ORIGINAL ARTICLE

Aberrant expression of VEGF-C is related to grade of cervical intraepithelial neoplasia (CIN) and high risk HPV, but does not predict virus clearance after treatment of CIN or prognosis of cervical cancer

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Aims: Increased angiogenesis leads to invasion in cervical cancer. Vascular endothelial growth factors (VEGFs) are involved in angiogenesis, but molecular links to the most important aetiological agent, human papillomavirus (HPV), need clarifying.

Material/Methods: Archival samples—150 squamous cell carcinomas (SCCs) and 152 cervical intraepithelial neoplasia (CIN) lesions—were examined immunohistochemically for anti-VEGF-C antibody and for HPV by polymerase chain reaction (PCR). Follow up data were available for all SCC cases, and 67 CIN lesions were monitored with serial PCR to assess HPV clearance/persistence after treatment.

Results: High risk (HR) HPV types were closely associated with CIN (odds ratio, 19.12; 95% confidence interval, 2.31 to 157.81) and SCC (27.25; 3.28 to 226.09). There was a linear increase of VEGF-C expression—weak in CIN1 and intense in CIN3 and SCC (20.49; 8.69 to 48.26). VEGF-C upregulation was a sensitive (93.5%; 95% CI, 90.1% to 96.9%) marker of HR-HPV type (4.70; 2.17 to 10.21), but lost its significance in multivariate regression—p16^{INK4a} and survivin were equally strong independent predictors of HR-HPV. Aberrant expression of VEGF-C did not predict clearance/persistence of HR-HPV after treatment of CIN. In cervical cancer, VEGF-C had no prognostic value in univariate or multivariate survival analysis. After adjustment for HR-HPV, FIGO stage, age, and tumour grade, only FIGO stage and age remained independent prognostic predictors.

Conclusions: VEGF-C is an early marker of cervical carcinogenesis, with linearly increasing expression starting from low grade CIN. VEGF-C expression is closely related to HR-HPV in cervical lesions, probably because of its p53 independent upregulation by the E6 oncoprotein of HR-HPV.

In large epidemiological studies, the high risk human papillomavirus (HR-HPV) types are associated with cervical cancer in almost 100% of cases, in contrast to the low risk HPV (LR-HPV) types, which are rarely found in cervical cancer and its precursors.^{1–6} The different oncogenic potential of LR-HPV and HR-HPV seems to be linked, at least in part, to the different functions of two viral oncogenes, E6 and E7, and their interactions with two cell cycle regulatory proteins, p53 and the retinoblastoma protein (pRB).^{2–5, 7–8} Whereas the E6 oncoprotein initiates degradation of the p53 tumour suppressor protein, HPV E7 binds to pRB and triggers the release of E2F-like transcription factors, resulting in G1–S transition of the cell cycle.^{2–4, 7–9} Recently, angiogenesis has been shown to play an important role in the development of an invasive phenotype in cancer precursors (cervical intraepithelial neoplasia; CIN). Angiogenesis is stimulated by members of the vascular endothelial growth factor (VEGF) family, which are also upregulated by the HR-HPV E6 oncoprotein.^{10–14}

“Recently, angiogenesis has been shown to play an important role in the development of an invasive phenotype in cancer precursors (cervical intraepithelial neoplasia)”

VEGF is the primary member of a family of growth factors, which together with its homologues conveys regulatory signals via KDF/Flk-1, Flt-1, and Flt-4 receptors.¹⁴ VEGF is

one of the most potent known angiogenic factors. The *VEGF* gene is located on chromosome region 6p12, and is amplified in most human malignancies.^{10–15} The well established upregulators of VEGF in malignant tumours include hypoxia, mutations of *ras*, receptor tyrosine kinases (such as, epidermal growth factor receptor, erbB-2/Her-2), non-receptor kinases (such as *src*), several protooncogenes encoding transcription factors (such as, *myc*, *c-fos*, *c-jun*),^{11–14} and members of the cyclooxygenase (COX) family (for example, COX-1 and COX2).^{14–15} Another important step in VEGF upregulation was shown to be the loss of wild-type p53 function,¹⁶ raising the possibility that HPV16 E6 was also actively involved.¹¹ Indeed, these authors established that HPV 16 E6 upregulated VEGF via a promoter region that contains four Sp-1 sites, and importantly, that this occurred in a p53 independent manner.^{10–14}

During the past few years, the role of VEGF in cervical carcinogenesis has been studied. It is now well established that the expression of VEGF is closely related to microvascular density (MVD) in invasive carcinomas and in

Abbreviations: CI, confidence interval; CIN, cervical intraepithelial neoplasia; COX, cyclooxygenase; ERK, extracellular signal regulated kinase; HPV, human papillomavirus; HR, high risk; IHC, immunohistochemistry; LR, low risk; MVD, microvascular density; NPV, negative predictive value; OR, odds ratio; PCR, polymerase chain reaction; PPV, positive predictive value; pRB, retinoblastoma protein; SCC, squamous cell carcinoma; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor

