Modern Pathology (2011) 24, 855–865

Role and predictive strength of transglutaminase type 2 expression in premalignant lesions of the cervix

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The demonstration that type 2 transglutaminase (TG2) can incorporate polyamine into the E7 oncoprotein of human papillomavirus (HPV) type 18 has led to the hypothesis that TG2 can have a role in the host cellular response to HPV infection. The aim of this study was to investigate whether HPV-related pathology, in infected human cervical epithelium, was associated with modulation of TG2 expression. Normal controls and HPV-infected cervical biopsies were analyzed for the expression of TG2, and the findings were compared with lesion grade. The correlation between TG2 expression and p16, a marker for HPV-induced dysplasia, and the retinoblastoma protein (Rb), a target of the E7 protein of HPV, was also investigated. Results obtained showed that TG2 was absent in normal squamous mucosa, whereas it was present in 100% CIN I lesions. Low-grade lesions showed significantly higher TG2 expression than high-grade lesions ($P<0.0001$). In 94% of CIN I more than 50% of the cells were positive for TG2, with a strong staining intensity (+3), whereas a decreased staining intensity and a low number of positive cells were found in CIN II/III. In CIN I cases, both nuclear and cytoplasmic staining were found in cells exhibiting classical morphological features of HPV infection. In addition, during progression from low-grade squamous intraepithelial lesions to severe dysplasia, TG2 expression was inversely correlated with p16 (Pearson: −0.930), whereas a positive correlation was observed between the expression of TG2 and pRb (Pearson: 0.997). TG2 is expressed in HPV infection as an early phenomenon, not restricted to high-risk genotypes. TG2 upregulation is probably part of host cell reaction against HPV-induced tissue modification. It may act as a cellular antioxidant defense factor, playing an important role in counteracting oxidative damage in neoplastic disease.
Modern Pathology (2011) **24,** 855–865; doi:10.1038/modpathol.2011.40; published online 25 March 2011

Keywords: cervical intraepithelial neoplasia; HPV; p16; pRb; TG2

Persistent infection by a large number of human papillomaviruses (HPV) types has been associated with benign and malignant epithelial lesions of the cervix.[1–3] The effects of papillomavirus infection depends on the infecting HPV type and site of infection, as well as on host factors that regulate virus persistence, regression, and latency. In cells in which the virus replicates the host cell cycle regulators (pRb, p53, p16), expression is altered and DNA replication machinery is reactivated; this allows high-level amplification of the viral genome, leading to virus synthesis in the upper epithelial layers (reviewed in ref. 4). The expression of the viral oncogenes, E6 and E7, enables the infected cells to expand, increasing the number of cells,

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Received 28 July 2010; revised 22 December 2010; accepted 29 December 2010; published online 25 March 2011

which then go on to produce infectious virions.[5] E7 associates with pRb (retinoblastoma tumor suppressor protein) and other members of the pocket protein family and disrupts the association between pRb and the E2F family.[6] A primary role of E6 is its association with p53 preventing growth arrest or apoptosis in response to E7-mediated cell cycle entry in the upper epithelial layers, which might otherwise occur through activation of the ADPribosylation factor pathway.[7] Evidence suggests that several regulatory pathways must be subverted before host cells are transformed.

Type 2 transglutaminase (TG2) has been proposed as a cellular-interfering factor in HPV infection.[8] TG2 is a multifunctional enzyme involved in a variety of biological functions, including cell death, signaling, cytoskeleton rearrangements, and extracellular matrix stabilization.[9–13] Aberrant activation of TG2 or deregulation of its function(s) is involved in a variety of human diseases, such as celiac disease, diabetes, neurodegenerative diseases, multiple sclerosis, and rheumatoid arthritis;[14] a role in inflammatory disorders and septic shock has also been shown.[15,16] A function for TG2 in viral pathogenesis has also been recently suggested by the discovery of a growing number of viral proteins, and the cellular proteins with which they interact, found to be modified by TG2.[17–19] The study of Jeon and coworkers[20] have established that the catalytic activity of TG2 can incorporate polyamine into HPV18 E7, and thereby inhibit its binding to pRb. It is of interest to note that TG2 can translocate from the cytosol into the nucleus incorporating polyamines into the retinoblastoma protein (Rb) to protect it from caspase-mediated degradation.[21] Further data showed that TG2 interact with pRB both in the cytosol and nucleus.[22]

Prompt by these data, and considering that very recently TG2 has been proposed as a biomarker for cervical neoplasia,[23] we decided to accurately document the expression of TG2 in different diagnostic groups to better understand the correlation between TG2 expression and the dynamic changes of cervical intraepithelial lesions. To this aim, the presence of TG2 in HPV-infected human cervical epithelium was analyzed and compared with pRb and p16.

Materials and methods

Patients and Tissue Samples

We performed a retrospective analysis of 60 formalin-fixed cervical biopsies obtained from women who underwent biopsy, between 2007 and 2008, at the INMI 'L Spallanzani' in Rome, after an abnormal Papanicolaou (Pap) test result and abnormal colposcopy. The uterine cervical samples included CIN I ($n=37$) and CIN II–III ($n=23$) lesions. The study included patients who were sexually active, were not pregnant, and who were all positive for HPV DNA test, but negative for other sexually transmitted diseases. The ages of the women ranged between 20 and 50 years (mean age 35 years). No women included in this study had a previous history of cervical neoplasia. Healthy controls ($n=20$) were obtained from the Department of Pathology of the Regina Elena Institute, collecting sections of normal cervical epithelium from patients undergoing hysterectomy for various non-malignant reasons. Controls were age-matched.

One section from all samples was stained with hematoxylin–eosin for routine histopathology and duplicate serial sections used for determination of HPV infection and for immunohistochemistry.

