ORIGINAL ARTICLE

Down-regulated nucleoside diphosphate kinase *nm23-H1* expression is unrelated to high-risk human papillomavirus but associated with progression of cervical intraepithelial neoplasia and unfavourable prognosis in cervical cancer

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Objective: One of the factors leading to an invasive phenotype is the *nm23* family of metastases-associated genes. Of the six known members, *nm23-H1* is the most frequently studied potential anti-metastatic gene in cervical cancer. However, the possible molecular links to oncogenic human papillomavirus (HPV) are completely unexplored as yet.

Materials and methods: As a part of the HPV-Pathogen Istituto Superiore di Sanità study, a series of 150 squamous cell carcinomas (SCCs) and 152 cervical intraepithelial neoplasia (CIN) lesions were examined by immunohistochemical staining for *nm23-H1*, and tested for HPV by polymerase chain reaction (PCR) with three sets of primers (MY09/11, $GP5^+/GP6^+$ and short PCR fragment). Follow-up data were available on all patients with SCC, and 67 CIN lesions were monitored by serial PCR for clearance or persistence of HPV after cone treatment.

Results: A linear decrease ($p=0.001$) was observed in *nm23-H1* expression, starting from CIN1 (85% with normal expression), with the most dramatic down regulation on transition from CIN2 (70% normal) to CIN3 (39%) and further to SCC (25%). Reduced expression was associated with CIN3 or cancer at an odds ratio 8.72 (95% confidence interval 4.13 to 18.41). *Nm23-H1* was of no use as a marker of the high-risk human papillomavirus (HR-HPV) type, and it did not predict clearance or persistence of HR-HPV after treatment of CIN. Importantly, *nm23-H1* expression was a significant prognostic factor in cervical cancer, reduced expression being associated with lower survival ($p=0.022$) in univariate analysis. In the multivariate (Cox) regression model, however, only the International Federation of Gynecology and Obstetrics stage ($p=0.001$) and age ($p=0.011$) remained independent prognostic predictors.

Conclusions: Down-regulated *nm23-H1* expression is markedly associated with progression from CIN2 to CIN3, and predicts poor prognosis in cervical cancer. *Nm23-H1* down regulation is probably orchestrated by mechanisms independent of HR-HPV oncoproteins and is possibly related to the emergence of a proteolytic phenotype.

The high-risk human papillomavirus (HR-HPV) types are associated with cervical cancer in almost 100% of cases, in contrast with the low-risk HPV (LR-HPV) types that are rarely found in cervical cancer and its precursors.[1–6] The different oncogenic potentials of LR-HPV and HR-HPV seem to be linked, at least in part, to the functions of two viral oncogenes, *E6* and *E7*, and their different interactions with the key regulatory proteins of the cell cycle, p53 and Prb.[2–5 7 8] Although the E6 oncoprotein of HR-HPV (but not LR-HPV) initiates degradation of the p53 tumour suppressor protein, HPV E7 of HR-HPV (but not LR-HPV) binds to pRB, resulting in G1–S transition of the cell cycle.[2–4 7–9] These HPV-type-dependent functions of E6 and E7 are in contrast with those of another HPV oncoprotein E5, which activates potent nuclear transcription factors (*c-fos*, *myc*, *Ets1*, *Ets2*, *Elk-1*, *c-jun*) through the extracellular signal-regulated kinase (ERK)–mitogen-activated protein kinase signalling pathway, but in an HPV-type-independent manner, as suggested by the fact that ERK1 expression is completely unrelated to types of HR-HPV.[10]

Apart from angiogenesis,[11] the development of an invasive phenotype from cervical cancer precursors (cervical intraepithelial neoplasia (CIN)) has been recently ascribed to a group of proteins encoded by the *nm23* family of metastases-associated genes.[12 13] The human *nm23* gene family includes several members (*nm23-H1*, *nm23-H2*, *DR-nm23*, *nm23-H4*, *nm23-H5* and *nm23-H6*),[14] with particular interest focused on *nm23-H1*. Since its characterisation, *nm23-H1* has been extensively studied as a potential anti-metastatic gene in several human tumours,[15–19] including cervical cancer.[12 13 20–25] The products of the *nm23-H1* and *nm23-H2* genes are identical to human nucleoside diphosphate (NDP) kinases A and B, respectively, both catalysing the phosphorylation of nucleoside diphosphates to nucleoside

Abbreviations: CIN, cervical intraepithelial neoplasia; ERK, extracellular signal-regulated kinase; FIGO, International Federation of Gynecology and Obstetrics; HPV, oncogenic human papillomavirus; HR-HPV, high-risk human papillomavirus; IHC, immunohistochemistry; ISS, Istituto Superiore di Sanità; LR-HPV, low-risk human papillomavirus; MMP, matrix metalloproteinase; NDP, nucleoside diphosphate; NPV, negative predictive value; PCR, polymerase chain reaction; PPV, positive predictive value; SCC, squamous cell carcinoma; VEGF, vascular endothelial growth factor

triphosphates.[14 26] Evidence suggests that the nm23 protein can function in the transcriptional regulation of c-myc expression.[26]