CIN.^{13 17–24} Furthermore, there are reports suggesting that VEGF expression and MVD increase almost in parallel with the increasing severity of CIN lesions, being highest in invasive disease.^{13 20 21 23–27} However, the role of VEGF expression as an independent prognostic predictor in cervical cancer remains a controversial issue, whereas MVD seems to be such a prognostic factor.^{28–33} Similarly, despite the fact that HR-HPV E6 oncoprotein is a powerful upregulator of VEGF, there are no studies analysing the relations between VEGF expression and HPV detection or viral genotyping in cervical lesions, or associations between VEGF and viral events (such as, integration, persistence, and clearance), known to be useful intermediate endpoint markers of cervical cancer.^{1 2 4 5}

As a part of our systematic search for new potential biomarkers in HPV related cervical carcinogenesis,^{8 34–36} we analysed a series of cervical carcinomas and CIN lesions to assess whether VEGF-C expression might be of use in predicting: (1) the grade of CIN, (2) HR-HPV type, (3) clearance of the virus after eradication of CIN, or (4) the prognosis of cervical cancer. VEGF-C expression was studied using immunohistochemistry (IHC) in CIN lesions treated by conisation and monitored by serial polymerase chain reaction (PCR) assays for HPV clearance, and survival data of cervical cancer were related to VEGF-C expression in surgical samples.

MATERIAL AND METHODS

Materials

The tissue used in our study comprises the retrospective component of the HPV-Pathogen ISS project.³⁷ It was collected from the files of the pathology departments of two Italian hospitals (Azienda Ospedaliera S. Orsola Malpighi, Bologna, and Ospedale Maggiore, University of Trieste). This prospective biopsy material comprised 302 samples from patients with either an invasive cervical squamous cell carcinoma (SCC) or CIN diagnosed and treated in these two hospitals between 1986 and 2002. Of these 302 cases, 114 CIN and 38 SCC cases were provided by Bologna, and 38 CIN lesions and 112 SCCs were available from Trieste. The mean age of the patients with CIN was 35.5 (range, 18–79) years, and that of those with SCC was 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 in separate recent studies,^{38–40} whereas the samples from Trieste were tested for HPV in our present study. Complete follow up data were available for all 150 patients with SCC, with a mean follow up of 51.7 months (range, 1–218). Furthermore, all patients with CIN from Bologna had been followed up at six month intervals after cone treatment (mean, 10.5 months; range, 2.4–27.6) and subjected to repeated colposcopy, Papanicolaou smear, and biopsy (if residual disease suspected). A minimum of two serial PCR analyses were available from 67 cases, and recently reported as part of a larger study on HPV clearance.⁴⁰ Clinical FIGO stage of the disease was available for 125 of the patients with SCC.

METHODS

Biopsy

Both the colposcopic biopsies and the surgical samples were fixed in 10% buffered formalin, embedded in paraffin wax, cut into 5 μ m thick sections, and stained with haematoxylin and eosin 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, or CIN3. The histological diagnosis of SCC was confirmed in all cases, and two

Figure 1 Normal squamous epithelium of the cervix stained with anti-vascular endothelial growth factor C (VEGF-C) antibody. No specific staining is detectable. (Immunohistochemistry for VEGF-C; original magnification. $\times 100$.)

adenocarcinomas originally present were excluded from this series.

IHC for VEGF-C

IHC for VEGF-C expression was carried out according to standard IHC procedures. VEGF-C was cloned in 1996 and found to have a region that is 30% homologous to VEGF-A and 27% homologous to VEGF-B. The ~ 23 kDa VEGF-C is a ligand for both VEGF receptor 2 (VEGFR-2; KDR/FLK1) and VEGFR-3 (FLT4) tyrosine kinases. In brief, the 5 μ m paraffin wax sections cut on to poly-L-lysine coated microscope slides were first dewaxed and rehydrated in graded alcohols. The sections were heated in citrate buffer (0.01M, pH 6.0, Dako target retrieval solution; Dako, Glostrup, Denmark) in a microwave oven (85–95°C, 3×5 minutes), followed by blocking of non-specific binding sites with goat/rabbit serum. Sections were incubated with the primary antibody, polyclonal rabbit anti-VEGF-C antibody (Zymed Laboratories Inc, South San Francisco, California, USA; 1/100 dilution), in a humidified chamber for one hour at room temperature. This polyclonal (IgG) antibody was raised in rabbits against the synthetic peptide corresponding to the C-terminus of human VEGF-C conjugated to carrier protein. Rabbit anti-VEGF-C is purified from rabbit antisera and diluted in phosphate buffered saline, pH 7.4, and 1% bovine serum albumin with 0.05% sodium azide as a preservative.

