regions, the first covering the E6–E7 region (65–875) and the second covering the E2–E5–L2 region (3697–4761). In 15 out of 16 CIN III lesions and 19 out of 19 tumours, there were no signals detected with the E2–E5–L2 probe, while signals were detected with the E6–E7 probe in all cases. The PCR analysis showed an amplification with the URR and E6 primers and no amplification with the E2 primers in 15 out of 16 CIN III lesions and 19 out of 19 tumours (Table 1). In contrast, in CIN I/II lesions all three primer sets amplified the HPV-16 DNA fragments. Fig. 1(*a*) represents an RNA *in situ* hybridization analysis of CIN I/II, CIN III and tumours using probes for the E6–E7 and E2–E5–L2 region respectively. We

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Table 1. HPV-16 gene expression and cellular changes in cervical lesions

Assay	CIN I and II	CIN III	Tumours
URR PCR	8/8	16/16	19/19
E6 PCR	8/8	16/16	19/19
E2 PCR	8/8	1/16	1/19
E6–E7 RNA *in situ*	8/8	16/16	19/19
E2–E5–L2 RNA *in situ*	8/8	1/16	1/19
Cytokeratin 14 and 10	18/18; 18/18	4/15; 10/15	7/15; 11/15
Cytokeratin 19 and 8·13	12/18; 14/14	10/15; 18/21	15/15; 12/12
Cytokeratin 18 and 4	18/18; 2/14	9/15; 12/21	3/15; 0/12
Cytokeratin 8 and 13	2/14; 2/14	1/21; 2/21	3/12; 3/12
Integrin subunit $\beta 1$	18/18 + + +*	12/15 +	13/15 +
Integrin subunit $\beta 4$	18/18 +	13/15 + + +	13/15 + + +
Integrin subunit $\alpha 2$	16/19 + +	5/15 +	8/15 +
E-cadherin	18/18 + +	0/15	0/15
CD44	18/18 + +	13/15 + + +	15/15 + + + +

* + + + +, very high; + + +, high; + +, medium; +, low.

detected E6–E7 transcripts (magenta-coloured staining) in all grades of lesions, using the antisense E6–E7 probe with the strongest signal detected in tumours. The corresponding sections tested with the sense probe did not reveal any significant staining (pale pink). Using the antisense E2–E5–L2 probe, we detected significant staining only in the CIN I/II lesions, with no signal detected using the sense probe at any stage. Dürst *et al*. (1992), in their study of high-grade CIN lesions, clearly detected some E2–E5–L2 transcripts. We detected very faint levels of transcripts using the E2–E5–L2 probe in the higher layers of some CIN III lesions (Fig. 1*a*). Our visualization system may not be as sensitive as the radioactive approach undertaken by Dürst and colleagues. However, our previous RT–PCR analysis (Daniel *et al*., 1995) showing that there were no full length E2 transcripts detectable in CIN III lesions and tumours, along with the RNA *in situ* analysis undertaken in this study, suggest that high-grade CIN lesions predominantly have transcripts for the E6–E7 oncogenes. We believe that this pattern of transcription would be consistent with the absence of detectable episomes in high-grade CIN lesions and tumours. An alternative possibility that we cannot rule out at this stage is that mutations or minor deletions alter transcriptional levels, as suggested by Dong and colleagues following their observations on deletions and mutations in the YY1 binding sites of HPV-16 DNA (Dong *et al*., 1994).

Using standard immunohistochemical protocols, we were interested in determining the status of cytokeratins and cell surface molecules involved in cell adhesion and motility in the progression of cervical lesions. We find cytokeratin 10-positive staining in the majority of CIN III lesions and tumours, 14-positive staining in less than half of CIN III lesions and tumours combined and cytokeratin 19-positive staining in virtually all CIN III lesions and tumours. From low-grade precursors onwards, most lesions all the way up to cervical tumours were negative for cytokeratin 4, 13 and 18. In Fig. 1(*b*), we illustrate the cytokeratin 10-positive (yellowish brown-coloured staining) and cytokeratin 14-negative staining from representative CIN I/II, CIN III lesions and invasive tumours. In the CIN I/II lesions, which are cytokeratin 10-positive, there are unstained cells in the lower layers which are substantially decreased in CIN III lesions (Fig. 1*b*). The 8.13 pan cytokeratin antibody served as a positive control for all sections. The overall cytokeratin analysis suggests that the differentiation status of tumour cells is selected for in CIN III lesions, with no dramatic differences in cytokeratin patterns as the lesions progress.