In cervical cancer and its precursor lesions, *nm23-H1* has been analysed in a sizable number of studies,[12 13 21–25 27–31] but the results are conflicting. Although some studies have established a definite anti-metastatic effect for *nm23-H1*,[24 28 29] others failed to do so,[12 13] or the number of cases was too small to draw definite conclusions. Regarding CIN lesions, some studies report an inverse relationship between *nm23-H1* expression and CIN grade,[23] whereas others report increased *nm23-H1* expression with increased high-grade CIN.[12 13 25] Similarly, the prognostic value of *nm23-H1* in cervical cancer remains a highly controversial issue.[12 13 20–22 24 27 28 30–32] Unexpectedly, none of these studies have assessed *nm23-H1* expression in relation to HPV in cervical lesions, and no in vitro studies are available on the possible interactions between HPV and the *nm23* gene family either.

As a part of our HPV-Pathogen Istituto Superiore di Sanità (ISS) study devoted to a systematic search for new potential biomarkers in HPV-related cervical carcinogenesis,[8 10 11 33] we analysed a series of cervical carcinomas and CIN lesions to assess whether *nm23-H1* expression may be of any use in predicting several of the intermediate endpoint markers of cervical cancer—namely (a) the grade of CIN; (b) the type of HR-HPV; (c) clearance of the virus after eradication of CIN; or (d) prognosis of cervical cancer. Expression of *nm23-H1* was studied in CIN lesions treated by conisation and monitored by serial polymerase chain reaction (PCR) assays for clearance or persistence of HPV, and survival data of patients with cervical cancer were related to *nm23-H1* expression in surgical samples.

MATERIALS AND METHODS

Materials

The materials of this study comprise the retrospective component of the HPV-Pathogen ISS project,[34] and were collected from the files of the pathology departments of two Italian hospitals (S Orsola Malpighi Hospital, Bologna, Italy and Maggiore Hospital, University of Trieste, Trieste, Italy). Altogether, this prospective biopsy material was taken from 302 patients with either invasive cervical squamous cell carcinoma (SCC) or CIN diagnosed and treated in these two hospitals between 1986 and 2002. Of these 302 patients, 114 patients with CIN and 38 patients with SCC were from S Orsola Malpighi Hospital, and 38 patients with CIN and 112 with SCC were from Maggiore Hospital. The mean age of all patients with CIN was 35.5 (range 18–79) years and that of patients with SCC was 59.2 (range 27–89) years ($p = 0.001$).

Available data

All the patients from S Orsola Malpighi Hospital had their HPV status determined by PCR, as reported before,[35–37] whereas the patients from Maggiore Hospital were tested for HPV in our study. Complete follow-up data were available on all 150 patients with SCC, with a mean follow-up of 51.7 (range 1–218) months. Furthermore, all patients with CIN from S Orsola Malpighi Hospital had been followed up at 6-month intervals after cone treatment (mean 10.5, range 2.4–27.6 months), and subjected to repeated colposcopy, cervical smear test and biopsy (if residual suspected). A minimum of two (up to seven) serial PCR analyses were available from 67 patients and recently reported in a study on HPV clearance.[37]

Methods

Biopsy

Both the colposcopic biopsy samples and surgical samples were fixed in 10% buffered formalin, embedded in paraffin wax and processed to obtain 5-μm-thick sections that were stained with haematoxylin–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 and CIN3. The histological diagnosis of SCC was confirmed in all cases, and two adenocarcinomas present in the original cohort were excluded from this series.

Immunohistochemistry for *nm23-H1*

Immunohistochemical (IHC) staining for the NDP kinase nm23-H1 (also known as NME1) expression was completed following standard IHC procedures. Briefly, 5-μm-thick sections of paraffin wax cut on poly-L-lysine-coated microscopy slides were deparaffinised and rehydrated in graded alcohols. The sections were heated in citrate buffer (0.01 mol/l, pH 6.0, Dako Target Retrieval Solution, Dako (Carpinteria, California, USA)) in a microwave oven (85–95℃, 3×5 min), followed by blocking the non-specific binding sites with normal rabbit serum. Sections were incubated with the primary antibody, polyclonal rabbit antihuman nm23 protein (Catalogue #A0096, DakoCytomation Denmark A/S, Glostrup, Denmark; dilution 1:50), in a humidified chamber for 1 h at room temperature. This affinity-isolated antibody was isolated using an immobilised peptide from the nm23 protein, which corresponds to a carefully selected synthetic peptide from the human nm23 protein used for immunisation. The antibody reacts with *nm23-H1* and *nm23-H2* gene products. Incubation with the primary antibody was followed by incubation with the biotinylated secondary antibody, polyclonal goat antirabbit immunoglobulin G (#6720, Abcam, dilution 1:200 (Abcam, Cambridge, UK)). Slides were then processed with universal LSAB-2 single reagents (peroxidase) kit (DakoCytomation), and expression of *nm23-H1* was localised by incubation with diaminobenzidine. As a final step, the slides were stained with light haematoxylin counterstaining. Negative controls were similarly processed by omitting the primary antibody, and biopsy samples from breast cancer were used as positive controls.

