Down-regulation of E-cadherin is closely associated with progression of cervical intraepithelial neoplasia (CIN), but not with high-risk human papillomavirus (HPV) or disease outcome in cervical cancer

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Summary

Objective: E-cadherin plays a pivotal role in maintenance of normal adhesion in epithelial cells but has also been shown to suppress tumour invasion and participate in cell signalling. Known to be capable of reversing the invasive phenotype of high-risk human papillomavirus (HPV)-transformed keratinocytes, E-cadherin is down-regulated in CIN and cervical cancer (CC), but still incompletely studied as an intermediate endpoint marker in this disease.

Material and Methods: As part of our HPV-PathogenISS study, a series of 150 CCs and 152 CIN lesions were examined using immunohistochemical (IHC) staining for E-cadherin, and tested for HPV using PCR with three primer sets (MY09/11, GP5+/GP6+, SPF). Follow-up data were available from all squamous cell carcinoma (SCC) patients, and 67 CIN lesions were monitored with serial PCR for HPV after cone treatment.

Results: Expression of E-cadherin was reduced in parallel with the increasing grade of CIN, with major down-regulation upon transition to CIN3 and further to invasive cancer (OR 6.95; 95% CI 2.67-18.09) (p = 0.0001). Negative markedly reduced E-cadherin expression was a 90.9% specific indicator of CIN, with 97.4% PPV, but suffered from low sensitivity (27.0%) and NPV (9.1%). E-cadherin expression was completely unrelated to HR-HPV (p = 0.982), and did not predict clearance/persistence of HR-HPV after treatment of CIN. Similarly, E-cadherin expression was not a prognostic predictor of CC in univariate or multivariate analysis.

Conclusions: Down-regulation of E-cadherin was closely associated with progressive CIN and cell proliferation. It is tempting to speculate that part of this cell proliferation is mediated through the canonic Wnt signalling pathway, after liberation of transcriptionally competent β-catenin from the E-cadherin/catenin complex, most notably orchestrated by E6 and E7 oncoproteins of HR-HPV. Such a liberation of β-catenin would abrogate the negative transcriptional control of E-cadherin on the Lef/TCF/β-catenin responsive genes. The exact role of HR-HPV oncoproteins E6 and E7 in this process remains to be seen in future studies.

Key words: E-Cadherin; Cell adhesion; Oncogenic human papillomavirus; CIN; Cervical cancer; Prognosis; Virus clearance; Persistence; CIN treatment.

Introduction

The different oncogenic potential of low-risk (LR) and high-risk (HR) human papillomaviruses (HPV) in inducing cervical cancer (CC) and its precursor cervical intraepithelial neoplasia (CIN) lesions is attributable to the different interactions of the two viral oncoproteins, E6 and E7, with the key regulatory cellular proteins, p53 and pRb [1-7]. While the E6 of the HR-HPV (but not LR-HPV) initiates degradation of the p53 tumour suppressor protein, HPV E7 of HR-HPV (but not LR-HPV) binds to pRb resulting in G1/S transition of the cell cycle [2, 5-8]. These type-dependent functions of E6 and E7 are different from those of the third HPV oncoprotein E5, which activates potent nuclear transcription factors through the ERK/MAPK signalling pathway in an HPV-type independent manner [2, 3, 8-10]. The complex process of developing an invasive phenotype is intimately linked with the adhesion properties of epithelial cells, mediated by a wide variety of adhesion molecules, e.g. cadherins and catenins, currently suspected to be interfered with by HR-HPV by as yet unknown mechanisms [1, 2, 5, 7, 11].

Revised manuscript accepted for publication January 16, 2006

Of all cell adhesion molecules, E-cadherin is among the most intensely studied in human cancer [11]. Also known as ovomorulin (L-CAM, cell-CAM 120/80, Arc-1), E-cadherin is a 120 kDa transmembrane glycoprotein, of which an extracellular 80 kDa tryptic fragment is released in the presence of Ca^{2+} [11-13]. The E-cadherin encoding gene, CDH1, is located in chromosome 16q22.1, frequently showing promoter methylation in human cancer [14-16]. E-cadherin is uniformly present at the lateral cell surfaces, tightly bound with two other proteins (α- and β-catenins) forming a cadherin-catenin complex, in which α-catenin connects the complex to actin-cytoskeleton and β-catenin interacts with both the cytoplasmic part of E-cadherin and α-catenin, stabilizing the formation of cell-cell junctions, known as adherens junctions (AJ) [14-17]. Apart from these important cell adhesion properties, E-cadherin has been recently shown to possess properties of a suppressor of tumour invasion [17-19], and importantly, it also seems to participate in some key intracellular signalling pathways [17]. Indeed, this recent evidence on the dual role for E-cadherin has important implications in cervical carcinogenesis, and offers potential new interactions with the oncogenic HPV in this process [17-21].