Primary antibody was followed by incubation with the biotinylated secondary antibody, polyclonal goat antirabbit IgG (Abcam, Cambridge, UK; 1/200 dilution). Slides were then processed with universal LSABTM-2 single reagents (peroxidase) kit (DakoCytomation, Glostrup, Denmark), and expression of VEGF was localised by incubation with diaminobenzidine. As a final step, the slides were lightly counterstained with haematoxylin. Negative controls were similarly processed but the primary antibody was omitted, and breast cancer biopsies were used as positive controls.

Evaluation of IHC

IHC was assessed using a light microscope (Leitz Diaplan; Leitz, Wetzlar, Germany), equipped with a digital camera (Leica DG300). No staining for VEGF-C was detected in normal squamous or columnar cervical epithelium. When originally grading the IHC staining, a semiquantitative score using four categories was used, as follows: 0, negative (equivalent to normal epithelium); 1, weak staining (positively stained scattered cells or a more widespread but weak

Figure 2 Normal transformation zone demonstrating the process of immature squamous metaplasia. The metaplastic squamous epithelium remains negative for vascular endothelial growth factor C (VEGF-C) expression. (Immunohistochemistry for VEGF-C; original magnification, $\times 100$.)

reaction in the epithelium); 2, moderately increased staining (more abundant positively stained cells); and 3, intense staining (almost all cells diffusely staining throughout the lesion) (figs 1–6). In statistical analysis, the staining results were used as dichotomous categorical variables combining the above categories as negative–weak/moderate–intense (0–1/2–3), or using the four tier categorisation.

HPV testing

The 114 patients with CIN and 38 patients with SCC from Bologna had already been tested for HPV for other purposes using PCR, as reported in separate studies.^{38–40} In our present study, the 150 paraffin wax embedded sections (112 SCC and 38 CIN) delivered from Trieste were subjected to HPV testing, as described below.

DNA extraction

Paraffin wax embedded tissues (5 μ m thick sections, not weighing more than 25 mg) were treated with xylene to

Figure 3 A low grade cervical intraepithelial neoplasia (CIN1) lesion, showing the characteristic morphological features of human papillomavirus infection (koilocytes) in the intermediate and upper layers of the epithelium. Weak vascular endothelial growth factor C (VEGF-C) cytoplasmic staining is present in the epithelial cells, whereas the underlying stroma remains completely negative. (Immunohistochemistry for VEGF-C; original magnification, $\times 100$.)

Figure 4 Detail of a large high grade (cervical intraepithelial neoplasia 3; CIN3) lesion with full thickness involvement of basaloid-type cells with abnormal mitotic figures and apoptotic cells. The whole lesion shows strong positivity for vascular endothelial growth factor C (VEGF-C) in all layers of the CIN3 lesion, and complete lack of staining in the underlying connective tissues. Immunostaining is mostly cytoplasmic, but also some nuclei seem to express VEGF-C. (Immunohistochemistry for VEGF-C; original magnification, $\times 100$.)

remove the paraffin wax, digested with ATL lysis buffer (Qiagen, Hilden, the Netherlands) and proteinase K overnight at 56°C in a thermomixer, and DNA was extracted according to the manufacturer's instructions (QIAamp DNA mini kit; Qiagen).

Polymerase chain reaction

To verify the extraction and the quality of DNA, 5 μ l of each sample was amplified with a primer set recognising the β actin gene (sense: 5'-GGCGGCACCACCATGTACCCT-3', antisense: 5'-AGGGGCCGGACTCGTCATACT-3'). The PCR mix contained 200 μ M each dNTP, 1.5 mM $MgCl_2$, 1 $\times$ PCR buffer, 40 pmol sense and antisense primer, and 1.25 U AmpliTaq Gold (Applied BioSystem, Branchburg, USA). The PCR conditions were as follows: an initial denaturation at 94°C for 10 minutes, followed by 25 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, with a final elongation at 72°C for seven minutes.

The samples were then amplified for the presence of HPV using different sets of degenerate primers, as described previously for MY09/MY11,⁴¹ GP5⁺/GP6⁺,⁴² and the biotinylated SPF primer mix located within the L1 region of the HPV genome.⁴³ The PCR conditions for the My09/My11 primer set were: 94°C for 10 minutes, followed by 40 cycles of 94°C for 30 seconds, 55°C for 45 seconds, and 72°C for 30 seconds, with a final extension at 72°C for seven minutes. The PCR mix contained 200 μ M each dNTP, 40 pmol each primer, 2 mM $MgCl_2$, 1 $\times$ PCR buffer, and 1.25 U AmpliTaq Gold. For the GP5⁺/GP6⁺ primers, the following conditions were used: 94°C for 10 minutes, followed by 40 cycles of 95°C for 30 seconds, 44°C for 60 seconds, and 72°C for 90 seconds, with a final extension at 72°C for seven minutes. Amplification with the SPF primer mix was carried out as follows: 94°C for 10 minutes, followed by 40 cycles of 94°C for 30 seconds, 52°C for 45 seconds, and 72°C for 45 seconds, with a final extension at 72°C for seven minutes. Positive and negative controls were included in each amplification.

