ORIGINAL ARTICLE

Correlation between laminin-5 immunohistochemistry and human papillomavirus status in squamous cervical carcinoma

G A V Boulet, I Schrauwen, S Sahebali, C Horvath, C E Depuydt, A Vereecken, D Vanden Broeck, E A Van Marck, J J Bogers

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See end of article for authors' affiliations

Correspondence to: Dr S Sahebali, Ambior-IX, Laboratory of Cell Biology and Histology, University of Antwerp, Groenenborgerlaan 171, BE-2020 Antwerp; shaira.sahebali@ua.ac.be

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Background: Human papillomavirus (HPV) plays a critical role in the carcinogenesis of squamous cervical carcinoma. Integration of viral DNA into the host genome is a major contributing factor to malignant transformation. Viral load may influence integration.
Aims: To compare HPV status (type, viral load, integration status) between normal samples, carcinoma in situ and invasive carcinoma in order to elucidate the role of HPV in progression to invasive lesions.
Methods: The study population comprised 10 biopsy samples from each diagnostic group. Laminin-5 immunohistochemistry was performed to distinguish invasive carcinoma from non-invasive high-grade lesions. Real-time PCR was used to detect specific HPV types, viral load and integrated HPV, with quantification of viral E2 and E6 genes.
Results: Invasive carcinomas contained a higher number of laminin-5 immunoreactive cells as compared to non-invasive lesions. Almost all samples contained HPV, with a higher viral load and copy number of HPV16 integrated in E2 in cases of laminin-5 immunoreactivity and cases of invasive carcinoma. High HPV16 viral load was associated with more integrated copies in E2.
Conclusions: HPV is important in progression from carcinoma in situ to invasive carcinoma. Viral load and HPV integration influence the development of cervical cancer towards invasiveness. Overall HPV status may be more predictive of patient outcome and may influence patient management.

Cervical cancer is the third most common malignancy among women worldwide.[1] The critical role of human papillomavirus (HPV) in the carcinogenesis of cervical squamous cell carcinoma (SCC) is well established.[2 3] Persistent infection with a high-risk type is a prerequisite, but other viral, environmental and host factors are of importance[1 3–6] in the progression to malignancy.

In high-risk HPV the viral oncogenes E6 and E7 are essential for malignant transformation of host cells. Control over their expression is often lost during integration, as this event frequently causes disruption of the E2 gene, an important regulatory protein of E6 and E7.[4 7 8] Integration is therefore one of the major contributing factors to malignant transformation. Viral load may influence integration, as the chance of integration should increase with increasing viral copy numbers present in the host cells.[7 9] Viral load has been suggested to be a marker of persistent infection and is associated with increased risk of dysplasia and carcinoma in situ (CIS),[10–12] but scant data exist concerning the connection to invasive cancer. The role of integration in the progression of cervical lesions towards invasiveness is unclear too, as invasive carcinomas sometimes develop containing only episomal, non-integrated HPV.[13 14] It may even be hypothesised that, once a certain threshold or phase in malignant progression has been reached, HPV is no longer necessary to stimulate the evolution of the malignant phenotype of the host cells. This would account for those cases of invasive carcinoma in which no HPV was found.

This study investigated and compared HPV status (type, viral load, integration status) between normal cervical samples, CIN3 and SCC in order to elucidate the role of HPV in progression from non-invasive to invasive lesions. Gene expression and translation of the extracellular matrix protein laminin-5 is up-regulated in SCC, especially in cells of the basal epithelial layer, which enables distinction between invasive and non-invasive high-grade lesions by laminin-5 immunohistochemistry.[15–17]

MATERIALS AND METHODS

Study population

Formalin-fixed, paraffin-embedded archival material from cone biopsies and hysterectomies taken during treatment and follow-up of cervical abnormalities during the period January 2000 to January 2005 was used. Sample selection was based on histopathological assessment. Ten normal cases, 10 cases of severe cervical intraepithelial neoplasia (CIN3) and 10 invasive cervical squamous cell carcinomas (SCC) were taken at random. Selection was performed in accordance with the guidelines of the Ethical Committee of the University of Antwerp.

