Differential expression of ANXA6, HSP27, PRDX2, NCF2, and TPM4 during uterine cervix carcinogenesis: diagnostic and prognostic value

MI Lomnytska*,1,7, S Becker2, I Bodin3, A Olsson3, K Hellman4, A-C Hellström4, M Mints5, U Hellman6, G Auer2 and S Andersson1

1Department of Obstetrics and Gynecology, Institute for Clinical Science and Technology, CLINTEC, Karolinska University Hospital, Huddinge, Karolinska Institutet, Stockholm SE-14186, Sweden; 2Unit of Cancer Proteomics, Department of Oncology and Pathology, Karolinska Institutet, Karolinska University Hospital, Stockholm SE-171 76, Sweden; 3Department of Obstetrics and Gynecology, Karolinska Institutet, Karolinska University Hospital, Stockholm SE-171 76, Sweden; 4Department of Gynecologic Oncology, Radboudumc, Karolinska University Hospital, Stockholm SE-171 76, Sweden; 5Department of Obstetrics and Gynecology, Karolinska University Hospital, Solna, Karolinska Institutet, SE-17176 Stockholm, Sweden; 6Ludwig Institute for Cancer Research, Ltd, Uppsala University, Box 595, Uppsala SE-75124, Sweden; 7Department of Oncology and Medical Radiology, Lviv National Medical University, Pekarska street, 69, Lviv 79010, Ukraine

BACKGROUND: Cytology-based diagnostics of squamous cervical cancer (SCC) precursor lesions is subjective and can be improved by objective markers.

METHODS: IHC-based analysis of ANXA6, HSP27, peroxiredoxin 2 (PRDX2), NCF2, and tropomyosin 4 (TPM4) during SCC carcinogenesis.

RESULTS: Expression of ANXA6, HSP27, PRDX2, and NCF2 in the cytoplasm of dysplastic cells increased from cervical intraepithelial neoplasia 2/3 (CIN2/3) to microinvasive cancer. Invasive SCC showed lower expression of TPM4 than CIN and normal epithelium. CIN2/3 with the highest sensitivity and specificity differed from normal epithelium by cytoplasmic expression of HSP27. Patients with cytoplasmic HSP27 expression in SCC deviating from that observed in normal epithelium had worse relapse-free (P = 0.019) and overall (P = 0.014) survival. Invasive SCC with the highest sensitivity and specificity differed from normal epithelium by expression of PRDX2 and TPM4 in the cytoplasm, from CIN2/3 by the expression of ANXA6 and TPM4 in the cytoplasm, and from microinvasive SCC by the expression of PRDX2 and ANXA6 in the cytoplasm. The number of sporadic ANXA6+ cells between the atypical cells increased from CIN2/3 to invasive SCC.

CONCLUSION: Detection of expression changes of the proteins ANXA6, HSP27, PRDX2, NCF2, and TPM4 in SCC precursor lesions may aid current cytological and pathological diagnostics and evaluation of prognosis.

Keywords: cervical cancer precursors; squamous cervical cancer; diagnostics; marker protein patterns; immunohistochemistry

Cervical cancer (CC) is the second most commonly diagnosed cancer among women worldwide (Garcia et al., 2007). The introduction of cytology-based screening has reduced incidence of the most common squamous cell cervical carcinoma (SCC), but CC remains a major problem in the developing world.

The aetiological role of human papillomaviruses (HPVs) for CC and precursor lesions has been established (Wallin et al, 1999; Munoz et al, 2003; Castellsague, 2008; Zur Hausen, 2009). Persistent infection with HR-HPV and expression of the viral oncoproteins E6 and E7 are critical for malignant transformation (Zur Hausen, 2009), making detection and monitoring of HR-HPV carriers upon primary detection of abnormal cervical cytology an attractive approach (Nobbenhuis et al, 1999; Brismar et al, 2009). However, more than half of patients with normal cervical histopathology demonstrate infection with HR-HPV although gain of the 3q26 chromosome region, where the human telomerase RNA gene (hTERC) is located, is an early event during CC carcinogenesis (Alameda et al, 2009; Andersson et al, 2009). Detection of hTERC amplification discriminates low- and high-grade squamous intraepithelial lesions and identifies patients with histologically confirmed cervical intraepithelial neoplasia (CIN) and SCC (Andersson et al, 2009).

In 1928, both Babés and Papanicolaou (Babés, 1928; Papanicolaou, 1928) described the potential for desquamated cell material from the uterine cervix to serve as a diagnostic tool for detection of CC. However, a high false-negative rate remains a major clinical problem today. The accuracy of Pap smear technique for early detection of CC and precursor lesions has been evaluated and the results varied with false-negative rates ranging from 5% to 40% (Näslund et al, 1986). When compared with conventional Pap smears, LBC showed neither higher sensitivity nor specificity for detection of CIN (Arbyn et al, 2008).