None of the samples was positive exclusively with the primer set MY09/MY11, whereas the GP5⁺/GP6⁺ primer set and the SPF primer mix alone gave positive results in 12 and 34 cases, respectively. Of the remaining cases, 42 samples were positive for HPV DNA when amplified with the SPF and GP5⁺/GP6⁺ primers and another 44 when using the triple set

Figure 5 A low power overview of an invasive squamous cell carcinoma. This is an example of intense expression of vascular endothelial growth factor C (VEGF-C). All the islets of the invasive cancer cells show strong and diffuse dark brown immunostaining, whereas the surrounding tumour stroma remains completely negative. (Immunohistochemistry for VEGF-C; original magnification, $\times 40$.)

of primers (MY09/MY11, SPF mix, and GP5⁺/GP6⁺). The amplified products were electrophoresed on a 2% agarose gel and visualised under ultraviolet light.

HPV typing

HPV typing was done using the reverse hybridisation assay. The denatured biotinylated amplified product (10 μ l) was hybridised with specific oligonucleotide probes, which are immobilised as parallel lines on membrane strips (InnoLiPA, Innogenetics, Ghent, Belgium).⁴³ 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 visualised line, it is possible to determine the HPV genotype.⁴³

Statistical methods

Statistical analyses were performed using the SPSS® and STATA software packages (SPSS for Windows, Version 12.0.1, and STATA/SE 8.2). Frequency tables were analysed using the χ^2 test, with Pearson correlation and/or likelihood ratio being used to assess the significance of the correlation between the categorical variables. Bivariate correlations between ordered variables were analysed using Spearman correlation analysis (Spearman ρ). Differences in the means of continuous variables were analysed using non-parametric tests (Mann-Whitney) or ANOVA (analysis of variance).

Figure 6 Medium power view of another invasive squamous cell carcinoma. Compared with the lesion in fig 5, this tumour shows only weak expression of vascular endothelial growth factor C (VEGF-C). In contrast to the intense diffuse staining, only weak cytoplasmic staining is present in some cancer cells, making it difficult to distinguish between the neoplastic areas and the surrounding stroma, where some of the microvessels show positive staining in their endothelial cells. (Immunohistochemistry for VEGF-C; original magnification, $\times 250$.)

Logistic regression models using stepwise backward approach and the likelihood ratio statistic for removal testing were used to analyse the power of different covariates as predictors of the outcome variables (CIN, HR-HPV), calculating crude odds ratios (ORs), and 95% confidence intervals (CIs). Performance indicators of VEGF as a marker of CIN or HR-HPV were calculated using the conventional contingency tables to calculate sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV), with 95% CI based on the F distribution ($\pm 1.96 \times \text{SE}$). Univariate survival (life table) analysis for the outcome measure (HPV clearance, HPV persistence, cancer survival) was based on the Kaplan–Meier method. Multivariate survival analysis was run using Cox's proportional hazards model in a backward stepwise manner with the log likelihood ratio significance test, and using the default values for enter and exclusion criteria. The assumption of proportional hazards was controlled by log minus log survival plots. In all tests, $p < 0.05$ was regarded as significant.

RESULTS

Table 1 shows the expression of VEGF-C in relation to the grade of the lesion in the cone (CIN) or surgical specimen (SCC). There was a direct relation between increasing grade of the lesion and the intensity of staining ($p = 0.0001$ for linear trend). Using the two tier category of staining (negative–weak and strong–intense), strong–intense

Table 1 Expression of VEGF-C and grade of cervical lesions

Lesion grade	VEGF-C expression							
	Negative		Weak		Moderate		Intense	
	N	%	N	%	N	%	N	%
No CIN	9	100.0	0	00.0	0	00.0	0	00.0
CIN1	13	65.0	6	30.0	1	5.0	0	00.0
CIN2	4	20.0	10	50.0	6	30.0	0	00.0
CIN3	1	1.1	24	25.8	21	22.6	47	50.5
SCC	5	3.3	23	16.3	38	27.0	75	53.2
Total (n = 283)	32	11.3	63	22.3	66	23.3	122	43.1

Chi square test, Pearson $p = 0.0001$ (also for linear trend).

CIN, cervical intraepithelial neoplasia; SCC, squamous cell carcinoma; VEGF-C, vascular endothelial growth factor C.