HeLa cells (30–40 copies of HPV18) and SiHa cells (1–2 copies of HPV16) were embedded in paraffin, processed identical to the biopsy specimens and used as positive controls.

Immunohistochemistry

Sections of 4 μm were deparaffinised and rehydrated. Staining was performed according to the manufacturer's protocol with some minor modifications.[15 16] Application of the primary monoclonal anti-laminin-5 (laminin γ 2 chain) antibody (1:50; DakoCytomation, Glostrup, Denmark) was followed by application of horseradish peroxidase-labelled secondary antibody (DakoCytomation) for 30 min. Negative controls were treated similarly, without using the primary antibody. An invasive lesion was used as positive control.

Epithelial cells with distinct cytoplasmic immunoreactivity on light microscopy were considered immunopositive. The result was expressed as the percentage of immunoreactive cells in the basal epithelial layer. Individual immunoreactive epithelial cells in the stromal tissue were considered separately as either present or absent. Staining intensity was not graded.

Abbreviations: CIN, cervical intraepithelial neoplasia; CIS, carcinoma in situ; HPV, human papillomavirus; SCC, squamous cell carcinoma

Table 1 Primer sequences (Tib Molbiol) used in real-time PCR integration assay[7]

Target	Sequence
HPV16 E2 hinge region	Forward: 5′-AACGAAGTATCCTCTCCTGAAATTATTAG-3′ Reverse: 5′-CCAAGGCGACGGCTTTG-3′
HPV16 E6	Forward: 5′-GAGAACTGCAATGTTTCAGGACC-3′ Reverse: 5′-TGTATAGTTGTTTGCAGCTCTGTGC-3′
GAPDH housekeeping gene	Forward: 5′- CCACATCGCTCAGACACCAT-3′ Reverse: 5′-GTGACCAGGCGCCCAAT-3′

HPV, human papillomavirus; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

HPV testing, typing, determination of viral load and integration

For each sample, three 10 μm sections were cut and placed in DNase-free and RNase-free Eppendorf tubes. DNA isolation was performed with the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

HPV testing, typing and viral load determination were performed with ABI Prism 7500 and 7700 (Applied Biosystems, Foster City, California, USA). Type-specific primers were used, targeting the E6 or E7 sequences of HPV types 6, 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66 and 68.[18 19] To quantify the number of host genomes, a fragment from the β-globin gene was amplified in parallel using GH20 and PC04 primers.[18 20] The Taqman Universal Master mix was used according to the manufacturer's protocol (Applied Biosystems).

Ct values were calculated by determining at which point fluorescence exceeded a background limit. Viral load was determined as the number of HPV DNA copies per β-globin gene copy.

All HPV16 positive samples were tested for disruption of the HPV16 E2 gene. E2 and E6 genes of HPV16 were quantified by LightCycler (Roche, Mannheim, Germany) using the LightCycler Faststart DNA Master^{PLUS} SYBR Green I kit (Roche). Control DNA for the E6 primer set was extracted from SiHa cells. The E2 primers were designed to recognise the E2 ''hinge'' region, which is most often deleted on HPV16 integration (see table 1).[7 21] Because of the deletion of this target region in SiHa cells, control DNA for the E2 primer set was extracted from a clinical sample containing complete HPV E2. Control DNA was isolated using the standard protocol and was used as calibrator to normalise for sample inhomogeneity and variability of detection. Calibrator-normalised relative quantification with efficiency correction was performed by LightCycler Relative Quantification Software V.1.0.

Statistical analysis

Data were analysed using SPSS V.12.0 for Windows 2000 (SPSS Inc., Chicago, Illinois, USA) and Prism 4 for OSX (Graphpad Software Inc., San Diego, California, USA). Kruskal–Wallis, Mann–Whitney U and Spearman's correlation r-tests were performed. A p-value $\leqslant 0.05$ was considered to be statistically significant. Analysed data were expressed as mean ± standard error of the mean (SEM). Only samples with diagnosis CIN3 and SCC were included in the comparison of laminin-5 immunopositivity to viral load and integration.