Immunohistochemical Analysis

The CINtec™ $p16^{INK4a}$ Histology kit (DAKOCytomation, Carpinteria, CA, USA), the Rb Ab-1 (DAKO-Cytomation), and the monoclonal anti-TG2 antibody (NeoMarkers, Fremont, CA, USA) diluted at 1:25 were used for our study. For immunohistochemistry, formalin-fixed, paraffin-embedded cervical epithelia sections were used. Three-micrometer sections were mounted on slides, deparaffinized in xylene, incubated for 5 min each in 100, 90, 80, 70, and 50% ethanol for rehydration, and immersed in 10 mM sodium citrate, pH 6.0, and microwaved for antigen retrieval. Endogenous peroxidase activity was blocked by 3% H_2O_2 for 5 min. After rinsing in phosphate-buffered saline (PBS), nonspecific antibody binding was reduced by incubating the sections with normal goat serum for 5 min.

Sections were washed in PBS/1% BSA buffer and incubated with primary antibody. Reactions were visualized by using a streptavidin–biotin–immunoperoxidase system with DAB (Biogenex, San Ramon, CA, USA) as chromogen substrates. Negative control staining was performed by omitting the primary antibody. Sections were counterstained in Mayer's acid hemalum.

Interpretation and Quantification of the Staining

The extent of immunoreactivity in the samples was assessed by two authors, using the same microscope by using a $\times 40$ objective with a field diameter of 0.52 mm. The official histopathology classification of the samples was disclosed after the scoring was performed. Cells were noted as positive for pRb when they showed nuclear immunoreactivity; nuclear as well as cytoplasmic reactivity was considered positive for p16 and TG2. For the interpretation of the results, we determined the positivity considering the different layers of the epithelium: superficial, middle, and deep layers.

Staining intensity was interpreted and scored on a semiquantitative subjective scale as follows: none, (+) weak, (++) moderate, and (+++) strong. Immunohistochemical results were evaluated

considering the overall proportion of positive cells: no staining; 0–10% positive cells; 10–50% positive cells; and >50% positive cells. In addition, for each case a Allred score was determined. The Allred scoring system is a semiquantitative system that takes into consideration the proportion of positive cells (scored on a scale of 0–5) and staining intensity (scored on a scale of 0–3). The proportion and intensity were then added to produce total scores of 0 or 2–8. A score of 0–2 was regarded as negative, whereas 3–8 as positive.[24]

HPV Typing

Formalin-fixed, paraffin-embedded tissues were processed with a QIAamp Tissue kit according to the manufacturer's instructions (Qiagen). The specific primers for the amplification of a fragment of *β-globin* gene were used as a control of DNA extraction.[25] Samples that were found to be negative for β-globin DNA by PCR were considered inadequate for analysis. For detection of HPV DNA, we used the consensus primers MY09/MY11, which amplify a highly conserved 450-bp segment in the L1 region.[26] In HPV-DNA-positive samples, restriction fragment length polymorphism (RFLP) was used to identify more than 40 HPV genital types.[27] Samples that did not have a discernible pattern in RFLPs analysis were retested/retyped using the kit Clinical Arrays Human Papillomavirus (Genomica, Madrid, Spain), which is a commercially available HPV genotyping microarray test and makes it possible to detect simple/single or mixed infection with 35 HPV types.[28] The kit was used according to the manufacturer's protocol; in PCR reaction, we used 10 μl purified DNA for each specimen.

Evaluation and Statistical Analysis

Expression rates of $p16^{INK4a}$ pRb and TG2 were calculated as the proportion of positive reactivities within total cells per high-power field. Statistical analysis was carried out using the Student's *t*-test and Pearson's correlation test. The χ^2 test was used to test for a potential association between study variables of interest. Differences were considered significant when *P*-values were <0.05.

Results

HPV Distribution in Cervical Lesions

A total of 80 samples were included in this study. Except for normal tissues (20 cases), which were negative for HPV infection, all dysplastic lesions (60 cases) showed HPV genotype positivity (Table 1). Cervical samples were assayed for 25 HPV types, 14 types were of high oncogenic risk (HR) (16, 18, 31, 33, 34, 35, 39, 45, 51, 52, 53, 56, 58, and 59) and 11 were of low oncogenic risk (LR) (6, 11, 40, 43, 44, 61, 70, 72, 81, 83, and 84). In the CIN I, HR-HPV genotypes were found in nine of the 37 lesions tested (24.3%), whereas 28 lesions were infected by LR types (75.6%). All the CIN II/III lesions, except one, displayed HR genotypes. Multiple HPV infection was detected in 19 (51.3%) CIN I cases and in 13 (56.5 %) CIN II/III cases (Table 1).

Table 1 Frequency of HPV types in CIN, as single or multiple types

	Histological grading	
HPV type	*CIN I (n = 37)*	*CIN II/III (n = 23)*
High oncogenical risk		
HPV16	4 (10.8%)	7 (30.4%)
HPV18		3 (13%)
HPV31, 33, 52, 58	3 (8.1%)	7 (30.4%)
Multiple other HR (34, 35, 39, 45, 51, 53, 56, 59)	2 (5.4%)	5 (21.7%)
Low oncogenical risk		
HPV6	11 (29.7%)	
HPV11	3 (8.1%)	
Multiple other LR (40, 43, 44, 61, 70, 72, 81, 83, 84)	14 (37.8%)	1 (4.3%)

CIN, cervical intraepithelial neoplasia.

Changes in p16 and pRb Expression

We first analyzed the expression of p16 and the Rb in relation to the lesion grade, compared with normal squamous cervical epithelium from controls.

p16 immunostaining was classified as positive when intense diffuse cytoplasmic and nuclear immunoreactivity was observed. In normal squamous epithelium, p16 was negative (Figure 1). In CIN I lesions, p16 was detected in 14/37 cases (37.8%) (Table 2). The staining pattern in CIN I lesions was focal or diffuse, confined in the middle layer of the epithelium or localized within the basal to intermediate layer (Figure 1). The immunoreactivity was found in a proportion of cells ranging from 10 to 50% in the majority (83%) of the positive cases (Table 2). In high-grade CINs, p16 positivity was found in all the epithelial layers (Figure 1). A strong staining was observed in a proportion of cells >50% in 89% of CIN II/III cases (Table 2).