Table 2 VEGF-C expression in relation to HR-HPV types in the lesions

HR-HPV*	VEGF-C expression							
	Negative		Weak		Moderate		Intense	
	N	%	N	%	N	%	N	%
Present	13	6.5	45	22.5	47	23.5	95	47.5
Absent	18	24.7	18	24.7	17	23.3	20	27.4
Total (n = 273)	31	11.4	63	23.1	64	23.4	115	42.1

*Includes cases that were HPV negative; χ^2 test, Pearson $p = 0.0001$.

HPV, human papillomavirus; HR, high risk; VEGF-C, vascular endothelial growth factor C.

VEGF-C expression was associated with CIN3/cancer (OR, 20.49; 95% CI, 8.69 to 48.26; $p = 0.0001$).

Only 11.1% of the lesions without CIN were HR-HPV positive (data not shown), compared with 70.5% of those with CIN. This figure was even higher in SCC: 77.6% were HR-HPV positive, 4.9% were negative, and in 17.5% the HPV type could not be determined. HR-HPV positivity was associated with SCC (OR, 27.25; 95% CI, 3.28 to 226.09) and with CIN (OR, 19.12; 95% CI, 2.31 to 157.81).

Table 2 shows the expression of VEGF-C in relation to the presence/absence of HR-HPV in the lesions. The VEGF-C staining intensity was significantly higher in HR-HPV positive than in HR-HPV negative lesions ($p = 0.0001$), 24.7% of which were VEGF-C negative. Negative-weak and strong-intense staining was 29.0% and 71.0%, respectively, in HR-HPV positive cases and 49.3% and 50.7%, respectively in HR-HPV negative cases ($p = 0.002$). In CIN lesions, strong-intense VEGF-C expression was associated with HR-HPV (OR, 2.38; 95% CI, 1.37 to 4.13; $p = 0.002$).

We calculated the performance indicators (sensitivity, specificity, PPV, and NPV) for VEGF-C staining (positive/negative) as a marker of CIN and HR-HPV. As shown in table 3, positive VEGF-C staining is a 100% specific indicator of CIN, with 100% PPV. Negative staining, however, does not rule out CIN—the NPV is only 33.3%. When using CIN3 as the cut off point, the test indicators are completely different, with sensitivity increasing to 97.4% and NPV to 81.2%. The OR for being CIN3 and VEGF-C positive is 42.95 (95% CI, 16.02 to 115.12). VEGF-C staining is also a sensitive test for detecting HR-HPV (93.5%), but suffers from low specificity and an NPV of 58%. The OR for being HR-HPV and VEGF-C positive is 4.70 (95% CI, 2.17 to 10.21).

Of the HPV positive women treated for CIN and controlled by serial PCR, 41 of 67 cleared their HR-HPV infection by the last PCR assay, during a total of 705 women months at risk, resulting in a monthly clearance rate of 5.8%; that is, 58/1,000 women months at risk. Clearance was much less frequent (35 of 59) in VEGF-C positive women than in VEGF-C negative women (four of five) ($p = 0.345$). The corresponding figures for virus persistence were eight of 59 and one of five, respectively ($p = 0.544$). In univariate (Kaplan–Meier) survival analysis, positive VEGF-C staining was not a significant predictor of either virus clearance (log rank,

$p = 0.684$) or viral persistence ($p = 0.535$). Thus, VEGF-C expression does not predict clearance or persistence of HR-HPV type in the cervix after treatment of CIN by large loop excision of the transformation zone.

Finally, we analysed the value of VEGF-C expression as a predictor of disease outcome in patients with cervical cancer. Of the 150 patients with SCC, 91 (60.7%) were alive and 59 had died during the mean follow up of 52 months. Of the women with negative-weak VEGF-C staining, 60.7% were alive at the end of follow up, compared with 59.3% of those with strong-intense staining ($p = 0.534$). When the positive/negative dichotomy was used, this difference reversed, with 40.0% and 60.3% being alive among VEGF-C negative and positive women, respectively ($p = 0.394$). In univariate (Kaplan–Meier) analysis, the survival was not significant when VEGF-C staining was classified as negative-weak/moderate-strong (log rank, $p = 0.979$), but was marginally significant when the negative/positive dichotomy was used ($p = 0.034$). However, the data are based on five VEGF-C negative cases only. HR-HPV showed a slight positive effect on survival in that 64.0% of HR-HPV positive and 43.8% of HR-HPV negative women were alive ($p = 0.044$; OR, 2.28; 95% CI, 1.027 to 5.072). In Kaplan–Meier analysis, this difference was significant (log rank, $p = 0.0306$). As usual, FIGO stage was a powerful predictor of survival in Kaplan–Meier analysis (log rank test, $p = 0.0001$).