We have analysed a selected number of cell surface molecules involved in cell adhesion and motility, based on changes reported in a wide variety of tumours (for review see Stetler-Stevenson *et al*., 1993). Our results demonstrate clear differences in the levels of $\alpha 2$, $\beta 1$ and $\beta 4$ integrin subunits, CD44 and E-cadherin during the evolution of CIN lesions, with further changes in CD44 levels during the transition from CIN III to tumours. To represent this analysis, Fig. 1(*c*) illustrates the very faint CD44 expression in the normal cervix and the subsequent upregulation as the lesions progress.

In Table 1 we summarize the PCR analysis, RNA *in situ* hybridization patterns and immunocytochemical analysis of the markers of differentiation, adhesion and motility.

With the intention of identifying changes in proliferative cells as they progress from CIN lesions to tumours, we analysed the Notch pathway. Following the startling observations of Zagouras *et al*. (1995) demonstrating small focal patches of nuclear Notch in cervical tumours and possibly membrane-associated Notch in the superficial layers of the

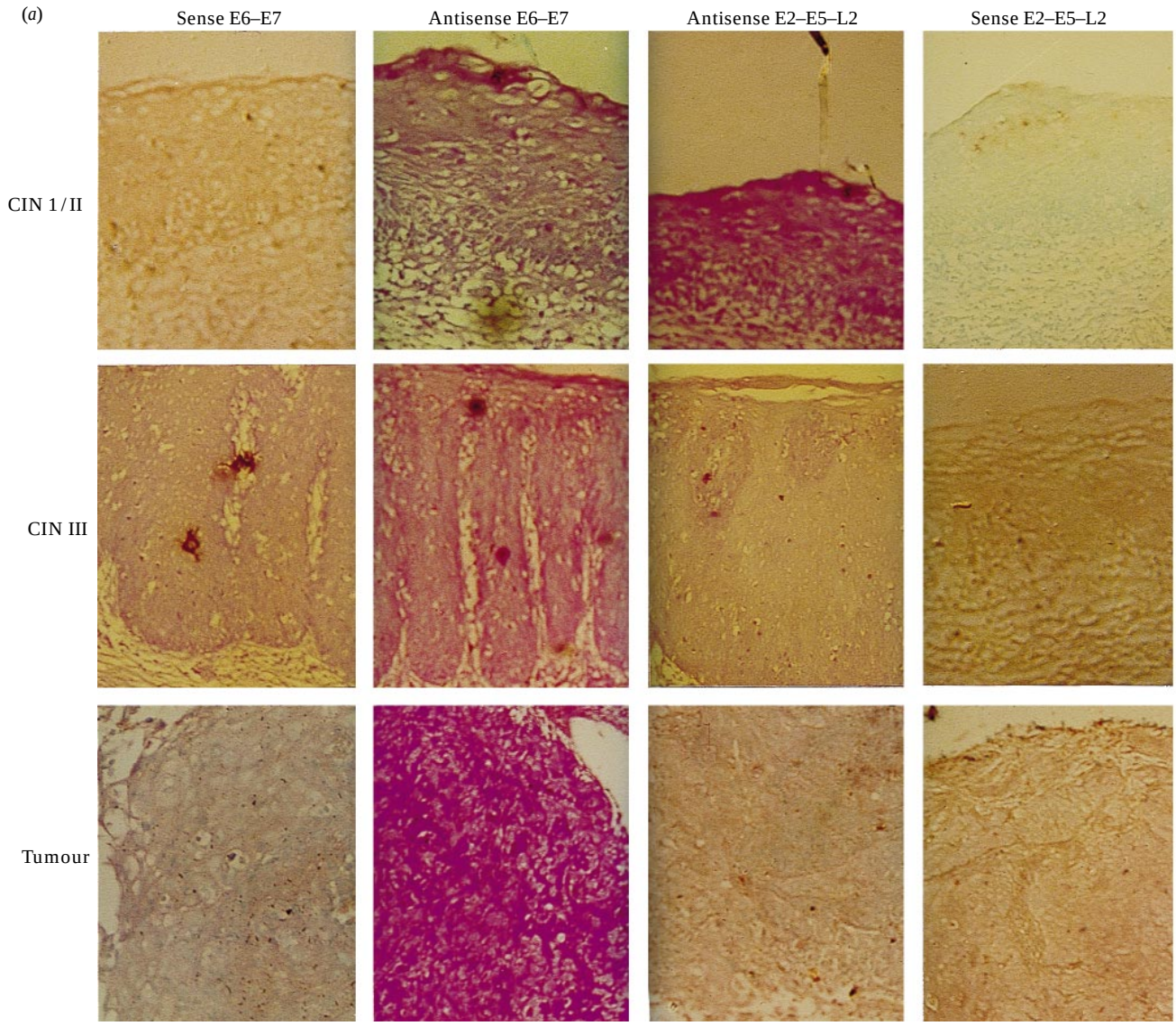

Fig. 1. (*a*) RNA *in situ* hybridization of cervical lesions. RNA *in situ* hybridization of CIN I/II, CIN III and tumours was undertaken using antisense oligonucleotide probes for the HPV-16 E6–E7 region (clone 056-26; 65–875 bp) and the E2–E5–L2 region (clone 056-14; 3697–4761 bp). The corresponding sense sequences served as negative control probes in all reactions. The probes were generated by undertaking *in vitro* transcription reactions using plasmids (Dürst *et al*., 1992) gifted to us by Mathias Dürst (German Cancer Centre, Heidelberg). All the sections represented here were developed using the RNA colour kit (Amersham). The sections were