Several years ago, it was shown that membrane E-cadherin expression was down-regulated in HPV16 E6/E7 transfected keratinocytes, with dramatic redistribution of α-, β- and γ-catenins [20]. Recently, E6/E7 was shown to cooperate with ErbB-2 in disruption of the normal E-cadherin/catenin complex, leading to cytoplasmic translocation of E-cadherin [21]. Recent observations also suggest that E-cadherin-dependent adhesion of dendritic and Langerhans cells to keratinocytes is defective in HPV-positive CIN lesions [22]. The latest evidence indicates that the cadherin-catenin complex may link intercellular adhesion and DNA transcription through the Wnt signalling pathway [17] and interestingly, activation of this canonical Wnt signalling pathway seems to be a prerequisite for transformation of HR-HPV-expressing human keratinocytes [23].

This Wnt pathway might offer the missing link between E-cadherin and HR-HPV in cervical carcinogenesis [22, 23]. Despite the sizeable number of studies on E-cadherin expression in cervical lesions [22, 24-38], surprisingly little attention has been paid to these possible associations between HPV and E-cadherin expression [22, 28, 32, 33]. Similarly, few studies have correlated E-cadherin expression with the clinical parameters [26, 31, 33, 36, 37], and even less data are available on E-cadherin as a prognostic factor in CC [27, 35, 39]. As a part of our systematic search for novel biomarkers in cervical carcinogenesis (HPV-PathogenISS study) [8, 9, 40, 41], we analysed a series of CIN and CCs to assess whether E-cadherin might be of any use in predicting the intermediate endpoint markers in cervical carcinogenesis: a) the grade of CIN, b) HR-HPV type, c) clearance of the virus after eradication of CIN, as well as d) survival of CC patients as the terminal event.

Our Research

The material of this study comprises the retrospective component of the HPV-PathogenISS project [42], and was collected from the files of the Pathology Departments of two Italian hospitals (S. Orsola Malpighi Hospital, Bologna and Maggiore Hospital, University of Trieste). Altogether, this prospective biopsy material comprises 302 patients with either an invasive cervical squamous cell carcinoma (SCC) or cervical intraepithelial neoplasia (CIN) diagnosed and treated in these two hospitals between 1986-2002. Of these 302 cases, 114 CIN and 38 CC cases were provided by Bologna, and 38 CIN lesions and 112 CCs were available from Trieste. The mean age of all CIN patients was 35.5 (range 18-79) years, and that of SCC patients 59.2 (range 27-89) years ($p = 0.0001$).

Available data

All the cases from Bologna had their HPV status determined by PCR, as reported before (43,44,45), whereas the samples from Trieste were tested for HPV in this study. Complete follow-up data were available from all 150 CC patients, with a mean follow-up of 51.7 months (range 1-218). Furthermore, all CIN cases from Bologna had been followed-up at 6-month intervals after cone treatment (for a mean of 10.5 mo, range 2.4-27.6 mo), and subjected to repeated colposcopy, PAP smear and biopsy (if residual was suspected). A minimum of two (up to 7) serial PCR analyses were available from 67 cases, as recently reported [45].

Methods

Biopsy

Both the colposcopic biopsies and surgical samples were fixed in 10% buffered formalin, embedded in paraffin, and processed for 5-mm-thick paraffin sections stained with haematoxylin-eosin (HE) for routine diagnosis. All slides were re-examined to confirm the diagnosis. On histological examination, the lesions were graded using the CIN nomenclature and categorised as CIN1, CIN2 and CIN3. The histological diagnosis of CC was confirmed in all cases, and two adenocarcinomas present in the original cohort were excluded from this series.

Immunohistochemistry for E-cadherin

Immunohistochemical (IHC) staining for E-cadherin expression was completed following standard IHC procedures. In brief, the 5-μm paraffin sections cut on poly-L-lysine-coated microscopy slides were first deparaffinised and rehy-

drated in graded alcohols. The sections were heated in citrate buffer (0.01 M, pH6.0, DAKO Target Retrieval Solution) in a microwave oven (85-95°C, 3x5 min), followed by blocking the non-specific binding sites with normal rabbit serum. Sections were incubated with the primary antibody, monoclonal mouse anti-human E-cadherin, clone NCH-38 (Catalogue #M3612, DakoCytomation A/S, Glostrup, Denmark), in a humidified chamber for one hour at room temperature (dilution 1:100). This purified mouse monoclonal (IgG1 kappa) antibody recognizes the 120 kDa mature form and 82 kDa fragment of human E-cadherin in Western blots of A431 lysates. The IHC technique has been extensively documented in different human tumours, and shown to perform well in paraffin-embedded sections. Primary antibody was followed by incubation with the biotinylated secondary antibody, polyclonal goat anti-mouse IgG (#6788, Abcam, Ltd, Cambridge, UK) (dilution 1:250). Slides were then processed with universal LSAB™-2 single reagents (peroxidase) kit (DakoCytomation), and expression of E-cadherin was localised by incubation with DAB (diaminobenzidine). As a final step, the slides were stained with a light haematoxylin counterstaining. Negative controls were similarly processed by omitting the primary antibody, and biopsies from breast cancer were used as positive controls.