Evaluation of IHC staining

IHC staining was examined with a light microscope (Leitz Diaplan, Leitz Wetzlar, Germany), equipped with a digital camera (Leica DG300). In normal squamous epithelium, *nm23-H1* staining was universally strong throughout the cervix. *Nm23-H1* staining was mainly cytoplasmic, but some nuclear staining was also detectable. In the original grading of IHC staining, a semiquantitative scoring into 4 categories was used: 0, negative (no *nm23-H1* staining); 1, weak staining (markedly reduced *nm23-H1* expression, with major negative areas or with a diffuse weak staining); 2, moderate staining (slightly to moderately reduced *nm23-H1*, with minor areas without staining or a diffuse staining weaker than normal); and 3, strong staining (equivalent to staining of the normal epithelium; figs 1–5). In statistical analysis, the staining results were treated as dichotomous categorical variables (strong *v* reduced), or using the four-tier categorisation.

HPV testing

The patients with CIN ($n = 114$) and SCC ($n = 38$) from S Orsola Malpighi Hospital had already been tested for HPV for other purposes by PCR, as recently reported.[35–37] In our study, the 150 sections embedded in paraffin wax (112 SCCs and 38 CINs) from Maggiore Hospital were subjected to HPV testing by PCR.

Polymerase chain reaction

To verify the extraction and the quality of DNA from the tissues embedded with paraffin wax, 5 μl of each sample was

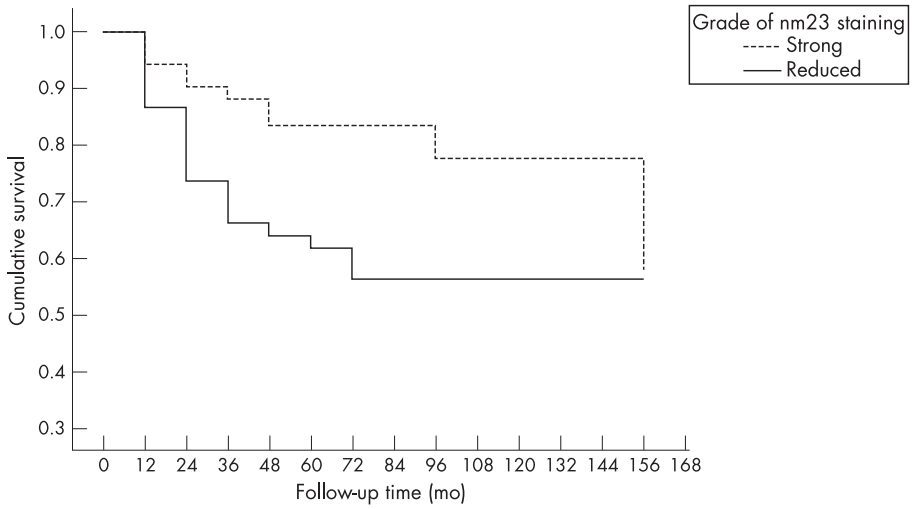

Figure 1 Cumulative survival related to *nm23-H1* expression in Kaplan–Meier analysis. mo, months.

amplified with a primer set recognising the *β-actin* gene (sense 5′-GGCGGCACCACCATGTACCCT-3′, anti-sense 5′-AGGGGCCGGACTCGTCATACT-3′). The PCR mix contained 200 μM of each deoxyribonucleotide triphosphate, 1.5 mM magnesium chloride ($MgCl_2$), 1× PCR buffer, 40 pmol sense and anti-sense primer, and 1.25 U AmpliTaq Gold (Applied BioSystem, Branchburg, New Jersey, USA). The PCR conditions were as follows: 94℃, 10 min for 1 cycle; 94℃, 30 s, 60℃, 30 s, 72℃, 30 s for 25 cycles; and finally, 72℃ for 7 min.

The samples were then amplified to test for the presence of HPV with different sets of degenerated primers, as described separately for MY09/MY11,[38] $GP5^+/GP6^+$[39] and biotinylated short PCR fragment primer mix located in the L1 region of the HPV genome.[40] The PCR conditions for the My09/My11 were as follows: 94℃, 10 min for 1 cycle; 94℃, 30 s, 55℃, 45 s, 72℃, 30 s for 40 cycles, followed by an extension step at 72℃ for 7 min. The PCR mix contained 200 μM of each deoxyribonucleotide triphosphate, 40 pmol of each primer, 2 mM $MgCl_2$, 1× PCR buffer and 1.25 U AmpliTaq Gold. For the $GP5^+/GP6^+$ primers, the following conditions were used: 94℃, 10 min for 1 cycle; 95℃, 30 s, 44℃, 60 s, 72℃, 90 s for 40 cycles; then a final extension step at 72℃ for 7 min. Amplification with short PCR fragment primer mix was carried out as follows: 94℃, 10 min, for 1 cycle; 94℃, 30 s, 52℃, 45 s, 72℃, 45 s for 40 cycles; then a final extension step at 72℃ for 7 min. Positive and negative controls were included in each amplification.