In multivariate survival (Cox regression) analysis, VEGF-C expression did not prove to be a significant independent prognostic factor, irrespective of the categorisation used. It was removed from the model when adjusted for age, HR-HPV, tumour grade, and FIGO stage. In the final Cox model, only FIGO stage and age proved to be independent predictors of patient survival, both at $p = 0.0001$. When FIGO stage 1 was used as the reference, OR for dying of the disease was 2.53 (95% CI, 0.79 to 8.12) in stage 2, 7.93 (95% CI, 2.63 to 23.85) in stage 3, and 80.93 (95% CI, 19.21 to 340.82) in stage 4. The mean age of women who were alive was 54.2 years compared with 66.7 years for those who died ($p = 0.0001$).

DISCUSSION

Several issues are important in relation to the prediction of cervical cancer and CIN.^{1–4} According to a recent task force on prognostic factors in cervical cancer, there is an urgent

Table 3 Performance indicators of VEGF-C as a marker of CIN and HR-HPV type

Outcome variable	VEGF-C performance* (95% CI)			
	Sensitivity	Specificity	PPV	NPV
CIN†	86.4% (80.7% to 92.3%)	100% (100% to 100%)	100% (100% to 100%)	33.3% (15.6% to 51.1%)
CIN3‡	97.4% (95.4% to 99.5%)	53.1% (39.1% to 67.0%)	90.8% (87.3% to 94.4%)	81.2% (67.8% to 94.8%)
HR-HPV	93.5% (64.7% to 77.3%)	24.6% (14.8% to 34.5%)	77.3% (72.0% to 82.6%)	58.0% (40.7% to 75.4%)

*VEGF-C staining (positive/negative); †any grade of CIN (SCC cases excluded); ‡CIN3 cut off (SCC cases included).

CI, confidence interval; CIN, cervical intraepithelial neoplasia; HPV, human papillomavirus; HR, high risk; NPV, negative predictive value; PPV, positive predictive value; SCC, squamous cell carcinoma; VEGF-C, vascular endothelial growth factor C.

need for more specific markers predicting disease outcome in individual patients.⁴⁴ With regard to the prediction of CIN, the role of persistent HR-HPV as a cause of treatment failure has achieved increased attention in the recent literature.⁴⁰⁻⁴⁵ Indeed, monitoring this risk of disease recurrence after radical cone treatment using a suitable molecular marker would be of considerable clinical value. We have recently started a systematic survey of such potential biomarkers in a retrospective series of our Pathogen ISS study.³⁵⁻³⁷ Until now, three of these markers of HR-HPV and CIN have been explored in detail, namely: p16^{INK4a},⁸ extracellular signal regulated kinase (ERK1),³⁴ and survivin.³⁶ As an indicator of aberrant function of the p16^{INK4a}-cyclin D-pRB pathway caused by interference by the E7 oncoprotein, p16^{INK4a} has been implicated as a specific marker of CIN and HR-HPV in several studies.⁷⁻⁹ ERK1, in turn, is linked to another HPV transforming protein, E5, the effects of which are mediated through the activation of potent transcription factors (c-fos, myc, Ets1, Ets2, Elk-1, c-jun) by the ERK/mitogen activated protein kinase signalling pathway.³⁴ Survivin, a member of the inhibitor of apoptosis family, is transcriptionally repressed by wild-type p53, and the well established function of the HR-HPV (but not LR-HPV) E6 oncoprotein in the degradation of p53 might explain why upregulation of survivin is so closely associated with HR-HPV types in cervical lesions.³⁶ These divergent molecular mechanisms also reflect the different associations of p16^{INK4a}, ERK1, and survivin with HR-HPV and CIN, as recently reported.⁸⁻³⁴⁻³⁶

“Vascular endothelial growth factor C overexpression proved to be a 100% specific marker of cervical intraepithelial neoplasia (CIN), because it was never found in biopsies without CIN”

Undoubtedly, one of the key mechanisms leading to the invasive phenotype in progressing CIN lesions is angiogenesis, which is induced by several angiogenic factors, such as those of the VEGF family.¹⁰⁻²⁰ VEGF-C was cloned in 1996 and found to have a region that is 30% homologous to VEGF-A and 27% homologous to VEGF-B.²⁷⁻²⁹⁻³² The 23 kDa VEGF-C molecule is a ligand for both VEGFR-2 (KDR/Flk-1) and VEGFR-3 (Flt-4) tyrosine kinases.¹⁴⁻¹⁵ During the past few years, VEGF and its tissue manifestation (MVD) have attracted increased attention in many different human malignancies, as major regulators of physiological and pathological angiogenesis and lymphangiogenesis in human tissues.¹⁴ This subject has become increasingly important since the recent discovery of the link between HPV and VEGF, suggesting that the HR-HPV E6 oncoprotein upregulates VEGF expression via a promoter region that contains four Sp-1 sites, and that it does this in a p53 independent manner.¹⁰⁻¹⁴ This prompted us to include VEGF-C among the panel of potential biomarkers of interest,³⁵ to assess whether VEGF-C expression might be of use in predicting any of the several outcome measures, namely: grade of CIN, HR-HPV type, clearance of the virus, and prognosis of cervical cancer.