Evaluation of the IHC staining

IHC staining was examined using a light microscope (Leitz Diaplan, Leitz Wetzlar, Germany), equipped with a digital camera (Leica DG300). E-cadherin staining was abundant in normal squamous epithelium, localised on the cell membrane and evenly distributed throughout the full epithelial thickness. This normal pattern was used as a reference to CIN lesions. Many of the low-grade CIN lesions retained this normal expression (at least in part), which was progressively disturbed upon progression towards high-grade CIN. The regular membrane staining was replaced by progressively increasing cytoplasmic staining, being most abundant in cancers. In this study, only the membrane (= normal) staining pattern was graded, using four categories as follows: 0 = negative or almost negative; 1 = markedly reduced expression (large areas without staining); 2 = moderately to slightly reduced expression (minor areas without staining); and 3 = normal expression (staining equivalent to normal epithelium) (Figures 1-6). In some statistical analyses, the staining results were also treated as dichotomous categorical variables, where separately indicated: markedly reduced to negative/normal to moderately reduced.

HPV testing

The 114 CIN and 38 SCC cases from Bologna had already been HPV tested for other purposes using PCR, as recently reported [43-45]. In the present study, the 150 paraffin-embedded sections (112 SCC and 38 CIN) delivered from Trieste were subjected to HPV testing by PCR.

Polymerase chain reaction

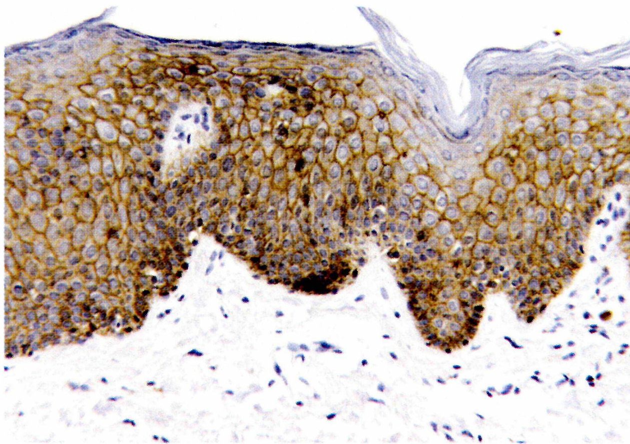
To verify the extraction and the quality of DNA from the paraffin-embedded tissues, 5 µl of each sample were amplified with a primer set recognizing the b-actin gene (sense: 5'-GGCGGCACCACCATGTACCCT-3', anti-sense: 5'-AGGGGCCGGACTCGTCATACT-3'). The PCR mix contained 200 µM each dNTP, 1.5 mM $MgCl_2$, 1X PCR buffer, 40 pmol sense and anti-sense primer, 1.25 U AmpliTaq Gold (Applied BioSystem, Branchburg, USA). The PCR conditions were as follows: 94°C, 10 min, for 1 cycle; 94°C, 30 sec, 60°C, 30 sec, 72°C, 30 sec, for 25 cycles, Finally, 72°C, for 7 min [45].

The samples were then amplified for the presence of HPV using different sets of degenerated primers as described separately for MY09/MY11 [46], $GP5^+/GP6^+$ [47], and biotinylated SPF primer mix located within the L1 region of the HPV genome [48], respectively. The PCR conditions for the My09/My11 were: 94°C for 10 min, one cycle; 94°C, 30 sec, 55°C, 45 sec, 72°C, 30 sec for 40 cycles, followed by an extension step at 72°C for 7 min. The PCR mix contained 200 µM each dNTP, 40 pmol each primer, 2 µM $MgCl_2$, 1X PCR buffer, 1.25 U AmpliTaq Gold [45]. For the $GP5^+/GP6^+$ primers, the following conditions were used: 94°C for 10 min, one cycle; 95°, 30 sec, 44°C, 60 sec, 72°C, 90 sec, for 40 cycles; then a final extension step at 72°C for 7 min. Amplification with SPF primer mix was carried out as follows: 94°C, 10 min, one cycle, 94°C, 30 sec, 52°C,45 sec, 72°C, 45 sec, for 40 cycles, and with the final extension step at 72°C for 7 min. Positive and negative controls were included in each amplification [46-48].

HPV typing

HPV typing was done using the reverse-hybridisation assay. The denatured biotinylated amplified product (10 µl) was hybridised with specific oligonucleotide probes, which are immobilised as parallel lines on membrane strips (Inno-LiPA, Innogenetics, Ghent, Belgium) [48]. After hybridisation and stringent washing, streptavidin-conjugated alkaline phosphatase was added and bound to any biotinylated hybrid previously formed. Incubation with BCIP/NBT chromogen yields a purple precipitate that can be visually interpreted. Based on the position of the visualized line, it is possible to determine the HPV genotype [48]. The following HPV types were included in the test panel: HPV 6,11,16,18,31,33,34,35,39,40,42,43,44,45,51,52,53,54,56,58,59,66,68,70,74.