HPV typing

HPV typing was carried out using the reverse-hybridisation assay. The denatured biotinylated amplified product (10 μl) was hybridised with specific oligonucleotide probes, which are immobilised as parallel lines on membrane strips (InnoLiPA, Innogenetics, Ghent, Belgium).[40] After hybridisation and stringent washing, streptavidin-conjugated alkaline phosphatase was added and bound to any biotinylated hybrid previously formed. Incubation with 5-bromo-4-chloro-3-indoyl phosphate–nitro blue tetrazolium chromogen yields a purple precipitate that can be visually interpreted. On the basis of the position of the visualised line, it is possible to determine the HPV genotype.[40] The following types of HPV were included in the test panel: HPV 6, 11, 16, 18, 31, 33, 34,

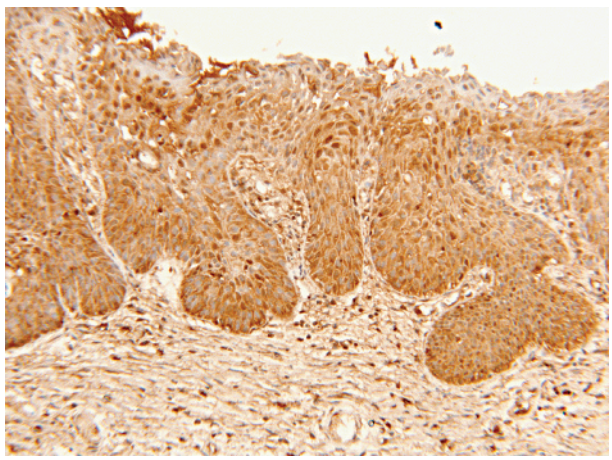

Figure 2 Normal transformation zone showing the process of immature squamous metaplasia. Also, the metaplastic squamous epithelium shows intense expression of nm23-H1 protein in all layers of the epithelium (immunohistochemistry for *nm23-H1*, original magnification ×100).

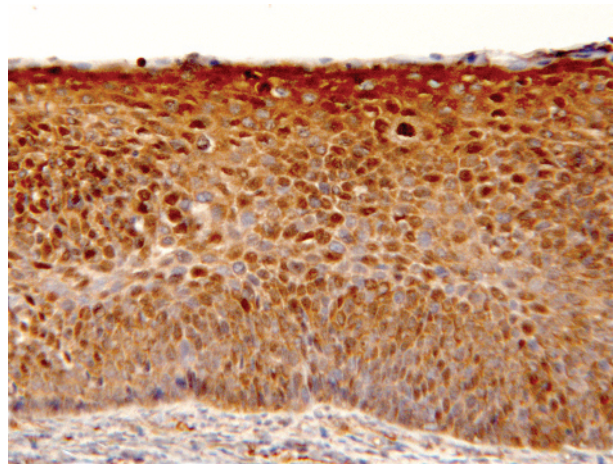

Figure 3 A low-grade cervical intraepithelial neoplasia 1 lesion, showing intense staining for *nm23-H1* throughout the full thickness of the lesion, being mainly cytoplasmic; many positive nuclei are also present. The underlying stroma is *nm23-H1* negative (immunohistochemistry for *nm23-H1*, original magnification ×200).

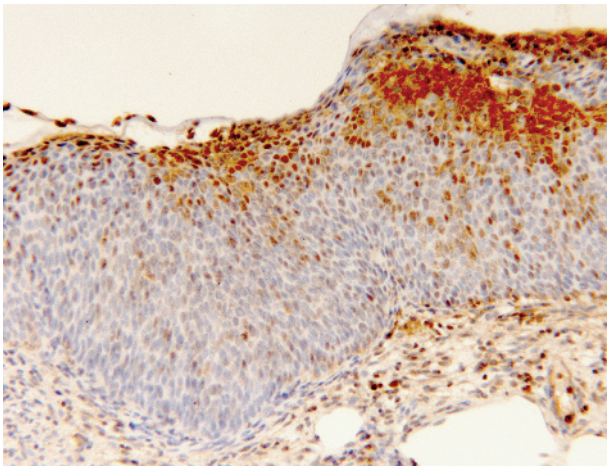

Figure 4 A high-grade cervical intraepithelial neoplasia 3 lesion, with markedly reduced *nm23-H1* expression. Positive staining is confined to the uppermost layers of the epithelium, whereas the lower two thirds remains entirely *nm23-H1* negative, except for some single scattered positive cells (immunohistochemistry for *nm23-H1*, original magnification ×100).