VEGF-C expression was completely absent in the nine lesions rediagnosed as not being CIN (table 1). This absence of VEGF-C in normal epithelium is consistent with the data established in several previous studies on CIN lesions and cervical cancer.²⁰⁻²¹⁻²³⁻²⁷ In these studies, the absence of VEGF expression is usually associated with minimal MVD, increasing in parallel with increasing CIN grade, and being first apparent in low grade CIN lesions.²⁰⁻²³ Indeed, we were able to establish an almost linear relation between the grade of CIN and the intensity of VEGF-C expression (table 1). Negative VEGF-C expression was inversely related to CIN grade, whereas moderate expression increased almost in

parallel with CIN grade ($p = 0.0001$ for the trend). Intense, diffuse expression was confined to CIN3 and SCC, so that there was a major difference in expression between CIN1 and CIN2 lesions, as pointed out recently.²⁰⁻²¹⁻²³⁻²⁶⁻²⁷ This suggests that VEGF expression is an early phenomenon in cervical carcinogenesis, being detectable in low grade CIN lesions, but being upregulated in high grade CIN3 lesions.

In fact, VEGF-C overexpression proved to be a 100% specific marker of CIN, because it was never found in biopsies without CIN (table 3). In addition, the PPV for predicting CIN was 100%. These figures are very similar to those obtained in our recent studies on p16^{INK4a},⁸ ERK1,³⁴ and survivin,³⁶ all three being 100% specific markers of CIN, with 100% PPV. However, minor differences were found in their sensitivities, being highest for VEGF-C (86.4%), followed by p16^{INK4a} (74.4%), survivin (71.6%), and ERK1 (70.3%).⁸⁻³⁴⁻³⁶ No comparative data have been published for VEGF-C, but in one study assessing the test for the detection of tumour recurrence, VEGF expression was 75% sensitive and 70% specific, with a PPV of 41% and an NPV of 92%.²⁸

We recently showed that p16^{INK4a} and survivin are specific biomarkers of cells harbouring HR-HPV infection,⁷⁻⁹⁻³⁶ whereas ERK1, as a marker of the ERK-mitogen activated protein kinase cascade (activated by HPV E5 protein), showed no specificity for HR-HPV types.³⁴ Interestingly, the present data for VEGF-C indicate that the expression of this angiogenic factor is significantly ($p = 0.0001$) related to HR-HPV types (table 2). Only 6.5% of lesions with HR-HPV were totally negative for VEGF-C, in contrast to 24.7% of the lesions without oncogenic HPV (OR, 4.70; 95% CI, 2.171 to 10.210). This discriminative power between HR and LR HPV types seems to be very similar to that of survivin and p16^{INK4a}, with almost identical ORs (around 4.5).⁸⁻³⁶ We recently entered these two markers in a multivariate regression model, and found that survivin and p16^{INK4a} were equally strong independent predictors of HR-HPV (OR, 3.23; 95% CI, 1.72 to 6.09 and OR, 3.45; 95% CI, 1.83 to 6.52, respectively).³⁶ When this analysis was repeated in our present study (data not shown), by entering VEGF-C in this multivariate model, survivin and p16^{INK4a} were not confounded, and VEGF-C was dropped from the model. This suggests that despite its power as a univariate predictor of HR-HPV, VEGF-C is not an independent predictor of HR-HPV, equivalent in power to survivin and p16^{INK4a}.³⁶ One plausible explanation might be offered by the fact that VEGF is upregulated by a multitude of pathways, including abrogation of wild-type p53 function.¹¹⁻¹⁴⁻¹⁶ HR-HPV E6 is involved in only one of these pathways, and seems to upregulate VEGF in a p53 independent manner. Thus, VEGF-C may be upregulated by other mechanisms, although in cervical lesions it appears to be closely linked with HR-HPV. However, the specificity of VEGF-C as such a marker seems to be much lower than that of survivin and p16^{INK4a}.⁸⁻³⁶

During the past few years, persistent HR-HPV infections have achieved increased attention as a cause of a greatly increased risk of treatment failure in CIN.⁴⁰⁻⁴⁵ 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 VEGF-C expression might be of predictive value for HR-HPV persistence/clearance. In univariate survival analysis, VEGF-C expression was not a useful predictor of these two viral events in the cervix after the treatment of CIN. Together with our previous data,⁸⁻³⁴⁻³⁶ this suggests that at least these four biomarkers (VEGF-C, survivin, ERK1, and p16^{INK4a}) cannot substitute for HPV testing in monitoring the risk of disease recurrence after cone treatment.