counter-stained with 2% fast green (Sigma). CIN I/II and CIN III lesions were photographed at 100× magnification, and the tumours at 400×, respectively. (*b*) Cytokeratin profiles of cervical lesions. Representative sections of CIN I/II, CIN III lesions and invasive tumours were tested for the presence of cytokeratins using antibodies against cytokeratin 10 (positive staining) and cytokeratin 14 (negative staining). All the anti-cytokeratin antibodies used were from Sigma. The sections were counter-stained with haematoxylin (100× magnification). (*c*) Changes in the levels of CD44 expression during the evolution of cervical lesions. Representative sections of normal cervix, CIN I/II, CIN III lesions and invasive tumours were tested for the presence of CD44 using antibodies against CD44 (400× magnification). The normal section only was photographed with a blue filter to enhance the detection of weak CD44 staining. All the antibodies used against CD44, $\alpha 2$, $\beta 1$ and $\beta 4$ integrin subunits and E-cadherin were from GIBCO BRL. The sections were counter-stained with haematoxylin.

normal cervix, we explored changes in the expression and cellular localization of Notch proteins in the transition from high-grade precursor lesions to tumours. We did not detect Notch staining in the proliferative cells in 7 out of 9 CIN I/II lesions. In 9 out of 12 CIN III lesions we detected membrane- and cytosolic-localized Notch proteins in different patches of the lesions (Fig. 2*a*). In 15 out of 15 tumours, we detected clear, widespread, strong nuclear staining (Fig. 2*a*). The tumours have a weaker cytoplasmic signal and no detectable membrane staining. As a comparison, we have included our analysis of a proliferation marker, PCNA, showing the presence of nuclear-localized PCNA from early CIN lesion onwards (Fig. 2*b*).