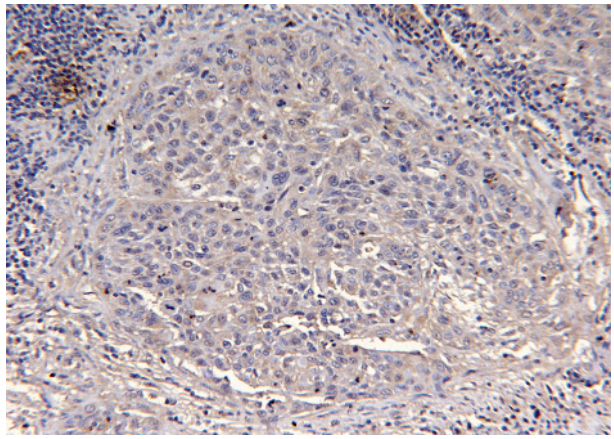

Figure 5 Details of an invasive squamous cell carcinoma. In contrast with the intense diffuse staining seen in cervical intraepithelial neoplasia 1 lesions, only a weak cytoplasmic staining is present in some single cancer cells, making it difficult to discern the invasive focus from the surrounding stroma. This represents a typical case of markedly reduced *nm23-H1* expression (immunohistochemistry for *nm23-H1*, original magnification ×100).

35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68, 70 and 74.

Statistical methods

Statistical analyses were carried out with the SPSS and STATA software packages (SPSS for Windows, V.12.0.1, and STATA/SE V.8.2). Frequency tables for categorical variables were analysed using the χ^2 test, the likelihood ratio or Fisher's exact test to assess the significance of the correlation. Bivariate correlations between ordered variables were analysed using Spearman's correlation analysis (Spearman's r). Differences in the means of continuous variables were analysed using non-parametric tests (Mann–Whitney U) or analysis of variance. Logistic regression models using the stepwise backward approach and likelihood ratio statistics for removal testing were used to analyse the power of different covariates as predictors of the outcome variables (CIN, HR-HPV), and to calculate crude odds ratios (ORs) and 95% confidence interval (CI). Performance indicators of *nm23-H1* as a marker of CIN or HR-HPV were calculated with the conventional contingency tables for sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV), with 95% CI based on the F-distribution ($\pm 1.96 \times$ standard error). Univariate survival (life-table) analysis for the outcome measure (clearance or persistence of HPV and overall survival) was based on the Kaplan–Meier method. Multivariate survival analysis was carried out using Cox's proportional hazards model in a backward stepwise manner with the log likelihood ratio significance test, and with the default values for entry and exclusion criteria. The assumption of proportional hazards was checked by log–minus-log survival plots. In all tests, $p<0.05$ was considered to be significant.

RESULTS

Table 1 shows the expression of *nm23-H1* related to the grade of the lesion in cone or surgical specimens. An inverse linear relationship was observed between the increasing grade of the lesion and the decreasing intensity of *nm23-H1* ($p = 0.001$ for linear trend). Reduced expression of *nm23-H1* was associated with CIN3 or cancer at OR 8.72 (95% CI 4.13 to 18.41; $p = 0.001$), using the two-tier category of staining (strong or reduced).

Table 1 Expression of *nm23-H1* and grade of cervical lesions

	Expression of *nm23-H1*					
	Markedly reduced		Slightly reduced		Strong	
Grade of lesion	n	%	n	%	n	%
Negative for: CIN	0	0.0	1	10.0	9	90.0
CIN1	0	0.0	3	15.0	17	85.0
CIN2	2	10.0	4	20.0	14	70.0
CIN3	21	21.9	37	38.5	38	39.6
SCC	51	38.3	48	36.1	34	25.6
Total (n = 279)	74	26.5	93	33.3	112	40.1

CIN, cervical intraepithelial neoplasia; SCC, squamous cell carcinoma. Fisher's exact test, p = 0.001 (also for linear trend).

Of the CIN lesions, 70.5% were HR-HPV positive, in contrast with only 11.1% of those without CIN. HR-HPV types were even more prevalent in patients with SCC (77.6%), the rest (22.4%) being either negative (4.9%) or those in whom the type of HPV could not be determined (17.5%). Detection of HR-HPV was associated with SCC at OR 27.25 (95% CI 3.28 to 226.09) and with CIN at OR 19.12 (95% CI 2.31 to 157.81). Among the grades of CIN, the most distinct separation is between CIN2 and CIN3, of which 52.4% and 78.6% are HR-HPV positive, respectively.

Table 2 shows the expression of *nm23-H1* related to the detection of HR-HPV in the lesions. *Nm23-H1* staining intensity was only marginally different in HR-HPV-positive and HR-HPV-negative lesions ($p = 0.047$), in that the strong expression (equivalent to normal epithelium) was slightly more common in HR-HPV-negative cases: 52.1% and 35.5% (OR 1.97, 95% CI 1.14 to 3.42; $p = 0.015$).