Fig. 1



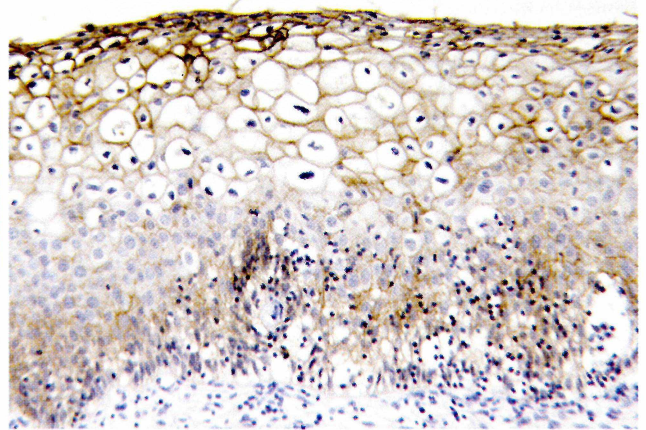
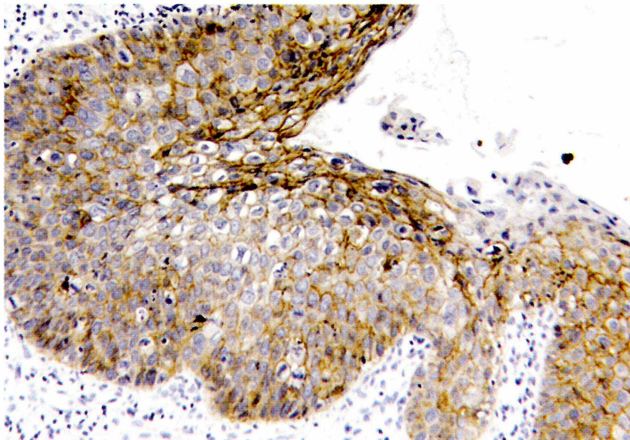


Fig. 3



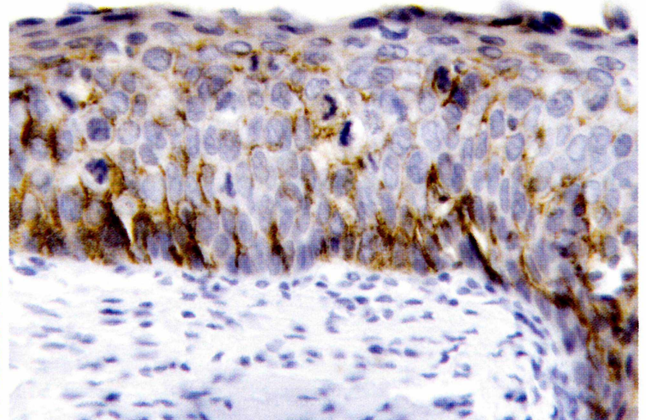
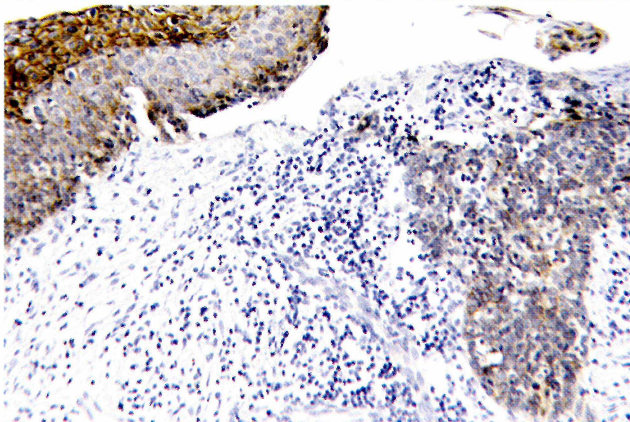


Fig. 5



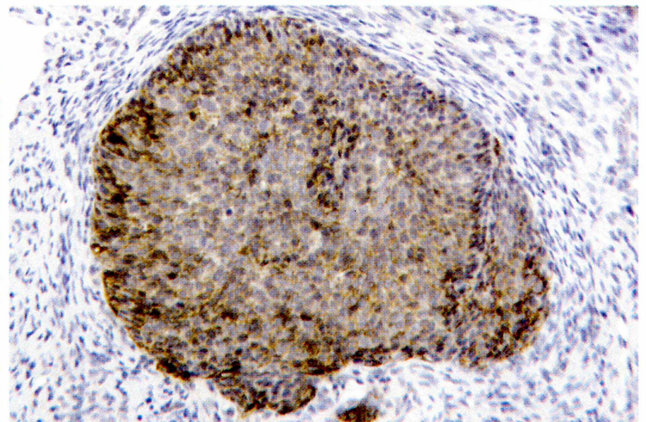


Figure 1. — Normal squamous epithelium with marked hyperkeratinisation. Intense membrane staining for E-cadherin is evenly distributed throughout the full thickness of the epithelium. This represents a characteristic E-cadherin expression in normal squamous epithelium, used as the reference for cervical lesions. (IHC for E-cadherin, original magnification x 100).