The pattern of pRB immunostaining is summarized in Table 2 and representative immunostainings are shown in Figure 1. Nuclear immunoreactivity of Rb was detected in all HPV-negative normal cervical epithelia. Highly proliferative basal cell layers displayed undetectable levels of pRb, whereas in 19/20 cases, pRb was expressed in parabasal cell layers and in the intermediate cell layers, decreasing in intensity with the grade of differentiation of the squamous epithelial cells (mature cells of the upper layer were completely negative for pRb protein

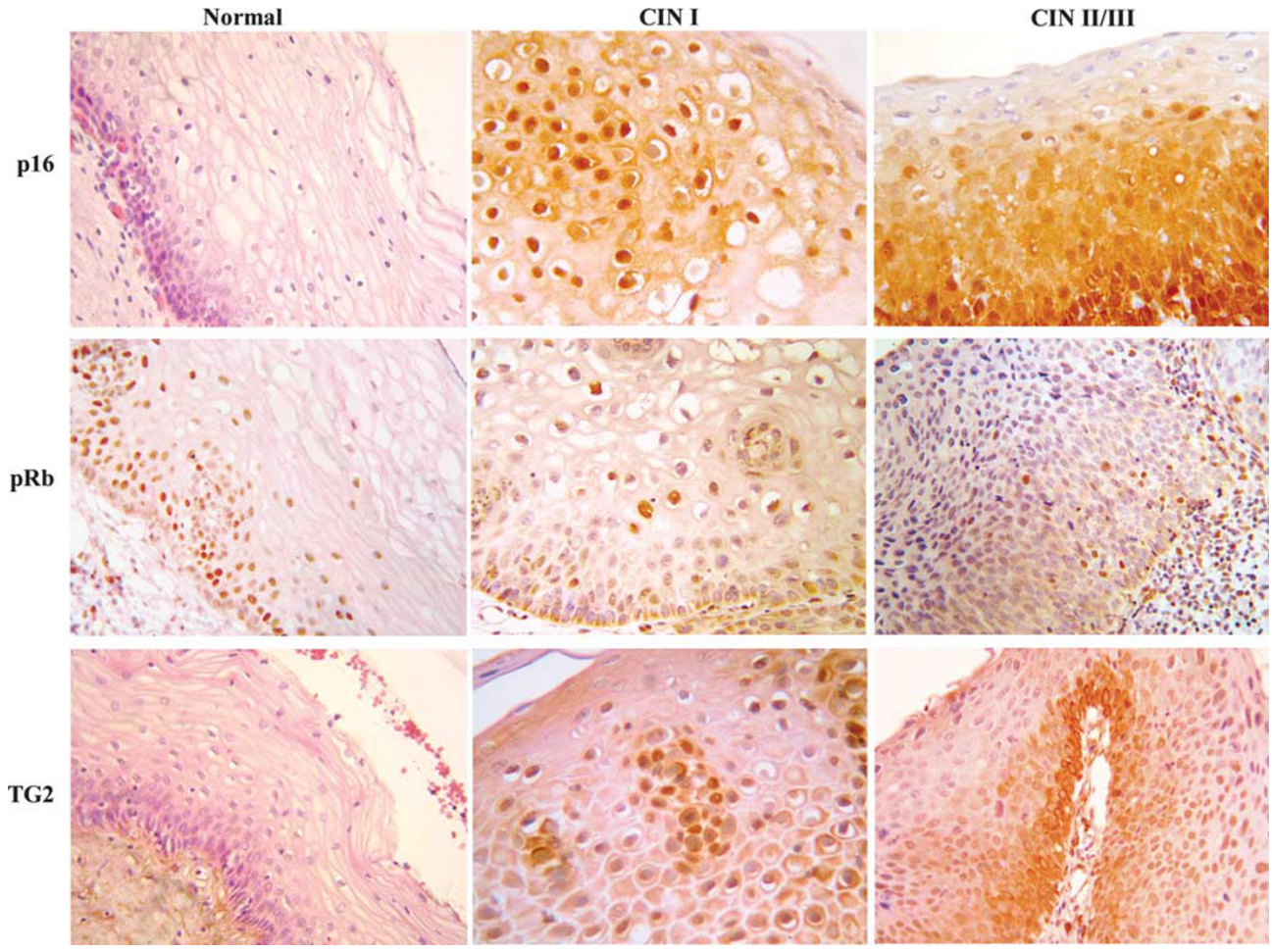

Figure 1 Immunohistochemical detection of p16, phosphorylated retinoblastoma protein (pRb), and type 2 transglutaminase (TG2) displays different features in normal cervical squamous epithelium (left column) in cervical intraepithelial neoplasia (CIN) I (central column) and CIN II/III (right column) lesions. Tissues were hematoxylin counterstained. Normal cervical epithelium exhibits negative p16 and TG2 staining, and a strong nuclear positivity for pRb, restricted to the parabasal layer (original magnification $\times 40$). In CIN I lesions (original magnification $\times 63$), p16 immunostaining (detected in 14/37 cases) appear diffusely positive, mainly confined to the nuclei; pRb exhibits nuclear immunoreactivity restricted to the middle layers; and TG2 staining, both nuclear and cytoplasmic, show a correlation with cell atypia. In CIN II/III (original magnification $\times 40$), p16 displays a diffuse and strong staining in both nuclei and cytoplasm; pRb appear irregularly expressed (10% positive cells) throughout the full thickness of the dysplastic mucosa; and TG2-positive cells are present in the deeper half of the epithelial layer.

expression) (Figure 1). Staining of the cells was strong (3+) in 13/20 cases (Table 3), and the proportion of the cells stained was, in most cases, between 10 and 50%. pRb expression was detected in 37/37 cases of CIN I, and in 20/23 (89%) cases of CIN II–III (Table 2). The proportion of positive cells was between 10 and 50% in most of low-grade dysplasia (Figure 1), whereas it became less than 10% in the majority of high-grade dysplasia, with scattered nuclear positivity (Figure 1).

TG2 Expression in Cervical Epithelium

TG2 expression was absent in normal squamous mucosa; scattered positivity for the protein was detected only in some stromal components of the cervical mucosa (Figure 1).