Table 3 shows the performance indicators calculated for *nm23-H1* as a marker of CIN and HR-HPV. Reduced *nm23-H1* staining is a 90% specific indicator of CIN, with 98.5% PPV, but strong staining does not rule out CIN, because NPV is only 11.5%. With the CIN3 cut-off, there is a drop in both specificity and PPV, and a slight gain in sensitivity and NPV. Evidently, *nm23-H1* staining is not a useful marker of HR-HPV in these lesions.

Table 2 Expression of *nm23-H1* related to high-risk human papillomavirus types in lesions

	Expression of *nm23-H1*					
	Markedly reduced		Slightly reduced		Strong	
HR-HPV*	n	%	n	%	n	%
Present	53	26.9	74	37.6	70	35.5
Absent	16	22.5	18	25.4	37	52.1
Total (n = 273)	69	25.7	92	34.3	107	39.9

HR-HPV, high-risk human papillomavirus.
*Also includes cases that were HPV negative; χ^2 test, Pearson's p = 0.047.

In all, 41 of 67 (61.2%) of the HPV-positive women treated for CIN and controlled by serial PCR cleared their HR-HPV infection by the last PCR assay, with a total of 705 women months at risk and a monthly clearance rate of 5.8% (58/1000 women months at risk). Clearance in 17 of 30 (56.7%) cases with strong *nm23-H1* expression was comparable with that of 22 of 34 (64.7%) cases with reduced staining (p = 0.610). The corresponding figures for virus persistence were 5 of 30 (16.7%) cases and 4 of 34 (11.8%) cases, respectively (p = 0.723). In univariate survival analysis, *nm23-H1* staining was not a significant predictor of either clearance (log rank, p = 0.514) or persistence (p = 0.243) of HR-HPV types in the cervix after treatment of CIN by large loop excision of the transformation zone.

Of the 150 patients with SCC, 91 (60.7%) were alive and 59 (39.3%) had died during the follow-up. *Nm23-H1* proved to be a significant predictor of survival in cervical cancer (table 4). The probability of survival was markedly higher (p = 0.022) for patients with strong (normal) *nm23-H1* expression than those with reduced expression (OR 2.82, 95% CI 1.16 to 6.83; p = 0.044). This is also evident in the Kaplan–Meier analysis, where survival was significantly determined by *nm23-H1* expression (p = 0.022; fig 1). HR-HPV also showed a slightly positive effect on survival in 64.0% of HR-HPV-positive women, and 45.2% of HR-HPV-negative women were alive (p = 0.062; OR 2.15, 95% CI 0.96 to 4.82). In the Kaplan–Meier analysis, this difference was not significant (p = 0.053). As usual, the International Federation of Gynecology and Obstetrics (FIGO) stage was the most powerful predictor of survival in the Kaplan–Meier analysis (p = 0.001).

In multivariate survival (Cox) analysis, *nm23-H1* expression did not prove to be a significant independent prognostic factor. It was removed from the model, when adjusted for age, HR-HPV, grade of tumour and FIGO stage. In the final Cox model, only the FIGO stage (p = 0.001) and age (p = 0.011) proved to be independent predictors of patient survival. When FIGO stage 1 was used as the reference, the OR for dying from the disease in stage 2 was 5.23 (95% CI 1.78 to 15.36), that in stage 3 was 9.29 (95% CI 3.18 to 27.13) and that in stage 4 was 23.33 (95% CI 6.85 to 79.46). The mean age of women who were alive was 54.2 years as compared with 66.7 years of those who died (p = 0.001).

DISCUSSION

We started a systematic survey of potential biomarkers of HR-HPV and CIN in our HPV-Pathogen ISS study.[34] Until now, four of the programmed 13 markers of HR-HPV have been completed: $p16^{INK4}$,[8] ERK1,[10] vascular endothelial growth factor (VEGF)-C[11] and survivin.[41] Although bearing some interesting links with HR-HPV and CIN, none of these proved to be an independent prognostic predictor in cervical cancer.[8 10 11 41] Recently, a group of proteins, known as the *nm23* family, has received considerable attention as a potential anti-metastatic gene in cervical cancer.[12 13 20–25] There is no full agreement yet on (a) the relationship between *nm23-H1* expression and grade of CIN, (b) its anti-metastatic effects or (c) its value as a predictor of survival in cervical cancer.[12 13 21–25 27–32] Because no previous data are available on the possible interactions between HPV and the *nm23* gene family either in vivo or in vitro,[1 2 4] we assessed whether *nm23-H1* expression may be of use in predicting any of the several outcome measures: grade of CIN, HR-HPV type, clearance of the virus and prognosis of cervical cancer, as reported for the other markers.[8 10 11 41]