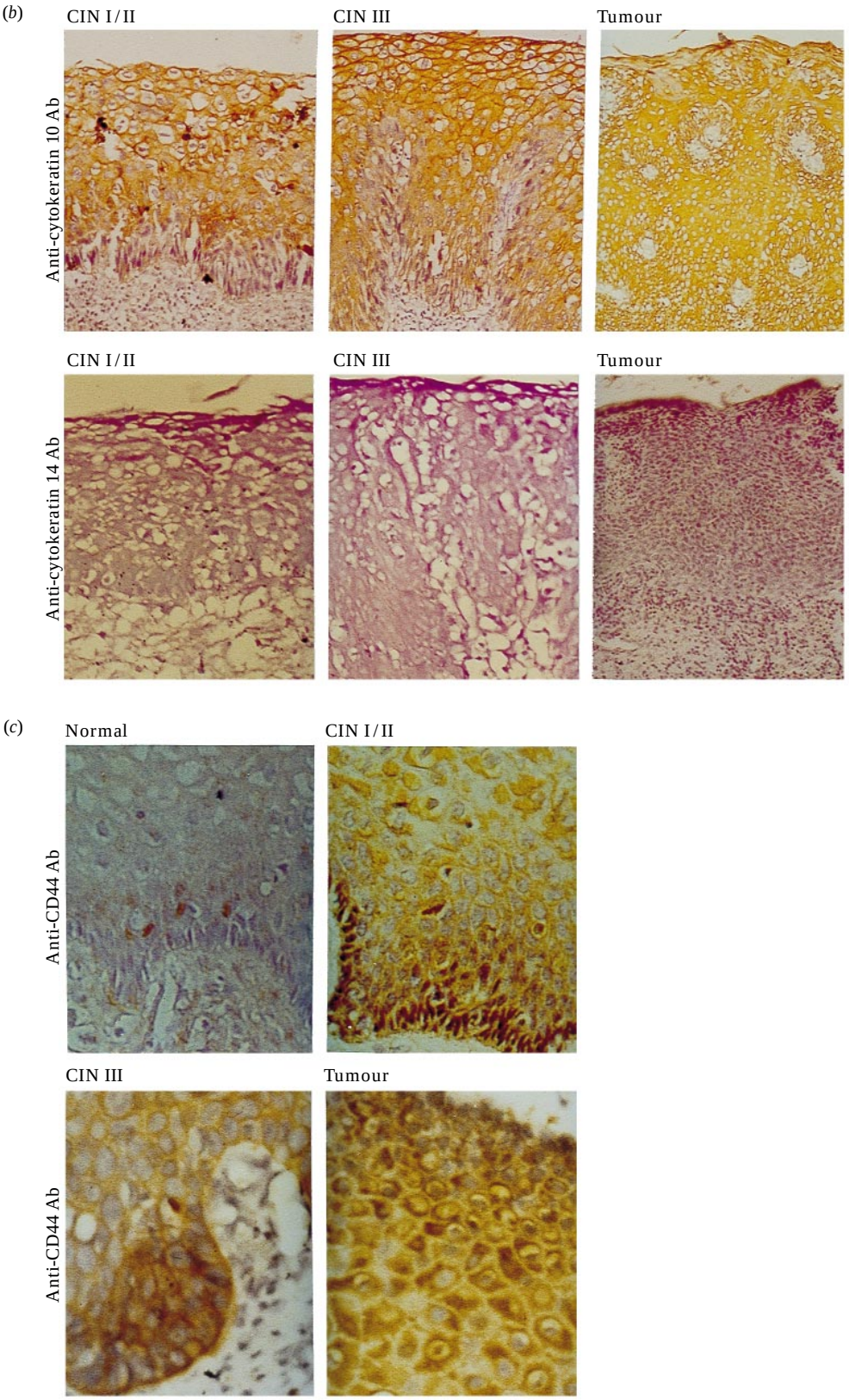

Fig. 1 *(b, c).* For legend see previous page

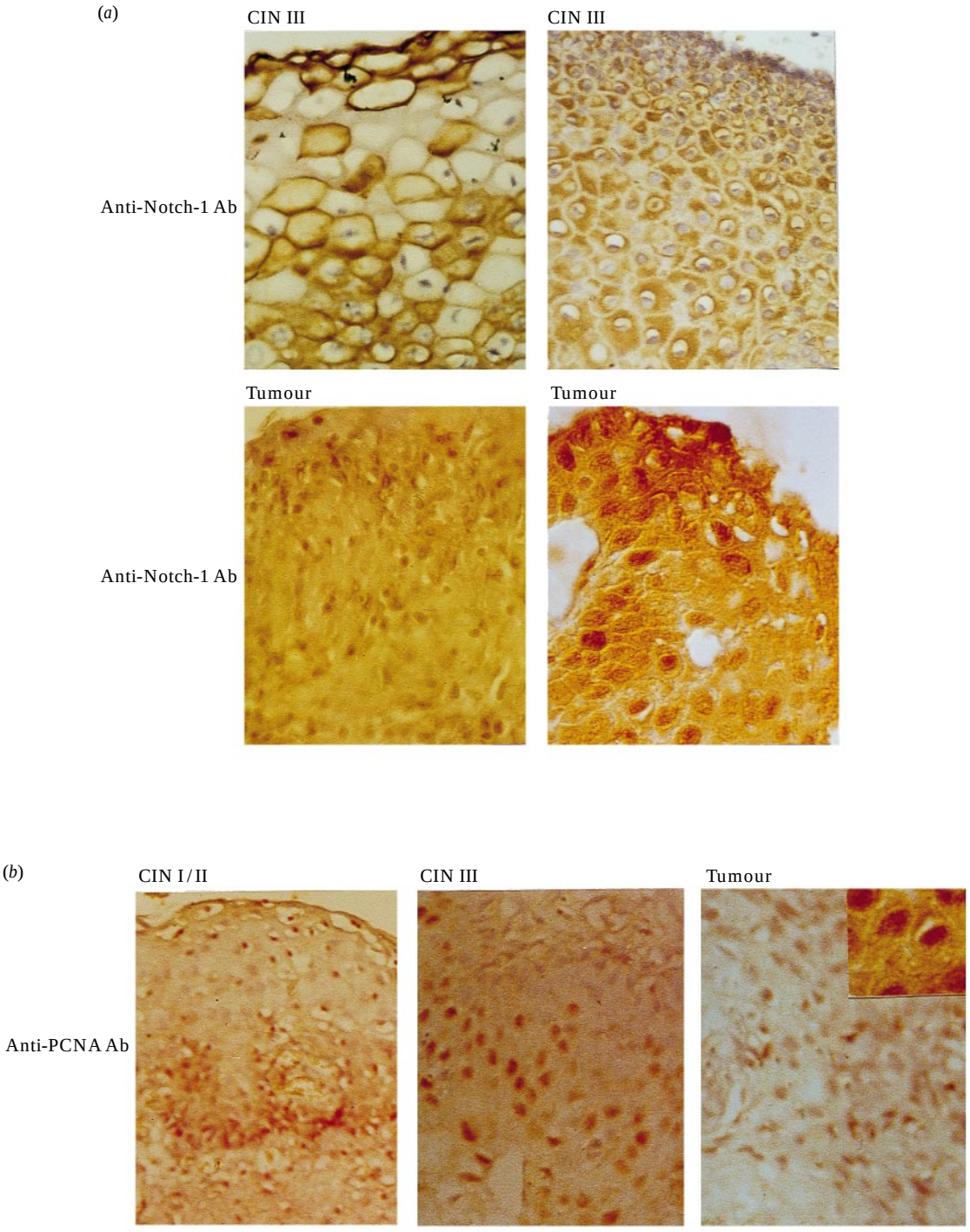

Fig. 2. (*a*) Changes in the levels of Notch cellular expression and cellular localization during the transition from high-grade precursor lesions to invasive tumours. Representative sections of CIN III lesions and invasive tumours (two different magnifications) were tested for the presence of Notch using antibodies against Notch. The anti-Notch monoclonal antibody used, TAN1-15A (Zagouras *et al*., 1995), was a gift from Spyros Artavanis-Tsakonas (Howard Hughes Institute and Yale University, Conn., USA). The sections were counter-stained with haematoxylin. The CIN III lesions were photographed at 400 × magnification and the tumour sections are represented at 200 × and 400 × respectively. (*b*) PCNA profile of cervical lesions. Representative sections of CIN I/II, CIN III lesions and invasive tumours were tested for the presence of PCNA using an antibody against PCNA (Sigma). The sections were counter-stained with haematoxylin. All the photographs are at 200 × magnification, with an inset for the tumour at 400 × .