In contrast, TG2 was detected in 100% of CIN I lesions (Table 2). Interestingly, TG2 expression correlated with the degree of HPV-related cellular atypia (Figure 1), whereas the superficial layer of the epithelium was negative or the staining was less intense. Of note, both nuclear and cytoplasmic staining was found in cells exhibiting classical morphological features of HPV infection, that is, koilocytes (Figure 2a). The staining pattern of TG2 in low-grade squamous intraepithelial lesions (LSIL) is shown in Figure 3; interestingly, the level of TG2 expression is not correlated to HPV genotype. Among the CINII–III lesions, 18/23 cases (78%) showed TG2 expression (Table 2). The immunoreactivity was found in deep layers (Figure 1). Cells exhibit cytoplasmic or nuclear immunoreactivity, or both, according to the position in the epithelium and to the HPV cytopathic effect (Figure 2b). The

Table 2 Expression of p16, pRb, and TG2 in normal squamous cervical mucosa and CIN

	Proportion of positive cells			
	Positive cases	*<10*	*10–50*	*>50*
Normal (n = 20)				
p16	0/20			
pRb	19/20		94.7%	5.3%
TG2	0/20			
CIN I (n = 37)				
p16	14/37	16.6%	83.4%	
pRb	37/37	34.4%	65.6%	
TG2	37/37		6.3%	93.7%
CIN II/III (n = 23)				
p16	23/23		11.2%	88.8%
pRb	20/23	75%	25%	
TG2	18/23	14.3	57.2%	28.5%

CIN, cervical intraepithelial neoplasia.

Table 3 Intensity of p16, pRb, and TG2 expression in squamous cervical mucosa

	Normal (n = 20)	*CIN I (n = 37)*	*CIN II/III (n = 23)*
p16			
Negative	20	23	0
Weak (+)	0	2	0
Moderate (++)	0	9	10
Strong (+++)	0	3	13
pRb			
Negative	2	0	3
Weak (+)	0	0	0
Moderate (++)	5	5	6
Strong (+++)	13	30	14
TG2			
Negative	20	0	5
Weak (+)	0	0	0
Moderate (++)	0	4	6
Strong (+++)	0	33	12

CIN, cervical intraepithelial neoplasia.

increase in CIN grade was accompanied by a strong TG2 positivity in the stroma (Figure 2c).

Relation between TG2 Expression and Lesion Grade

Analyzing the level of TG2 expression in cervical lesions, a highly statistically significant difference was found for TG2 expression and lesion grade ($P<0.0001$). More than 50% positive cells were observed in 35 out of 37 CIN I (94%) and in five of 23 CIN II/III cases (22%) (Table 4). Analysis of TG2 expression (Figure 4) revealed that the majority of CIN I cases (33/37) showed a strong intensity (3+) of immunoreactivity (Table 3), whereas a decrease in the strength of the staining was found in the

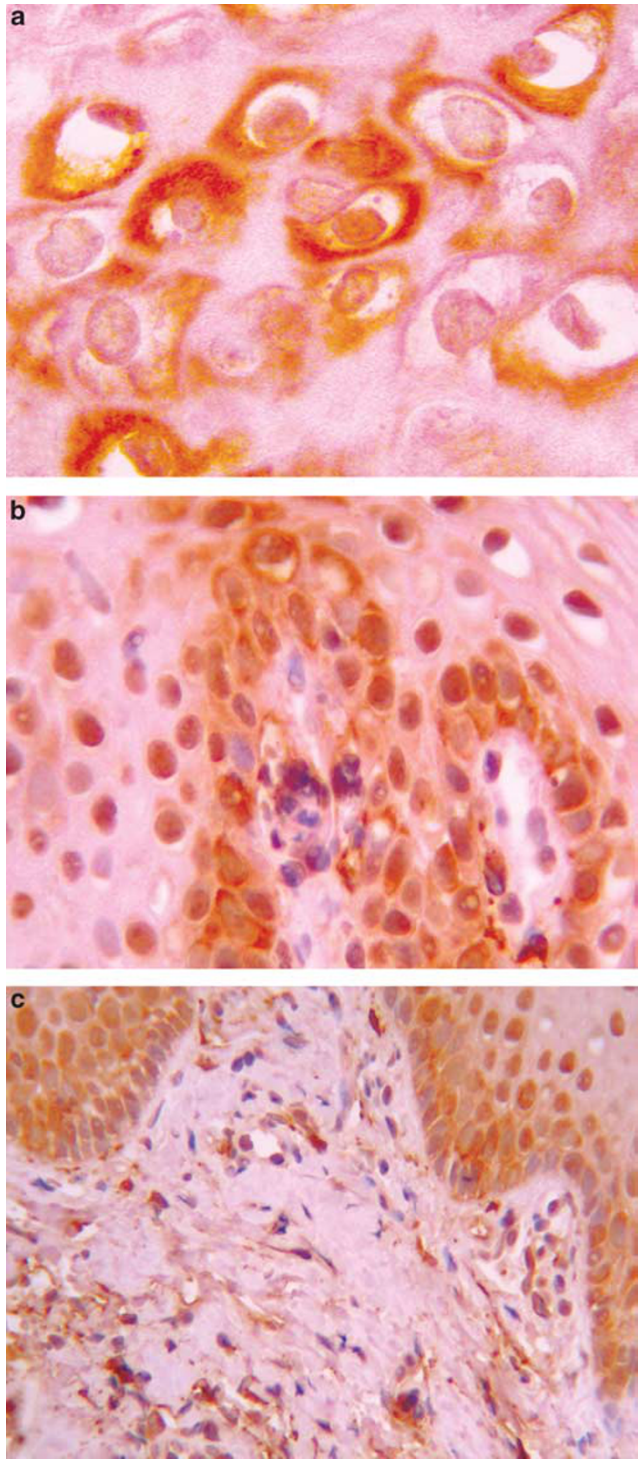

Figure 2 Immunohistochemical staining of type 2 transglutaminase (TG2) in human papillomavirus (HPV)-induced cervical dysplasia. (**a**) Typical koilocyte features with acentric, enlarged nucleus displaced by a large perinuclear vacuole surrounded by a thickened cytoplasm; these cells exhibit both nuclear and cytoplasmic TG2 immunostaining. (**b**) Diversity of TG2 staining in cells of high cervical intraepithelial neoplasia (CIN) lesion: the positivity is observed in the nucleus, in the cytoplasm, or both. (**c**) TG2 immunopositivity in cervical stroma is shown. Original magnifications: (**a**) ×63; (**b**, **c**) ×40.