Expression of *nm23-H1* as related to grade of CIN has been assessed in a few previous studies.[12 13 23 25] Although in some of these studies *nm23-H1* expression was found to increase in parallel with increasing grade of CIN,[12 13 25] Lee and Gad[23] reported a significant decreasing trend (p = 0.006) from normal epithelium to CIN3 and SCC. This observation is in perfect agreement with the findings of our study, where the reduced expression of *nm23-H1* was shown to increase towards high-grade CIN, being most frequent among carcinomas (table 1). Indeed, this reduced *nm23-H1* expression was associated with CIN3 or cancer at OR 8.72 (95% CI 4.13 to 18.41; p = 0.001), when compared with CIN1, CIN2 or normal epithelium. The most feasible explanations for these seemingly contradictory observations in the literature could be the (a) different IHC protocols used; (b) different *nm23-H1* antibodies; (c) different systems of grading the IHC staining; and (d) the known problems in grading CIN lesions, particularly the low-grade CIN.[1 2 4]

In the light of the available data on the biological functions, it seems feasible to predict that the constitutive expression of this anti-metastatic protein[15–19] should be down

Table 3 Performance indicators of *nm23-H1* as a marker of cervical intraepithelial neoplasia and high-risk human papillomavirus type

Outcome variable	Performance of *nm23-H1* (95% CI)*			
	Sensitivity, %	Specificity, %	PPV, %	NPV, %
CIN†	48.8 (40.4 to 57.3)	90.0 (71.4 to 100)	98.5 (95.6 to 100)	11.5 (4.4 to 18.6)
CIN3‡	68.5 (62.5 to 74.6)	80.0 (68.9 to 91.1)	94.0 (90.4 to 97.6)	35.7 (26.8 to 44.5)
HR-HPV	64.4 (57.7 to 71.1)	52.1 (40.4 to 63.7)	78.8 (72.6 to 85.2)	34.5 (25.6 to 43.5)

CIN, cervical intraepithelial neoplasia; HR-HPV, high-risk human papillomavirus; NPV, negative predictive value; PPV, positive predictive value.
**nm23-H1* staining (reduced or strong).
†Any grade of CIN (squamous cell carcinoma (SCC) cases excluded).
‡CIN3 cut-off (SCC cases included).

Table 4 Expression of *nm23-H1* and prognosis of cervical cancer

Expression of *nm23-H1*	Overall survival of patients with cervical cancer					
	Alive		Dead			
	n	%	n	%	OR (95% CI)	Significance
Strong	26	76.5	8	23.5	Reference	
Slightly reduced	24	50.0	24	50.0	0.40 (0.15 to 1.06)	p=0.044
Markedly reduced	29	56.9	22	43.1	0.30 (0.11 to 0.81)	
Strong	26	76.5	8	23.5	Reference	p=0.022*
Reduced	53	53.5	46	46.5	2.82 (1.16 to 6.83)	

*Logistic regression.

regulated in a considerable proportion of high-grade CIN and invasive cancer as compared with low-grade CIN and normal epithelium, exactly as reported before[23] and also confirmed by our results. In fact, the most dramatic change in *nm23-H1* expression seems to take place on transition from CIN2 to CIN3, when the proportion of normal (strong) expression drops from 70% down to 39% (table 1). These observations are identical with those of Lee and Gad,[23] who showed that *nm23-H1* staining was either absent or markedly reduced in all CIN3 lesions, and the difference between CIN1 and CIN3 was significant ($p = 0.013$). These data suggest that down-regulated expression of *nm23-H1* is a marker of progressive disease, probably associated with a clonal selection (among the CIN lesions) of cases capable of rapid progression to CIN3 and predestined to develop an invasive phenotype from CIN3.

As it is rarely (if ever) seen in early precancer lesions (CIN1), down-regulated *nm23-H1* seems to be a late marker of cervical carcinogenesis, and as such markedly differs from those previously analysed in our study.[8 10 11 41] In fact, strong or reduced expression of *nm23-H1* distinguished CIN with 90% specificity and 98.5% PPV, but was not particularly sensitive (48.8%; table 3). These figures are slightly inferior to those recently calculated for $p16^{INK4a}$,[8] ERK1,[10] survivin[41] and VEGF-C,[11] all being 100% specific markers of CIN, with 100% PPV. For *nm23-H1*, no comparative data have been previously published. We also tested these ''performance indicators'' for *nm23-H1* by changing the cut-off to CIN3, which improved the sensitivity and NPV but slightly compromised the specificity and PPV. In the future, the value of *nm23-H1* as a marker of CIN or cancer will be tested in combination with the other markers, as soon as the analysis of the 13 biomarkers is completed.[33 34]

In our study, reduced *nm23-H1* staining was associated with HR-HPV-positive cases with OR 1.97, 95% CI 1.14 to 3.42 ($p = 0.015$). This association is much weaker than previously reported for $p16^{INK4a}$ and survivin,[8 41] and *nm23-H1* readily dropped out from the multivariate model containing these two independent predictors of HR-HPV. Accordingly, there seems to be no role for *nm23-H1* as an independent marker of HR-HPV (table 3), in contrast with $p16^{INK4a}$, survivin and (to some extent) VEGF-C, which are all functionally regulated by HR-HPV oncoproteins.[8 11 41] This failure to show any HR-HPV specificity for *nm23-H1* suggests that this family of proteins is regulated by molecular mechanisms unrelated to oncogenic HPV. At this moment, these regulatory mechanisms remain obscure, however.[26 42 43]