Figure 2. — A low-grade CIN1 lesion with characteristic features of HPV infection (koilocytes). As compared to normal epithelium, the expression of E-cadherin is reduced. E-cadherin expression equivalent to that of normal epithelium is still present in the intermediate and superficial layers, whereas the intensity is markedly reduced in the dysplastic cells within the lowermost one-third of the epithelium. (IHC for E-cadherin, original magnification x 100).

Figure 3. — A high-grade CIN3 lesion with markedly reduced E-cadherin expression. Only the cells in the most superficial layers demonstrate strong expression of E-cadherin on their membrane, similar to that seen in Figure 2. In contrast, cells in lower levels only show a weak membrane staining for E-cadherin. (IHC for E-cadherin, original magnification x100).

Figure 4. — Another CIN3 lesion with higher power, making individual cells clearly visible. E-cadherin expression is absent in many of the cells, or present as incomplete membrane expression in some others. Some additional cells also demonstrate an abnormal intracellular (cytoplasmic) immunostaining. (IHC for E-cadherin, original magnification x 250).

Figure 5. — A focus of an invasive squamous cell carcinoma with an adjacent CIN3 lesion (on the left) to demonstrate the contrast. While the CIN3 lesion still retains some E-cadherin expression (similar as in Figure 3), immunostaining is practically absent in the invasive cancer focus. (IHC for E-cadherin, original magnification x 100).

Figure 6. — A single focus of another invasive carcinoma. E-cadherin expression pattern is highly abnormal and unevenly distributed. While a few cancer cells demonstrate weak membrane immunoreactivity to E-cadherin, most of the cells are negative. Towards the periphery of the cancer focus, there is an accumulation of cells with strong cytoplasmic expression. Surrounding connective tissue stroma is completely negative. (IHC for E-cadherin, original magnification x 100).

Statistical methods

Statistical analyses were performed using the SPSS® and STATA software packages (SPSS for Windows, Version 12.0.1, and STATA/SE 9.1). Frequency tables for categorical variables were analysed using the chi-square test, with likelihood ratio (LR) or Fisher's exact test to assess the significance of the correlation. Bivariate correlations between ordered variables were analysed using Spearman correlation analysis (Spearman rho). Differences in the means of continuous variables were analysed using non-parametric tests (Mann-Whitney) or ANOVA (analysis of variance). Logistic regression models using a stepwise backward approach and LR statistic for removal testing, were used to analyse the power of different covariates as predictors of the outcome variables (CIN, HR-HPV), calculating crude ORs (and 95% CI). Control for confounding was also performed by calculating the weighted average of the stratum-specific estimates, using the Mantel-Haenszel test for the common OR (with 95% CI). Performance indicators of PCNA as a marker of CIN, CIN3/CC or HR-HPV were calculated using the contingency tables for sensitivity, specificity, positive and negative predictive value (PPV, NPV), with 95% CI based on the F-distribution (±1.96 x SE). Univariate survival (life-table) analysis for the outcome measure (HPV clearance/persistence, overall survival) was based on the Kaplan-Meier method. Multivariate survival analysis was run by using Cox's proportional hazards model in a backward stepwise manner with the log-likelihood ratio (L-R) significance test, and using the default values for enter and exclusion criteria. The assumption of proportional hazards was checked by log-minus-log (LML) survival plots. In all tests the values $p < 0.05$ were regarded as statistically significant.

Results

Table 1 shows the expression of E-cadherin related to the grade of cervical lesions. There was a linear relationship between the increasing grade of CIN and decreasing intensity of regular membrane expression of E-cadherin, with a major down-regulation upon progression to CIN3 ($p = 0.0001$, also for linear trend). Using the two-tier category of staining, negative-markedly reduced E-cadherin expression was associated with CIN3/cancer with OR 6.95 (95% CI, 2.67-18.09) ($p = 0.0001$).

Of the CIN lesions, 70.5% were HR-HPV positive, as contrasted to only 11.1% of those without CIN. Of the CC lesions, 95.1% were HPV-positive, with HR-HPV types being characterised in 77.6%, while in the remaining 17.5%, HPV type could not be determined. HR-HPV detection was associated with CC with OR 27.25 (95% CI, 3.28-226.09) and to CIN with OR 19.12 (95% CI, 2.31-157.81).

Table 2 shows the expression of E-cadherin related to detection of HR-HPV in the lesions. E-cadherin staining intensity was completely unrelated to HPV-type detection in the lesions. In fact, distribution of the different E-cadherin expression categories was almost identical in lesions with and those without HR-HPV types ($p = 0.982$), and perfectly identical, when the 2-tier grading was used.