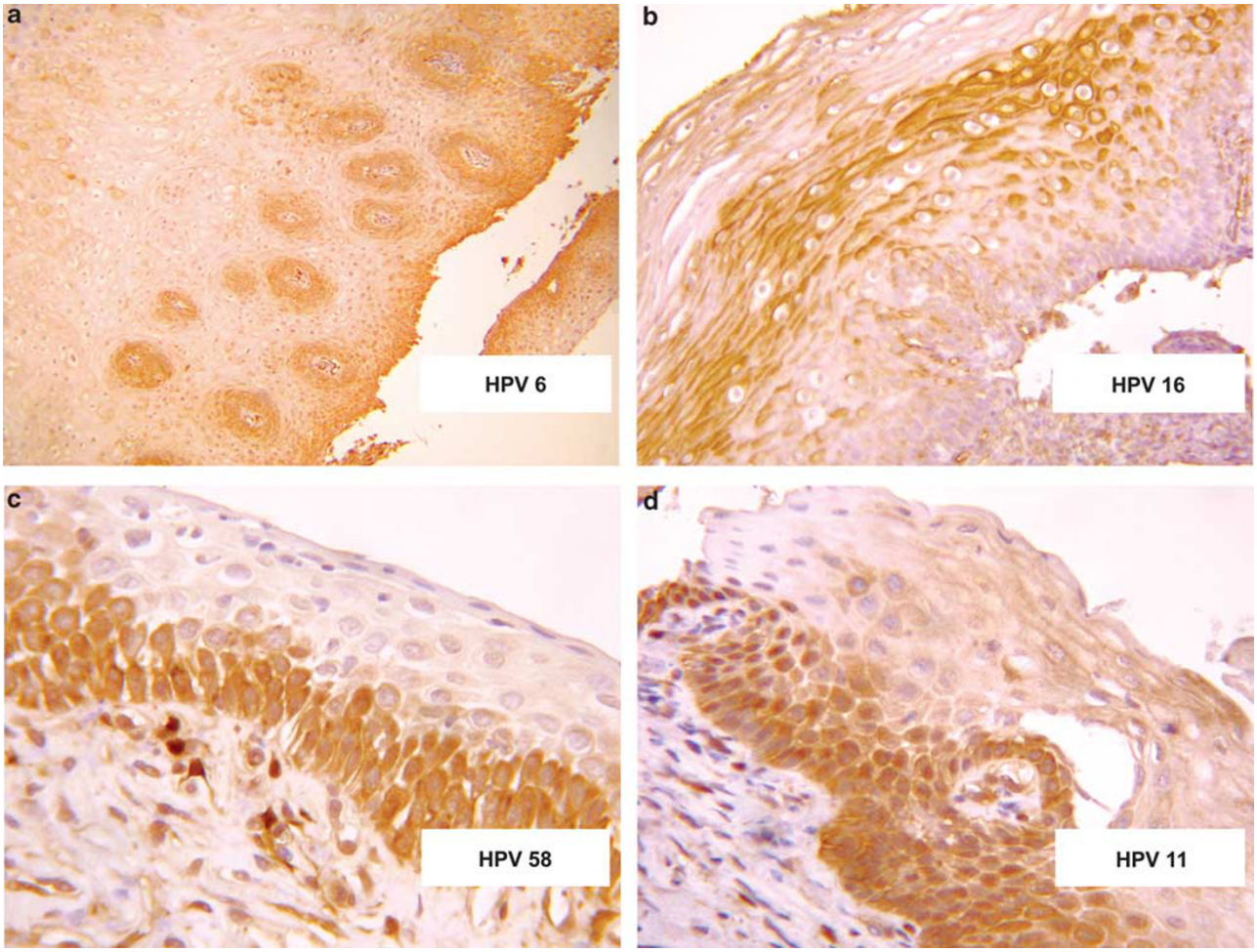

Figure 3 Type 2 transglutaminase (TG2) immunostaining in low-grade squamous intraepithelial lesions (LSIL): human papillomavirus (HPV)-induced lesions not qualified as cervical intraepithelial neoplasia (CIN) *vs* CIN I. HPV genotypes are indicated. (**a**) Condyloma acuminatum with acanthosis and papillomatosis: both nuclear and cytoplasmic TG2 positivity in koilocytes. (**b**) Flat condyloma: TG2-stained koilocytes in intermediate epithelial layer (**c**, **d**) in the CIN I lesions accompanied by koilocytotic atypia TG2 positivity is characteristically showed by basal and parabasal epithelial layers with a strong intensity in the majority of cells. The expression level of TG2 was independent of the HPV virotype.

Table 4 Comparison of TG2 immunostaining between low-grade and severe cervical *lesions*

TG2 expression	*CIN I*	*CIN II/III*	*Total*
≤50%	2	18	20
≥50%	35*	5*	40
	37	23	60

*$P<0.001$.

CIN II/III. A particularly high statistically significant association was obtained between the TG2 overexpression and CIN I lesion ($P<0.0001$). To avoid subjective or misleading interpretation, the immunohistochemical results were rescored according to the Allred method. The frequency distribution of TG2 immunohistochemical results based on the Allred score is shown in Figure 5. In 100% of CIN I

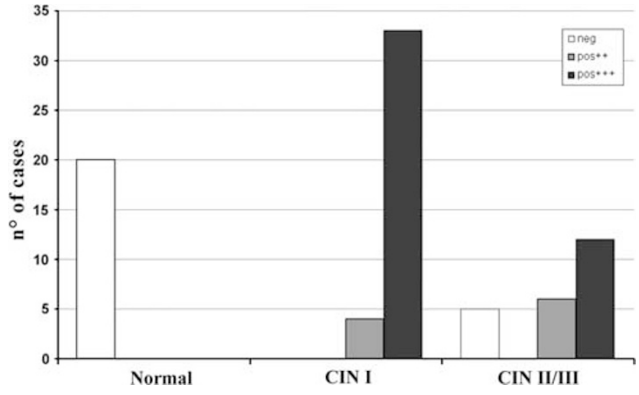

Figure 4 Distribution of type 2 transglutaminase (TG2) expression level according to the different lesion grades. Protein expression in normal and cervical intraepithelial neoplasia (CIN) lesions was analyzed by χ^2 test. A statistically significant association was found between TG2 overexpression and CIN I lesion ($P<0.0001$; confidence interval (CI) 95%).