The PCR analysis reiterates our previous observation regarding disruption of the E2 reading frame in high-grade CIN lesions, preceding the development of tumours. In this paper, we have matched every case with an RNA *in situ* analysis. Collectively, these two approaches lead us to the conclusion that high-grade CIN lesions have integrated HPV-16 genomes and may be similar to the early integration detected in precursor lesions with HPV 18 (Cullen *et al.*, 1991). We have found that there are differing levels of E6–E7 transcripts in high-grade CIN lesions and consistently high levels of E6–E7 transcripts in tumours. The well-categorized repression of HPV-16 URR by the E2 gene suggests that the most likely process of upregulation of URR is due E2 gene disruption. However, other mechanisms which lead to transcriptional regulation by virtue of chromosomal insertional position cannot be ruled out. Our cytokeratin analysis suggests that cells with suprabasal marker, namely cytokeratin 10, are the cells detected in tumours implying a possible selection for the differentiation status. Previous reports which have detected upregulation of E6–E7 transcription in higher epidermal layers in tissues with active viral assembly, might provide a rationale for the proliferation of partially differentiated cervical keratinocytes with increased E6–E7 expression. We have recently detected a clear correlation between the upregulation of URR of HPV-16 in cell lines with markers of suprabasal cells (V. Tergaonkar, D. Viji Mythili and S. Krishna, unpublished results). The upregulation of E6–E7 levels from high-grade CIN lesions to tumours is very likely to be due to the additional genetic events or altered responsiveness to ligands, as suggested by Bartsch *et al.* (1992) based on their cell fusion experiments.

It is intriguing that most of the changes in levels of cell surface molecules involved in adhesion and invasiveness were detected in the transition from early CIN lesions to late CIN lesions. Initial work with transformed lines had suggested that the levels of integrins is the key to differences in the adhesiveness and motility of tumours (Plantefaber & Hynes, 1989). A recent report suggesting that the ligand-binding activity of integrins can be altered in transformed cells without detectable changes in integrin levels per se (Zhang *et al.*, 1996), raises some interesting ideas on the possible manner in which integrins influence the course of tumourigenesis.

The changes detected in patterns of expression of cell surface markers from early CIN lesions to late CIN lesions might reflect chromosomal changes which are known to accumulate during the process of cervical carcinogenesis (for reviews see zur Hausen, 1991; Stanley & Sarkar, 1994). The more likely alternative possibility is that they represent specific developmental states in tumour progression, due to altered signalling pathways and the consequent changes in gene expression. The fairly identical changes in the patterns of invasiveness/cell adhesion molecules in virtually all CIN III lesions and tumours suggests that a switch-like mechanism might be operating at the transition from early CIN lesions to high-grade CIN lesions. Hanahan & Folkman (1996) recently suggested that the transition from low-grade cervical dysplasias (low-grade SILs or squamous intraepithelial neoplasias) to high-grade dysplasias (high-grade SILs) represents an angiogenic switch.

Our data show no detectable Notch protein in early CIN lesions, with striking differences in the cellular localization of the Notch protein in the transition from high-grade lesions to tumours. Based on studies principally from lower eukaryotes, the Notch proteins are cell surface molecules involved in cell fate decisions (for review see Artavanis-Tsakonas *et al.*, 1995). The human Notch gene, TAN-1, was broken by a chromosomal translocation in a T lymphoblastic leukaemia (Ellisen *et al.*, 1991). Our focus in future studies will be to analyse changes in the Notch pathway proteins in order to understand the changes in cellular localization and their potential synergistic role with HPV proteins in promoting cervical carcinogenesis.

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