As recently concluded by the prognostic factor committee of the European Society of Gynaecological Oncology,⁴⁴ there

is an urgent need for specific prognostic biomarkers in cervical carcinoma. In searching for such potential prognostic markers,³⁵ we recently showed that p16^{INK4a}, ERK1, and survivin expression was of no value as a predictor of disease outcome in cervical cancer.^{8 34 36} The prognostic value of VEGF in cervical cancer has been analysed in several studies, with conflicting results.^{15 28–33} In our present study, VEGF-C expression, graded as positive/negative or negative-weak/moderate-intense, was analysed using both univariate (Kaplan–Meier) and multivariate (Cox) survival analyses. In univariate analysis, only the positive/negative grading was of prognostic value (log rank, $p = 0.034$), but these data were based on five VEGF-C negative carcinomas only, and might not be reproducible with larger series. When entered in Cox multivariate model, VEGF-C expression was removed from the model, and of all the entered variables (HR-HPV, tumour grade, VEGF-C, FIGO stage, age), only the last two remained significant independent prognostic factors at the $p = 0.0001$ level. We also analysed the relation between VEGF-C expression and the histological differentiation of cancer and FIGO stage, but failed to establish a correlation ($p = 0.095$ and $p = 0.086$, respectively). These data imply that VEGF-C expression analysed by IHC has no independent prognostic value in cervical cancer. Similar data have been reported recently,^{15 31 33} whereas others have found VEGF to be an independent prognostic predictor in cervical cancer.^{28–30 32} Thus, this issue must still be considered unsolved, and larger series with appropriate follow up are needed to clarify this issue.

“Our data suggest that multiple interactions exist between the different molecular pathways in cervical carcinogenesis, as anticipated by the participation of the same molecules (such as p53 and high risk human papillomavirus E6) in several of these pathways”

Taken together, our results suggest that VEGF-C expression is a diagnostic marker for early stages of cervical carcinogenesis, increasing linearly from low grade CIN, with strong expression in high grade CIN and cancer lesions. Interestingly, VEGF-C upregulation is closely related to the presence of HR-HPV types in CIN and cervical cancer, being an equally powerful predictor to p16^{INK4a} and survivin^{8 36} in univariate analysis, but losing its power in the multivariate model. Apart from the well known mechanisms leading to upregulation of VEGF-C,^{11 14 16} another plausible mechanism leading to VEGF overexpression is through the E6 oncoprotein of HR-HPV types, which upregulate VEGF in a p53 independent manner.¹¹

Take home messages

- Vascular endothelial growth factor C (VEGF-C) is an early marker of cervical carcinogenesis
- Expression of VEGF-C begins in low grade cervical intraepithelial neoplasia (CIN) and increases as the lesion progresses towards cancer
- VEGF-C expression is closely related to high risk human papillomavirus (HR-HPV) in cervical lesions, probably because of its p53 independent upregulation by the E6 oncoprotein of HR-HPV
- We found that VEGF-C was not an independent marker of prognosis in cervical cancer or of viral clearance/persistence after treatment for CIN

In cervical cancer, VEGF-C expression has been closely related to the expression of other markers, including c-erbB-2,²⁴ matrix metalloproteinase 2 (MMP-2),^{14 32 46} proliferating cell nuclear antigen (PCNA),⁴⁷ and MMP-9.⁴⁸ Of these, PCNA and MMP-2 were included in the panel of biomarkers analysed in our present series,³⁵ and were checked for mutual correlations (data not in tables). Indeed, PCNA and VEGF-C seem to be closely related (Spearman ρ , 0.368; $p = 0.0001$). Interestingly, the same was true for MMP-2 and VEGF-C (Spearman ρ , 0.381; $p = 0.0001$). An even stronger correlation was found between survivin and VEGF-C (Spearman ρ , 0.530; $p = 0.0001$), and the correlations between VEGF-C and ERK1 and VEGF-C and p16^{INK4a} were also significant (Spearman ρ , 0.305 and 0.333, respectively). Our data suggest that multiple interactions exist between the different molecular pathways in cervical carcinogenesis, as anticipated by the participation of the same molecules (such as p53 and HR-HPV E6) in several of these pathways. This indicates that HR-HPV plays a central role in this process, and is capable of interfering with the normal regulatory pathways and thus contributing to the aberrant expression of the key molecules supporting normal cell growth, finally leading to development of a malignant (and invasive) phenotype. Which of these cellular proteins prove to be the most powerful predictors remains to be seen when the data from our 13 markers are available,³⁵ and are entered in a multivariate model to predict the intermediate end point markers and disease outcome in cervical carcinogenesis.

APPENDIX

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