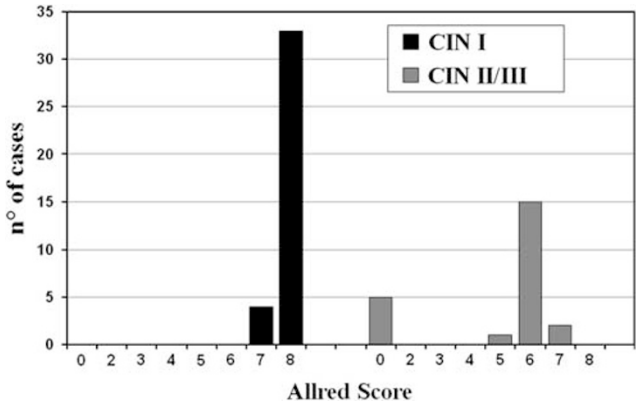

Figure 5 Frequency distribution of type 2 transglutaminase (TG2) immunohistochemical results based on the Allred score.

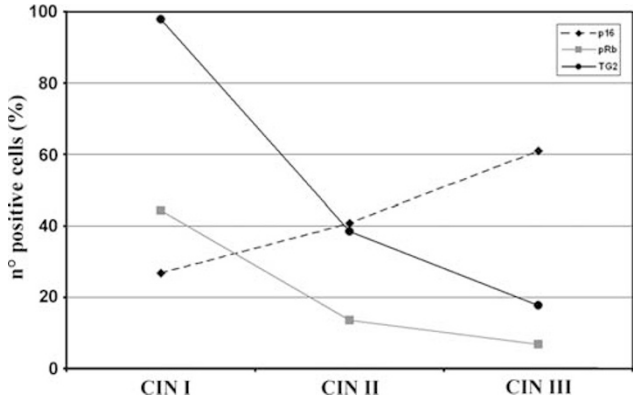

Figure 6 Correlation between p16, phosphorylated retinoblastoma protein (pRb), and type 2 transglutaminase (TG2) expression, and cervical intraepithelial neoplasia (CIN) grade. The number of p16-positive cells increases with an increasing CIN grade, whereas pRb- and TG2-stained cells decrease with an advanced CIN grade. A positive correlation was found between the expression of TG2 and pRb (Pearson value = 0.997).

cases, the Allred score was high (an Allred score of 8 was assigned in 89% of cases), again indicating a strong TG2 positivity in these lesions. Among the CIN II/III cases, five cases were completely negative (score 0); in only two cases was an Allred score of 7 assigned, whereas 16/23 (70%) cases showed an intermediate Allred score (scores 5 and 6).

Correlation between TG2, p16 and pRb Expression in Cervical Intraepithelial Lesions

The results of correlation between the expression of p16, pRb, TG2, and CIN lesions are illustrated in Figure 6. The expression of p16 increased with increasing CIN grade; in contrast, pRb and TG2 expression decreased with an advanced CIN grade. The percentage of positive cells within a given lesion showed that TG2 expression was inversely correlated with p16 (Pearson value = −0.930), whereas a positive correlation, during progression from LSILs to severe dysplasia, was observed between the expression of TG2 and pRb (Pearson value = 0.997). A consistent relationship between pRb and TG2 was found in the epithelial area of immunopositivity distribution; however, the percentage of stained cells was greater for TG2 (Figure 6). Significantly, although in normal cervical epithelium from healthy controls TG2 was not expressed, TG2 in the mature squamous epithelium contiguous with dysplastic area from HPV-positive patients was expressed in the cells of the middle layers (Supplementary Figure 1).

TG2 Expression Pattern in Atypical Squamous Epithelial Changes

To better determine the predictive value of TG2 for the diagnosis of low-grade dysplasia, different equivocal cases, which may cause diagnostic challenges (especially when superimposed onto HPV-associated changes), were evaluated for TG2 expression. Results obtained, concerning both reactive/inflammatory changes (Figure 7) and immature squamous metaplasia (Figure 8), showed that TG2 positivity is consistent with HPV-related changes, thus making it possible to distinguish between reactive squamous atypia and HPV-associated cervical dysplasia.

Discussion

In this study, we have analyzed the expression of TG2 in the epithelium of human cervix in relation to HPV-induced dysplastic modifications. Results obtained show that TG2 is undetectable in healthy normal tissue, whereas it is strongly upregulated in early cervical lesions in HPV infection.

HPV infection causes a number of changes in gene or protein expression within the infected host cells. Epithelial differentiation triggers the productive phase of the HPV life cycle, which includes genome amplification, activation of late gene expression, and assembly of mature virions. HPV-positive cells re-enter into the S phase upon differentiation due to the actions of the viral oncoproteins.[29] The binding of viral E6 with p53 has been shown to destabilize wild-type p53, which should also facilitate cell cycle progression. Similarly, the binding of E7 to Rb has been linked to its transforming capacity, possibly due in part to its upregulation of Akt activity.[30] This sequence can abrogate normal cellular checkpoints and interfere with processes intended to eliminate aberrant cells, including apoptosis/autophagy and senescence.