RESULTS

Laminin-5 immunohistochemistry

Immunoreactive cells were located at the invasive front (fig 1), with individual immunoreactive epithelial cells sometimes present in the stromal tissue. Histopathological review of tissue sections and comparison with immunohistochemistry led to the exclusion of one case from further analysis because it was an adenosquamous carcinoma. Two cases of invasive SCC were reclassified as CIN3, because the invasive lesion itself was no longer present in the slides used for immunohistochemistry and viral load and integration assays.

SCC contained significantly more laminin-5 immunoreactive cells as compared to the other two groups, without significant difference between CIN3 and normal samples ($p \leqslant 0.01$; fig 2). Normal samples did not contain any immunoreactive cells. Individual immunoreactive epithelial cells were more often present in the stromal tissue as the severity of the diagnosis increased ($p \leqslant 0.01$; data not shown).

HPV typing

DNA was amplified in all samples. In single infections types 16, 33 and 52 were found (table 2). Most samples contained multiple types in double or triple combinations: 16/58, 16/18, 16/67, 58/59, 52/58, 33/52, 16/18/67 and 16/18/58. The three negative samples were histologically normal.

There were no significant differences in laminin-5 expression between HPV positive and negative samples, nor for any specific HPV type (data not shown).

Viral load

The distribution of the mean viral load for each type, expressed as the viral copy number per cell, was as follows: 6.36 (HPV16), 0.01 (HPV18), 0.43 (HPV33), 13.18 (HPV52), 0.01 (HPV58), <0.01 (HPV59) and <0.01 (HPV67). Table 3 shows the

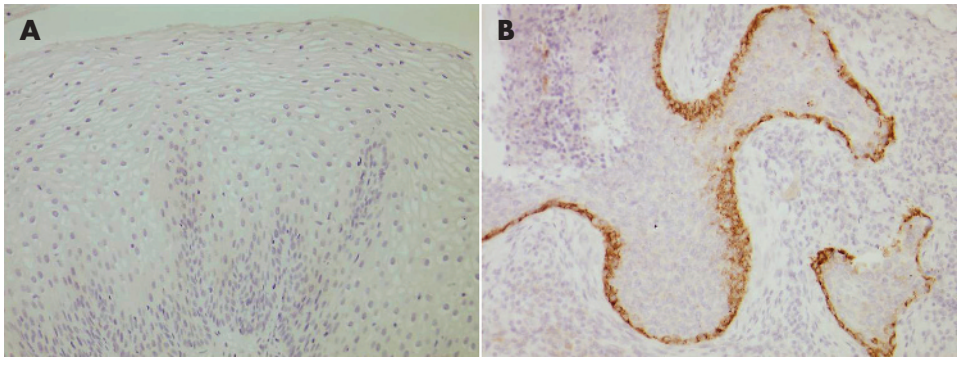

Figure 1 Laminin-5 immunohistochemistry. (A) Normal cervical squamous epithelium. (B) Squamous cell carcinoma. Both at 100× magnification.

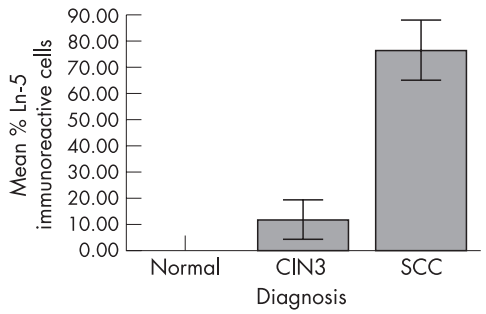

Figure 2 Mean percentage laminin-5 immunoreactive cells in each diagnostic category. Normal: 0.00 (0.00); CIN3 (cervical intraepithelial neoplasia): 11.83 (7.55); SCC (squamous cell carcinoma): 76.57 (11.42).

distribution of the total viral load as well as the distribution for the two most common types (16 and 18).