Table 1. — *Expression of E-cadherin as related to grade of the lesions.*

Lesion grade*	E-cadherin expression							
	Negative		Markedly reduced		Slightly-moderately reduced		Equivalent to normal epithelium	
	No	%	No	%	No.	%	No.	%
Negative for CIN	0	00.0	0	00.0	3	30.0	7	70.0
CIN1	1	5.0	1	5.0	4	20.0	14	70.0
CIN2	1	4.8	2	9.5	6	28.6	12	57.1
CIN3	6	6.2	27	27.8	32	33.0	32	33.0
SCC	30	20.4	42	28.6	54	36.7	21	14.3
Total (n = 295)*	38	12.9	72	24.4	99	33.6	86	29.2

*Fisher's exact test, p = 0.0001 (also for linear trend); SCC, squamous cell cancer.

Table 2. — *Expression of E-cadherin as related to high-risk HPV types in the lesions.*

HR type*	E-cadherin expression							
	Negative		Markedly reduced		Slightly-moderately reduced		Equivalent to normal epithelium	
	No	%	No	%	No.	%	No.	%
Present	27	13.1	47	22.8	71	34.5	61	29.6
Absent	9	11.5	19	24.4	27	34.6	23	29.5
Total (n = 284)**	36	12.7	66	23.2	98	34.5	84	29.6

*Cases that were HPV-negative; **Pearson, p = 0.982.

The calculated performance indicators for E-cadherin as a marker of CIN, high-grade CIN (CIN3 and above), and HR-HPV are shown in Table 3. Negative markedly reduced E-cadherin expression was a 90.9% specific indicator of CIN, with 97.4% PPV, but normal moderately reduced expression did not rule out CIN because NPV was only 9.1%. With the CIN3 cut-off, there was a slight drop in both specificity and PPV, but marked gain in sensitivity (43.0%) and NPV (24.9%). The performance indicators as a marker of HR-HPV are not useful.

Of the HPV-positive women treated for CIN and controlled by serial PCR, 41/67 (61.2%) had cleared their HR-HPV infection by the last PCR assay for a total of 705 women/months at risk (wmr), with the monthly clearance rate of 5.8% (58/1.000 WMR). Clearance (52.0%) (13/25) in cases with markedly reduced-negative E-cadherin expression was slightly less frequent than that (66.7%) (24/36) in the cases with normal-moderately reduced staining ($p = 0.250$). The corresponding figures for HPV

Table 3. — *E-cadherin staining as predictor of CIN, CIN3, and HR-HPV type.*

Outcome variable	*E-cadherin performance indicators (95% CI)							
	Sensitivity		Specificity		PPV		NPV	
CIN**	27.0%	(19.7-35.2)	90.9%	(58.7-99.7)	97.4%	(86.2-99.9)	9.1%	(4.5-16.1)
CIN3[1]	43.0%	(36.7-49.5)	90.2%	(78.6-96.7)	95.5%	(89.7-98.5)	24.9%	(18.8-31.7)
HR-HPV	35.9%	(29.4-42.8)	64.1%	(52.4-74.7)	72.6%	(62.8-80.9)	27.5%	(21.1-34.6)

*E-cadherin staining (negative-markedly reduced/normal-moderately reduced); **any grade of CIN (SCC cases excluded); [1]CIN3 cut-off (SCC cases included).

persistence were 15.4% (4/26) and 12.8% (5/39) (p = 0.770). In univariate (Kaplan-Meier) survival analysis, E-cadherin expression was not a significant predictor of HPV clearance/persistence in the cervix after treatment of CIN (log-rank, p =0.245).

Of the 150 CC patients, 91 (60.7%) were alive and 59 had died during the follow-up. The expression of E-cadherin was not a significant predictor of survival in SCC. The probability of being alive with normal-moderately reduced and negative-markedly reduced E-cadherin was only slightly different: 53.3% and 66.7%, respectively (p = 0.098). In Kaplan-Meier analyses, this survival difference was not significant (p = 0.0697). The presence of HR-HPV had a slightly positive effect on survival in that 64.0% of HR-HPV-positive and 43.8% of HR-HPV-negative women were alive (p = 0.042) (OR 2.28, 95% CI, 1.02-5.07). Of all variables analysed, FIGO stage was the most powerful predictor of survival in univariate analysis (p = 0.0001).

As anticipated, in multivariate survival (Cox) analysis, E-cadherin expression did not prove to be a significant independent prognostic predictor. It was removed from the model, when adjusted for age, HR-HPV, and FIGO stage, in a backward stepwise approach. In the final Cox model, FIGO stage (p = 0.0001) and age (p = 0.003) proved to be the only independent predictors of patient survival. When FIGO Stage 1 was used as the reference, HR (hazard ratio) for dying of the disease in Stage 2 was 4.29 (95% CI, 1.67-10.99), in Stage 3, HR was 7.58 (95% CI, 2.93-19.61) and in Stage 4, HR was 22.22 (95% CI, 7.77-63.56). The mean age of women who were alive was 54.2 years as compared to 66.7 years for those who died of their SCC (p = 0.0001).