Studies show that TG2 is involved in the regulation of cell death processes. TG2 is the most peculiar member of a large family of transamidating acyltransferases called transglutaminases.[13] Although mainly localized in the cytoplasm, TG2 can also be secreted outside the cell where it regulates

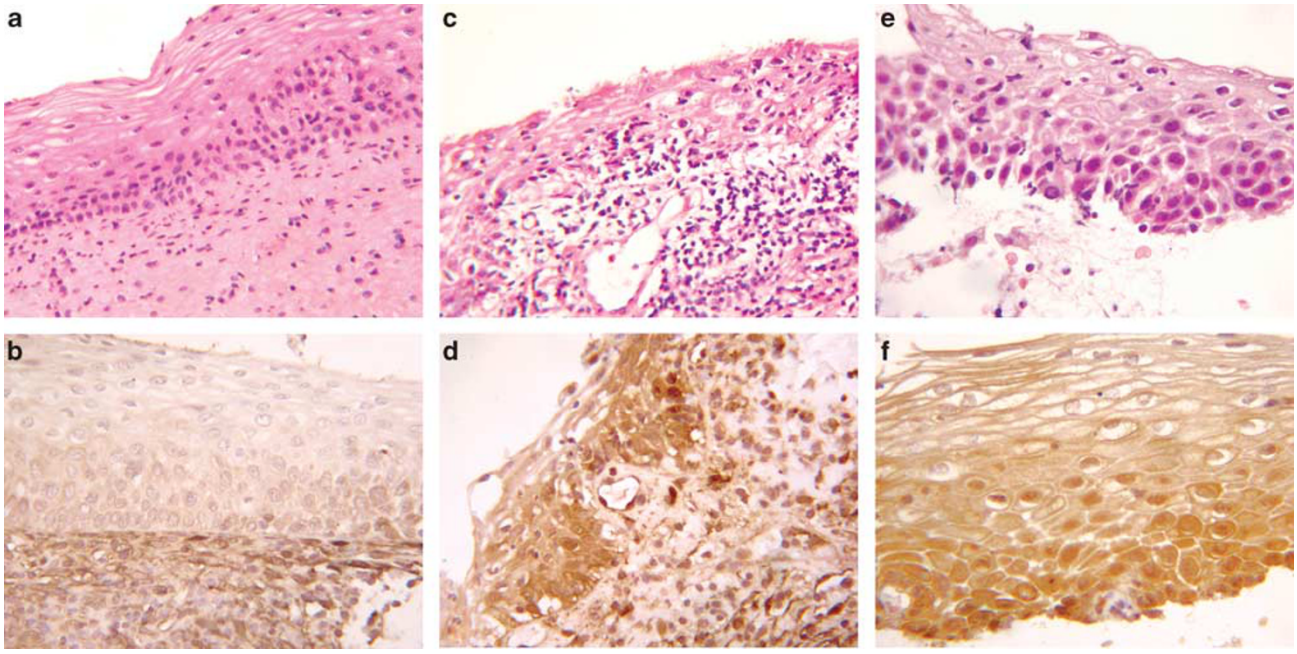

Figure 7 Squamous atypia: reactive *vs* dysplastic. (**a**) Hematoxylin (HE) staining of inflammatory atypia of the squamous epithelium, with exudates (lymphocytes) and basal cells hyperplasia. (**b**) Type 2 transglutaminase (TG2) negative or weak staining suggests an exocervical inflammatory/reactive atypia. (**c**) HE staining of squamous equivocal atypia. (**d**) TG2 strong positivity reveal low-grade squamous intraepithelial lesion human papillomavirus (HPV) related. (**e**) HE staining of disturbed epithelial maturation with nuclear abnormalities in the lower third of the epithelium. (**f**) Strong nuclear and cytoplasmic TG2 immunoreactivity evidentiates cervical intraepithelial neoplasia (CIN) I lesion accompanied by koilocytotic atypia. Original magnifications: $\times 40$.

cell–matrix interactions[31] and can translocate into the nucleus[32] by interacting with importin-α3 (ref. 33). The nuclear TG2 can incorporate polyamines into the pRb to protect it from caspase-mediated degradation.[34] In addition, under circumstances in which the TG2 kinase activity predominates and its transamidating activity is suppressed (eg, high ATP, low Ca^2 levels), translocation of TG2 to the nucleus could favor cell proliferation and protect against apoptosis by enhancing the Rb phosphorylation state.[35] The prevalence of a specific activity and the different localization inside the cell underlie TG2's pro- or an antiapoptotic function.[36,37] The results of this study showed a strong positive correlation in HPV-infected mucosal epithelium between TG2 expression and pRb. Rb is considered to be a major functional target of HPV E7 protein. The range of pRb functions is complex. The absence of functional pRb may result in apoptosis rather than in uncontrolled cell proliferation,[38] whereas overexpression of functional pRb may induce apoptosis or rescue cells from death, depending on the system.[39] Recently, it has been suggested that the TG2 interaction with Rb increases significantly concomitant with an attenuation of apoptosis.[40] Accordingly, the increased proliferation rate in the low-grade lesions of HPV-infected epithelium is only rarely associated with measurable apoptosis.[41]

In vitro studies have shown that the transamidating activity of TG2 can incorporate polyamine into HPV18 E7, and thereby inhibit its binding to Rb; in contrast, TG2 failed to modify E7 protein of HPV16.[20] Thus, the authors propose TG2 as a cellular-interfering factor of HPV-induced carcinogenesis, and suggest that TG2's inability to inactivate HPV16 E7 could explain the high prevalence of HPV16 in cervical cancer.[20] Indeed, to date, no evidence for polyaminated E7 being present in infected cervical epithelium has been reported. Our results on the expression levels and subcellular localization of TG2 in cervical biopsies have established that the presence of the enzyme is not related to the HPV genotypes; indeed, TG2 upregulation was found in precancerous lesions of both low- and high-risk HPV types.