There was a significant difference in viral load between each of the diagnostic categories ($p \leqslant 0.01$, fig 3A). Total viral load was significantly correlated both with the percentage of laminin-5 immunoreactive cells ($p \leqslant 0.01$), and with the presence of individual immunoreactive cells in the stromal tissue (fig 3B).

HPV16 viral load correlated significantly with the percentage of laminin-5 immunoreactive cells ($p \leqslant 0.01$, $r = 0.63$). There was a correlation between HPV16 viral load and the presence of individual immunoreactive cells in the stromal tissue (fig 4B), as well as significant differences in viral load between diagnostic categories ($p \leqslant 0.01$). A significant difference was seen between SCC and both other categories, but not between normal and CIN3 (fig 4A).

HPV16 integration status

HPV16 was present in 22 samples. Three of these exhibited aspecificity in the melting-curve analysis and were excluded. Table 4 is an overview of integration analysis results.

There was a correlation between the percentage of laminin-5 immunoreactive cells and the copy number of HPV16 integrated in E2 ($p \leqslant 0.03$, $r = 0.57$). There was a significant difference in the number of integrated copies between SCC and both other diagnostic categories ($p \leqslant 0.01$, fig 5A). Between normal and CIN3 there was no significant difference. Furthermore there was a correlation between the mean number of copies HPV16 integrated in E2 and the presence of laminin-5 immunoreactive cells in stromal tissue ($p \leqslant 0.05$, fig 5B).

There was a significant correlation between the copy number of HPV16 integrated in E2 and its viral load ($p \leqslant 0.01$, $r = 0.99$; fig 6).

DISCUSSION

In this study normal cervical samples, CIN3 and SCC cases, as identified by histology and immunocytochemistry for laminin-5, were investigated. Laminin-5 results were correlated with HPV status, comprising type, viral load and integration status in the E2 region.

Table 2 HPV typing results

	HPV type	n (%)
Single	16	11 (37)
	Other*	2 (7)
Multiple	Containing 16	11 (37)
	Other†	3 (10)
Negative		3 (10)
Total		30 (100)

*Types 33 and 52.
†Other double/triple combinations not containing HPV16.

Table 3 Viral load, diagnosis and laminin-5 expression

	Mean total viral load (n)	Mean viral load	
		HPV16 (n)	HPV58 (n)
Diagnosis			
Negative	0.010 (10)	0.003 (5)	0.015 (5)
CIN3	1.617 (12)	1.835 (10)	0.013 (3)
SCC	22.090 (7)	19.209 (6)	<0.001 (1)
Laminin-5 presence*			
Absent	0.900 (19)	1.271 (13)	0.016 (7)
Present	15.706 (10)	14.638 (8)	<0.001 (2)
Total†	6.004 (29)	6.362 (21)	0.013 (9)

HPV, human papillomavirus; CIN, cervical intraepithelial neoplasia; SCC, squamous cell carcinoma.
*Laminin-5 immunoreactivity in epithelium.
†Adenocarcinoma excluded.

Laminin-5 expression is closely tied to transition from pre-invasive lesions to invasive lesions and increases with severity of the lesion.[15 16] This enables an objective and precise distinction between CIN3/CIS and SCC. In this study two cases of SCC did not show laminin-5 immunoreactivity. During revision of the original H&E slides, it became apparent that the invasive lesion was no longer present in the new immunohistochemistry slides. These cases were reclassified as CIN3, because it was argued that the invasive cell clone itself was no longer present in the samples. Indeed in subsequent viral load and integration assays these samples had an HPV status similar to that of the other CIN3 cases.