Discussion

Despite a large number of studies analysing E-cadherin expression in CIN and CC using IHC [22, 24-38], little is known about its relationship with HPV [22, 28, 32, 33], and even less concerning its prognostic value in CC [27, 35, 39]. In the present study, we analysed E-cadherin expression as a predictor of several intermediate endpoint markers and its value as a prognostic factor, as part of our systematic search for prognostic markers in CC [8, 9, 40, 41, 49, 50]. Consistent with the published data [22, 24-38], we found normal squamous epithelium invariably positive for E-cadherin, demonstrating intense membrane staining throughout the entire thickness of the epithelium (Figure 1). This regular pattern was retained in metaplastic squamous epithelium, and in the majority of low-grade lesions as well. The staining pattern was completely different in high-grade CIN lesions and cancer, where the regular membrane E-cadherin expression was gradually replaced by the abnormal cytoplasmic staining (Figures 2-6). This is in agreement with most of the previous studies reporting a similar IHC staining pattern for E-cadherin in cervical lesions [22, 24-27, 30, 32-34, 36, 38].

In practically all published IHC studies, the regular membrane expression of E-cadherin was shown to decrease in parallel with the increasing grade of CIN [22, 24-27, 30, 32-34, 36, 38]. This is in perfect alignment with our observations in the present series, where E-cadherin expression showed a major down-regulation upon progression of CIN2 to CIN3, which further accentuated in invasive disease (Table 1). Thus, negative-to-markedly reduced E-cadherin expression was associated to CIN3/cancer with high OR, 6.95 (95% CI, 2.67-18.09) (p = 0.0001), implicating a close relationship between perturbed cellular adhesion and progressive cervical disease [24-27, 30, 32, 33, 36, 38]. This also indicates that IHC staining for E-cadherin gives highly reproducible results, despite the variety of antibodies used in these studies.

Because it retains its normal pattern in low-grade lesions, E-cadherin expression seems to be affected relatively late in cervical carcinogenesis. Indeed, negative-markedly reduced E-cadherin expression distinguished CIN lesions with 90.9% specificity and 97.4% PPV, but was not particularly sensitive (27%) (Table 3). As far as the authors could determine, these performance indicators for E-cadherin immunostaining in predicting CIN have not been calculated in any of the previous studies [22, 24-38]. Because applicable biomarkers in cancer screening are urgently needed [5, 41], any marker detectable by IHC could be a potential candidate to be applied in PAP smear screening. As a test with over 90% SP and 97% PPV in detecting CIN, IHC analysis of E-cadherin would seem a potential screening tool. Recently, cytoplasmic expression of β-catenin was suggested as such a screening tool, being a specific marker of the activated Wnt pathway [23]. In theory, a combination of E-cadherin assay with another test showing high SE and high NPV (e.g., HPV testing by Hybrid Capture 2), might look like a particularly attractive combination as a screening tool. Interestingly, retained normal expression of E-cadherin was shown to be associated with false-negative PAP smears, most likely due to interference with the regular exfoliation of epithelial cells [34]. This is closely related to the major limitation of E-cadherin staining, precluding its use as a screening test for CIN, i.e., the fact that E-cadherin expression is retained in many of the high-grade lesions making it impossible to differentiate normal cells from those derived from

CIN in Pap smears. The use of negative E-cadherin staining as a cut-off would suffer from far too low specificity to be of any practical value as a biomarker in SCC screening.

Persistent HR-HPV infections have recently achieved increasing attention as a major cause of treatment failures in CIN [1, 45]. Monitoring this risk of disease recurrence after cone treatment using a suitable marker would be of considerable clinical value, and we were interested to see whether E-cadherin expression might be of any predictive value for HR-HPV persistence/clearance. It was observed that the clearance events were slightly more frequent in cases with normal-moderately reduced E-cadherin than in those with negative-markedly reduced expression ($p = 0.250$). The same was true with the HPV clearance rates calculated per women/months at risk (wmr): 24/371 (64.69/1,000 wmr) and 13/320 (40.62/1,000 wmr) for normal/moderately reduced vs negative/markedly reduced E-cadherin expression, respectively (Mantel-Haenszel Rate Ratio, RR = 1.592, 95% CI, 0.81-3.10) ($p = 0.176$). Thus, E-cadherin adds another member in the growing list of markers analysed in this material ($p16^{INK4a}$, ERK-1, VEGF-C, survivin, nm23-H1, 67kD laminin receptor, topo IIα, NF-κB, MMP2, TIMP2, telomerase, PCNA) [8, 9, 40, 41, 49, 50], none of which was shown to possess any predictive value for HPV clearance/persistence in cone-treated cervix [45].