In a recent work, Gupta and co-workers[23] have proposed that TG2 immunostaining can supplement p16 immunostaining as an additional biomarker for the identification of cervical dysplasia. Our data concerning the expression of TG2 in cervical lesions show a statistically significant association between TG2 upregulation and LSIL, whereas TG2 expression was found to be strongly reduced in high CIN grade. TG2 appeared to be associated with the early changes in HPV-induced modifications. Our findings suggest that at the LSIL stage or before it, a process is initiated that leads to the expression of TG2. The reasons for this initial upregulation are not clear. It can be hypothesized that TG2 expression in early lesions is a cellular response to the presence of HPV. In the infected epithelium, some cells characteristically display enlarged hyperchromatic nuclei with cytoplasmic halos (koilocytotic atypia). The mature virus usually concentrates in this cell

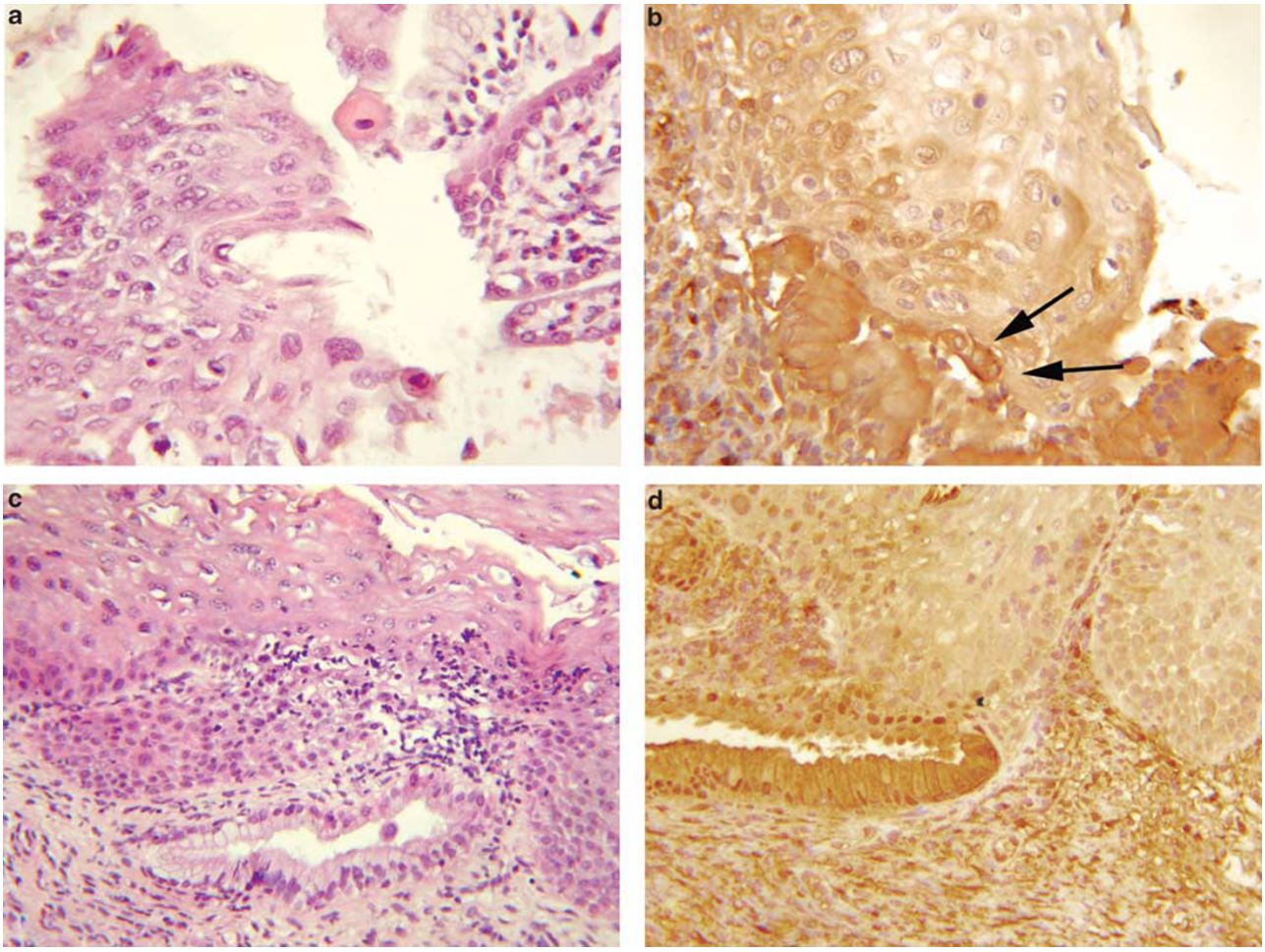

Figure 8 Squamous metaplasia. (**a**) Hematoxylin (HE) staining of complex pattern resulting from cervical immature squamous metaplasia with monocellular dyskeratosis. (**b**) A strong type 2 transglutaminase (TG2) cytoplasmic staining is visible in dyskeratotic cells (arrows) and endocervical epithelium as expression of human papillomavirus (HPV)-related changes. (**c**) HE staining of disturbed epithelial maturation and squamous metaplasia; in this case, TG2 immunoreactivity (**d**) with nuclear and cytoplasmic positivity shows immunohistochemical features of an HPV-related low-grade squamous intraepithelial lesions. Original magnifications: (**a**, **b**) $\times 63$; (**c**, **d**) $\times 40$.

population, with most of HPV proteins localized in the nucleus.[42] Interestingly, results reported here show that TG2 is mainly expressed in koilocytes. Of note, similar to pRb, TG2 in these cells, in addition to cytoplasmic positivity, showed a strong nuclear staining, thus supporting the view of a possible physical interaction leading to pRb inactivation.

TG2 is largely excluded from the nucleus, but translocates there in response to specific stressors. This translocation places TG2 in proximity to components of transcriptional machinery, especially those affecting cell survival after insults.[43] Oxidative damage is markedly increased in CIN.[44] It is possible that TG2 shuttles in the nucleus to protect the cells by modulating hypoxia-mediated transcriptional events, possibly by attenuating HIF activation of pro-cell death genes as observed during ischemic stroke.[45]

The purpose of our study has been to clarify the role of TG2 during the evolution of CIN lesions. The level of TG2 expression shows a strong association to the early changes in HPV-induced modifications.

The development of invasive cancer (HPV infection, development of lesions, and pre-cancer) occurs over a period of approximately 10 years. Thus, high-sensitivity markers to detect early-stage disease are needed for improved staging. We suggest that TG2 could be a useful tool for detecting disease in its early stages, thus increasing the predictive value of current screening methods. Additional studies are being carried out on patients' follow-up so as to evaluate the persistence of CIN lesions and to assess TG2's potential clinical relevance for progress prediction of cervical cancer and for the triage management of LSIL.

Disclosure/conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Modern Pathology website (http://www.nature.com/modpathol)