The causal role of persistent High-Risk (oncogeretic) Human Papilloma Virus (HR-HPV) infection in the carcinogenesis of cervical carcinoma is well established.[2 22] The present study could not identify an association between dysplasia and HR-HPV presence. This is probably due to the selection of the study population. Conisations and hysterectomies, which were part of the treatment and follow-up of cervical lesions, were used, yielding a population at high risk of HPV infection. Only three samples out of 30 were HPV negative (with normal histology) and 22 contained HPV16. In this respect the population is not representative of the general population. At the same time the very low number of HPV negative cases hampered statistical analysis.

The mean viral load for HPV16 and HPV58 was significantly higher than those of other types, irrespective of diagnosis. There was also a large difference for these types in CIN3 and SCC cases specifically. This confirms data from other authors, who identified a difference in viral load of 3000–4000 between HPV16 and HPV18, HPV31 and HPV45 in CIN2–3 lesions.[23] This quantitative difference may be the result of type-specific differences in replication and in the host immune response, leading to faster clearance and lower copy numbers for some types.

Total viral load increased with severity of the lesion, irrespective of type. This may be explained in part by the increasing viral load of HPV16, which made up the bulk of all types present. There was a remarkable discrepancy in viral load between SCC and both other categories. At the same time a significant positive correlation was seen between total viral load or HPV16 viral load and the percentage of laminin-5 immunoreactive cells in the cervical epithelium and the number of individual laminin-5 immunoreactive cells in the cervical stromal tissue.

Transition from CIN3 to SCC is therefore associated with an increase in viral load. This indicates that HPV still has a part to play in the progression from non-invasive to invasive lesions.

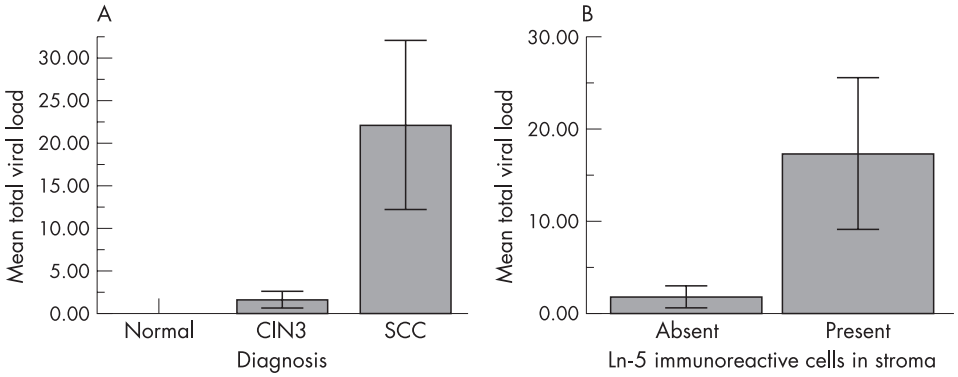

Figure 3 Total viral load. (A) Differences in viral load between diagnostic categories: normal: 0.01 (0.01); CIN3 (cervical intraepithelial neoplasia): 1.62 (0.97); SCC (squamous cell carcinoma): 22.09 (9.92). (B) Laminin-5 (Ln-5) immunoreactivity in individual cells in stromal tissue compared to mean total viral load: absent: 1.81 (1.17); present: 17.32 (8.21).