The prognostic value of E-cadherin in SCC has been incompletely studied [27, 35, 39]. In two of these studies, E-cadherin expression was of no prognostic value [27, 35], whereas in the third, abnormal expression correlated significantly ($p < 0.05$) with tumour grade, lymph node metastases and clinical stage, but no survival data were presented [39]. We recently failed to establish any value for E-cadherin as a predictor of treatment response to neoadjuvant chemotherapy in SCC [43]. On the other hand, there are some studies implicating that hypermethylation of the CDH1 gene promoter [14] is a sign of ominous prognosis in SCC [15, 16, 51]. This hypermethylation leads to CDH1 gene silencing and down-regulation of E-cadherin expression, which seems to take place unrelated to HR-HPV E6/E7 oncogenes [51]. In the present series of 150 patients, overall survival was very similar in women with normal/moderately reduced E-cadherin and those with negative/markedly reduced expression, implicating that E-cadherin expression is of no prognostic value even in univariate analysis. These data support the observations reported in the two previous studies [27, 35]. We also controlled whether E-cadherin down-regulation is a predisposing factor for more aggressive behaviour, as previously suggested [39], by correlating the expression to histological grade and FIGO stage. There was no relationship with the histological grade, and only a borderline difference in E-cadherin expression between the FIGO stages ($p = 0.046$, Fisher's exact test). This failure to confirm the data reported by Sun *et al.* [39] is most probably explained by the different grading systems used in these two studies. Thus, the present study does not provide any evidence that E-cadherin expression determined by immunohistochemical staining would be an independent prognostic predictor in CC.

All previous studies assessing E-cadherin expression and HPV detection have failed to establish any association between these two in cervical lesions [22, 28, 32, 33]. Fully consonant with these observations, we also failed to confirm any significant association between E-cadherin expression and HR-HPV detection in this series (Table 2). This lack of any association with HR-HPV is in sharp contrast to the data previously established for $p16^{INK4a}$ [8] and survivin [49], both being independent predictors of HR-HPV also in multivariate analysis. These data implicate that, in contrast to $p16^{INK4a}$ [8], survivin [50], VEGF-C [40], and also PCNA [52], E-cadherin expression analysed using IHC is of no value as a marker of HR-HPV. Considering that the latter are all known to be functionally regulated by HR-HPV oncoproteins [8, 40, 50, 52], the failure to establish any relationship between HR-HPV and E-cadherin in the present study, could suggest that this adhesion molecule is not under the direct control of HR-HPV.

In this respect, of interest are the recent observations on the dual role of E-cadherin in carcinogenesis [17-19]. Apart from its pivotal cell adhesion properties, E-cadherin was shown to participate in some key intracellular signalling pathways, most notably in the canonical Wnt pathway [17, 23]. This pathway could provide the means as to how normally expressed E-cadherin may link the cell-cell adhesion signals and DNA transcription control in the nucleus by sequestering the transcriptionally competent pool of β-catenin and effectively blocking the expression of Lef/TCF/β-catenin responsive genes [17]. However, this normal E-cadherin/catenin complex in the adherens junctions could be disrupted by the concerted action of E6/E7 and ErbB-2 oncogenes, leading to cytoplasmic translocation of E-cadherin and α-, β- and γ-catenins [20, 21]. Such a translocation of the normal membrane to cytoplasmic expression has been demonstrated for E-cadherin in high-grade CIN and CC by several studies [24-27, 30, 32, 33, 36, 38], and also confirmed in the present series (Figures 4 & 6). The exact mechanisms of how E-cadherin down-regulation affects β-catenin-mediated transcription are not clear, although elevated E-cadherin levels are known to suppress β-catenin-mediated signalling [17]. It was speculated that the response of the cells to E-cadherin down-regulation might depend on whether or not the signal is received through the canonical Wnt pathway [17]. Highly interestingly, activation of this canonical Wnt signalling pathway seems to be a prerequisite for transformation of human keratinocytes expressing HR-HPV [23].

To conclude, the present analysis confirms that normal membrane-type E-cadherin expression is retained late in cervical carcinogenesis, with major down-regulation being observed upon transition to CIN3. This down-regulation of E-cadherin parallels with increasing cell proliferation as shown by its significant inverse relationship to PCNA expression [52] (Spearman rho = -0.335, $p = 0.0001$, data not shown). It is tempting to speculate that part of this cell proliferation is mediated through the canonic Wnt signalling pathway, after liberation of transcriptionally competent β-catenin from the E-cadherin/catenin complex, most notably orchestrated by the E6 and E7 oncoproteins of HR-HPV

[17, 20, 21]. This liberation of β-catenin would abrogate the negative transcriptional control of E-cadherin on the Lef/TCF/β-catenin responsive genes and lead to cell proliferation. The failure to establish any direct relationship between E-cadherin and HR-HPV suggests that E-cadherin expression is not under the direct control of HR-HPV, but most probably mediated by other mechanisms, e.g., promoter methylation of the CDH1 gene [14-16, 51]. How the HR-HPV oncoproteins E6 and E7 are involved in this process, remains to be seen in the future studies.

Acknowledgements

This study has been supported by a grant from the Italian Ministry of Health (Ministero della Salute, Ricerca Corrente 1% 2002; Fasc. OG/C). The technical and secretarial assistance of Dr. R. Mancini, Mrs. S. Mochi, and Miss D. Crupi is gratefully acknowledged.

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