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- and 14-positive and 4-, 13- and 18-negative, is also detected in CIN III lesions. The changes in levels of α2, β1 and β4 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 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
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 only in the CIN I lesions, with no signal detected using the sense probe at any stage. Durst 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(b)). 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(b) 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. 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.

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<th>Assay</th>
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* + + + +, very high; ++ +, high; + +, medium; +, low.

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

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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. 2a). In 15 out of 15 tumours, we detected clear, widespread, strong nuclear staining (Fig. 2a). 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. 2b).

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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 (Durst et al., 1992) gifted to us by Mathias Durst (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, α2, β1 and β4 integrin subunits and E-cadherin were from Gibco BRL. The sections were counter-stained with haematoxylin.
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 (Ellissen 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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References


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