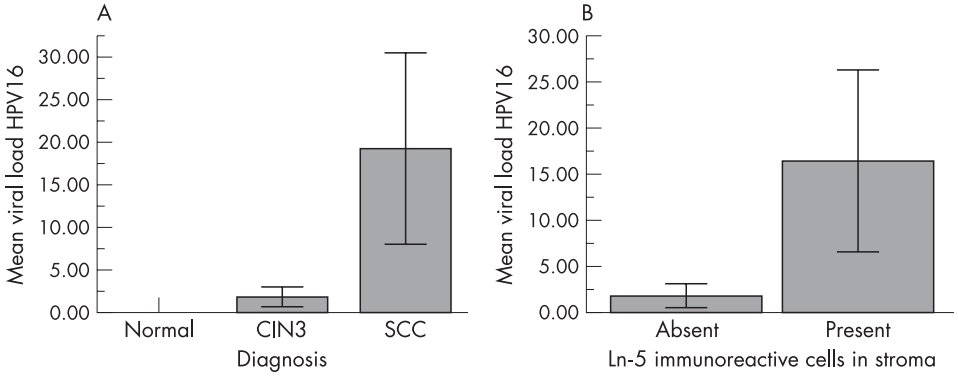

Figure 4 HPV16 (human papillomavirus) viral load. (A) Differences in HPV16 viral load between diagnostic categories: normal: 0.003 (0.031); CIN3 (cervical intraepithelial neoplasia): 1.835 (1.165); SCC (squamous cell carcinoma): 19.209 (11.237). (B) Laminin-5 (Ln-5) immunoreactivity in individual cells in stromal tissue compared to HPV16 viral load: absent: 1.960 (1.295); present: 16.567 (9.857).

As the large majority of samples contained HPV16, this hypothesis may not be readily applied to other types.

The real-time PCR method used to determine the integration status of HPV16 is rapid, reliable and very sensitive, finding small amounts of integrated HPV16 DNA even in the presence of excess episomal DNA. Therefore, it can be considered as an ideal technique for detecting the physical status of HPV in research and clinical applications. Conventional methods such

Table 4 HPV16 integration in E2 and viral load as compared to diagnosis and percentage of laminin-5 immunoreactive cells

Sample no	Diagnosis	Viral load (E6)*	% Integrated	Copies integrated†	% Laminin-5	Laminin-5 stromal
3	Normal	0.13	96	0.12	0	a
4	Normal	0.01	84	0.01	0	a
5	SCC	10.60	35	3.74	100	p
6	CIN3	0.02	100	0.02	0	a
8	SCC	3.77	66	2.49	100	p
9	SCC	16.60	90	14.86	100	p
11	CIN3	0.18	92	0.16	0	a
12	CIN3	0.79	68	0.54	10	p
13	CIN3	0.30	92	0.28	0	a
14	CIN3	0.03	98	0.03	0	a
16	SCC	0.81	95	0.76	60	p
19	CIN3	3.44	80	2.75	0	a
20	CIN3	0.85	90	0.76	0	a
22	CIN3	5.97	57	3.39	0	a
23	SCC	36.40	90	32.87	42	p
25	SCC	1.82	61	1.10	100	p
26	Normal	0.10	96	0.10	0	a
29	Normal	0.08	67	0.05	0	a
30	CIN3	1.37	68	0.94	80	a

HPV, human papillomavirus; CIN, cervical intraepithelial neoplasia; SCC, squamous cell carcinoma.
Laminin-5 immunoreactive cells in stromal tissue: a, absent; p, present.
*Normalised ratio.
†Number of copies integrated in E2 for one glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene.

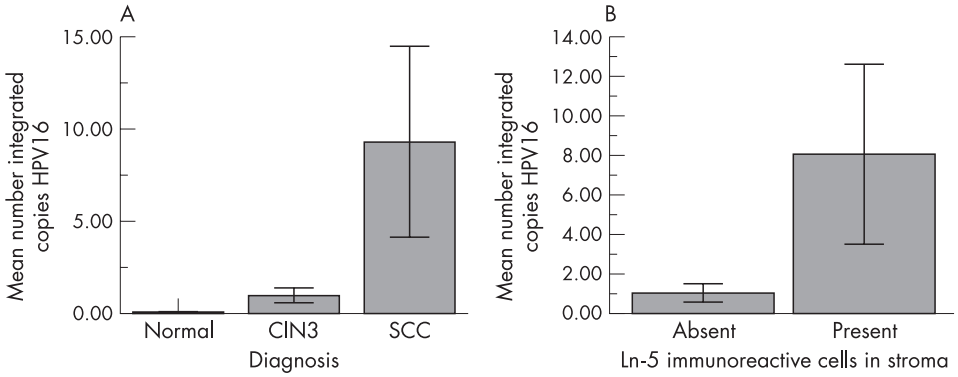

Figure 5 HPV16 (human papillomavirus) integration status. (A) Differences in HPV16 integration in E2 between diagnostic categories: normal: 0.07 (0.02); CIN3 (cervical intraepithelial neoplasia): 0.99 (0.41); SCC (squamous cell carcinoma): 9.30 (5.18). (B) Laminin-5 (Ln-5) immunoreactivity in individual cells in stromal tissue compared to HPV16 integration in E2: absent: 1.04 (0.46); present: 8.05 (4.55).