During the past several years, persistent HR-HPV infections have received increasing attention as a cause of markedly increased risk of treatment failure of CIN.[2 37] Monitoring this risk of disease recurrence after cone treatment with a suitable marker would be of considerable clinical value, and we were interested to see whether *nm23-H1* expression may be of any such predictive value for persistence or clearance of HR-HPV. In alignment with the failure to find any specificity for HR-HPV, *nm23-H1* expression did not provide any useful information as a predictor of clearance or persistence of HPV in the cervix after treatment of CIN. With our previous data,[8 10 11 41] this implicates that none of the five biomarkers analysed so far (nm23-H1, VEGF-C, survivin, ERK1 and $p16^{INK4a}$) can substitute HPV testing in monitoring the risk of disease recurrence after cone treatment.[37]

As pointed out earlier, there is no agreement on the value of *nm23-H1* as a predictor of survival in cervical cancer.[12 13 21–25 27–32] In our study, *nm23-H1* expression was analysed by both univariate (Kaplan–Meier) and multivariate (Cox) survival analysis. Importantly, *nm23-H1* proved to be a major predictor of survival, as 76.5% of the patients with strong *nm23-H1* expression were alive in contrast with 53.5% of those with reduced *nm23-H1* expression (table 4). In Kaplan–Meier analysis, survival was significantly determined by the *nm23-H1* expression with the two-tier grading ($p = 0.022$; fig 1). Thus, our results, confirming a positive prognostic value for strong expression of *nm23-H1*, are in alignment with the previous studies reporting similar observations in cervical SCC[12 13 23 27 29] or adenocarcinoma.[28 30] Other studies fail to ascribe any prognostic value for *nm23-H1* expression, however.[22 24 28 30 31] In our study, the prognostic value of *nm23-H1* was important only in univariate analysis, but lost its independent value when it was entered into the Cox multivariate model, where the FIGO stage was the single most powerful prognostic predictor. As pointed out, however, it seems most likely that the power of the FIGO stage as an independent prognostic predictor will be hard to exceed by any single marker, and, consequently, any new biomarker showing a predictive value in univariate analysis can be considered to be a potentially useful prognostic predictor in cervical cancer.[44]

Regarding the mechanisms of the anti-metastatic effects of *nm23-H1*, several possibilities have been implicated.[13 27] These known functions of *nm23-H1* include NDP kinase activity, transcription factor, as well as associations with differentiation, proliferation, cell motility and signal transduction.[27] It was recently suggested that the *nm23* gene instability is probably associated with regulation of tumour progression and would explain the divergent biological role of this protein in different tumours.[13] In cervical cancer, *nm23-H1* expression was recently linked with some other markers—that is, H-ras, cathepsin B (cystein proteinase), collagenases and plasminogen activators.[27] Of these, only type IV collagenase (matrix metalloproteinase (MMP)-2 or gelatinase A) is included in our panel of biomarkers[33 34] and was checked for bivariate correlations with *nm23-H1* out of curiosity. Interestingly, a significant inverse correlation was established between *nm23-H1* and *MMP-2* expression (Spearman r −0.167, $p = 0.008$; data not shown). As recently suggested, interactions between the proteolytic phenotype and expression of *nm23-H1* in cervical cancer seem to exist,[27] which would be consonant with this association of down-regulated *nm23-H1* with up-regulated MMP-2 (ie, increased proteolytic activity). More is known about the possible *nm23-H1* pathways in

other tumours—for example, in lung cancer, where *nm23-H1* up regulated mRNA and protein levels of β-catenin, E-cadherin, TIMP-1 and CD44s, and down regulated levels of MMP-2, CD44v6 and VEGF.[43] Apart from the above association between *nm23-H1* and MMP-2, we could readily also confirm the inverse correlation between *nm23-H1* and VEGF-C (Spearman's r −0.189; p = 0.002) in our series. It remains to be seen whether *nm23-H1* expression is markedly correlated with any of the other biomarkers to be analysed in this series.[33 34]

Taken together, no evidence was obtained to substantiate direct interactions between HPV and *nm23-H1*, the expression of *nm23-H1* being unrelated to HR-HPV. *Nm23-H1* seems to be a marker of progressive disease, being dramatically down regulated in high-grade CIN and invasive cancer. No plausible explanation linking this down regulation with HR-HPV was disclosed, but this down regulation was an adverse prognostic sign in cervical cancer. Whether eventual indirect molecular links to HPV will be disclosed remains to be seen, when the data of our 13 markers are ready for multivariate analysis to predict the intermediate endpoint markers and disease outcome in cervical cancer.

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