as Southern blotting, two-dimensional electrophoresis or chromosomal in situ hybridisation may also be applied, but studies using these techniques have shown that high-level episomal forms can mask the presence of low-level integrated HPV forms.[7 25]

In samples with a low viral load and a high integration percentage there will be less integrated copies than in samples with a high viral load and lower integration percentage. In order to normalise large variances in viral load and more easily compare samples, the copy number of HPV16 integrated in E2 per equivalent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was calculated; this is the copy number HPV16 integrated in E2 per cell.

Most samples contained both episomal and integrated HPV16. No sample was fully episomal and only one sample had 100% integration. The copy number of HPV16 integrated in E2 was significantly higher in samples containing laminin-5 immunoreactive cells, as well as in SCC. Again these data suggest a role for HPV in progression from CIS to SCC. Integration will lead to up-regulation of the E6 and E7 oncogene expression by E2 loss, but integration may have additional molecular consequences such as increased stability of E6 and E7 messages, modulating the behaviour of cervical cancer. Therefore increased integrated copy numbers will increase the chances of progression to malignancy and invasiveness.

As integration is a random event, one of the most important influencing factors would be viral load.[9] Integration will increase with an increase of the number of viral copies present in the host cell. This important observation was confirmed in this study, as a clear correlation was found between increased viral load and increased number of integrated HPV16 copies.

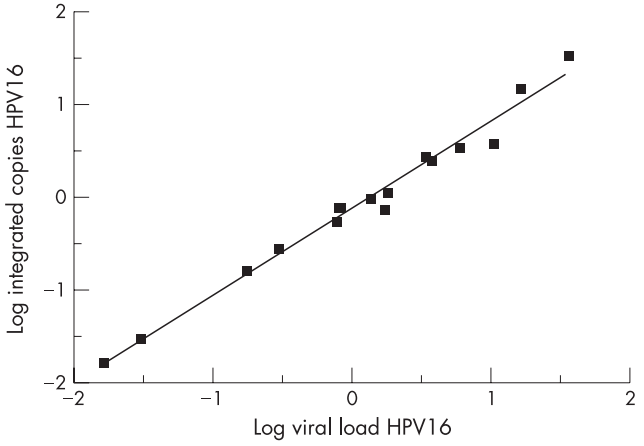

Figure 6 Correlation between HPV16 (human papillomavirus) viral load and number of copies integrated in E2.

Take-home messages

- Overall HPV status, comprising type, viral load and integration status, influences progression towards invasive malignancy in cervical carcinogenesis.
- Knowing overall HPV status may be more predictive of clinical patient outcome than mere knowledge of HPV presence.

Second, based on the present data and literature,[9 24] it can be concluded that overall HPV status, comprising type, viral load and integration status influences progression towards invasive malignancy in cervical carcinogenesis. Knowing the overall HPV status may be more predictive of clinical patient outcome, than mere knowledge of HPV presence.

Authors' affiliations
G A V Boulet, I Schrauwen, S Sahebali, C Horvath, J J Bogers, Ambior-IX, Laboratory of Cell Biology & Histology, University of Antwerp, Antwerp, Belgium
C E Depuydt, A Vereecken, Laboratory for Clinical Pathology (Labo Riatol), Antwerp, Belgium
E A Van Marck, Department of Pathology, University of Antwerp, Antwerp, Belgium
D Vanden Broeck, Laboratory of Human Biochemistry, University of Antwerp, Antwerp, Belgium

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