Correct diagnosis is important for choice of therapy and avoiding of under- and over-treatment. This indicates need for
additional objective markers of CC precursors in cervical cytology material that can be detected by professionals or assessed through automated technology, and thus improve early diagnostics.

One promising approach in the search for new cancer markers is proteomics. Proteomics is used in the analysis and identify differentially expressed proteins in tissue samples, and to validate their significance as disease markers (Kulasingam and Diamandis, 2008). Proteomics was used in several studies of CC markers to identify a number of differentially expressed proteins, but without analysing their potential diagnostic value (Bae et al., 2005; Choi et al., 2005; Zhu et al., 2009). We previously used proteomics analysis to compare SCC and squamous vaginal cancer (Hellman et al., 2004, 2009; Lomnytska et al., 2010), and we extend the analysis in a current study aiming to establish a marker protein pattern for objective detection of SCC precursor lesions. We performed an analysis of expression of ANXA6, HSP27, peroxiredoxin 2 (PRDX2), NCF2, and tropomyosin 4 (TPM4) on sequential steps of SCC carcinogenesis, that is, on CIN2/3, microinvasive, and invasive cancers. We evaluated cytoplasmic and nuclear expression of these proteins in differentiated, dysplastic, and cancer cells, compared expression of proteins, and discussed the potential clinical value of the differential expression of the studied marker protein patterns.

MATERIALS AND METHODS

Clinical material

The study was performed on formaldehyde-fixed paraffin-embedded material, collected at the Department of Gynecology, Karolinska University Hospital, Huddinge, Sweden, and at the Department of Oncology and Medical Radiology, Lviv National Medical University, Ukraine with informed consent and approval from the local ethics committees (Stockholm County Council – Dnr. 97-244, 00-068, 352/00; Ethics Committee of Lviv National Medical University – protocol No. 2; Table 1A). Histopathological presentation of selected cases was confirmed.

IHC

Paraffinised tissue blocks were cut to obtain 4 µm-thick tissue sections on SuperFrost (Braunschweig, Germany) slides and kept overnight at 55°C before deparaffinisation and rehydration that was carried out in a series of xylene and ethanol baths of decreasing concentration. Antigen was retrieved by boiling in 0.1 M Na citrate buffer (pH 6.0). Endogenous peroxidase was inhibited by 0.5% H2O2. Samples were incubated in 5% serum of the species from which the secondary antibody was obtained to avoid non-specific binding. Monoclonal antibodies to ANXA6 (1:400), PRDX2 (1:100), HSP27 (1:400), NCF2 (1:150), TPM4 (1:100), vimentin (1:200, V5255, Sigma-Aldrich, St Louis, MO, USA), and CD68 (1:400, PG-M1, DAKO, Carpenteria, CA, USA) in 1% BSA were applied and incubated overnight at + 4°C (Table 1B). The secondary antibody was coupled to DAB via a biotin–avidin complex for visualisation (VectorStain, Vector, Burlingame, CA, USA). Tissue samples were counterstained with hematoxylin, washed in lukewarm water, dehydrated in a series of increasing concentrations of ethanol and xylene, mounted using a permanent mounting medium, and covered. All steps were carried out in a moist chamber.

Evaluation of staining

Images were captured at a Leica DM4500B light microscope (camera DFC320, ocular 10 ×, objectives 20 ×/0.50 HC PL, and 40 ×/0.13 HC PL, Leica Application Suite software (version 2.4.0, Wetzlar, Germany), 16-bit depth.tif format images with 48 bit/µm image resolution). Expression of the analysed proteins was scored based on the intensity of staining, location of staining in individual cells, and on the number of positively stained cells. Intensity of colour expression was scored as: 0, negative; 1 +, weak; 2 +, moderate; and 3 +, strong. The number of positively stained cells was scored as 0 if no staining was observed or was present in <5% of cells; 1 +, positive staining in 5–25% of cells; 2 +, positive staining in 25–75% of cells; and 3 +, positive staining in more than 75% of cells. Expression of a protein was evaluated as a

Table 1 Characterisation of clinical material (A) and primary antibodies used for immunohistochemistry (IHC) (B)

(A)

| Diagnosis, FIGO | Histopathology | Abbreviation | Number of cases (%) |
|----------------|----------------|-------------|---------------------|
| Normal cervix  | Normal cervix  | SCE         | 8 (11.94)           |
| CIN 2          | Moderate cervical intraepithelial dysplasia | CIN 2 | 4 (5.97)       |
| CIN 3          | Severe cervical intraepithelial dysplasia | CIN 3 | 9 (13.43)       |
| SCC, stage IA1 | Squamous cervical carcinoma in situ | MiSCC | 12 (17.91)       |
| SCC, stage IA2 | Invasion >5 mm in depth or >7 mm horizontally | InvSCC | 2 (2.99)     |
| SCC, stage IB1 | Visible lesion ≤ 4 cm in greatest dimension | 17 (25.37) |
| SCC, stage IB2 | Visible lesion > 4 cm | 2 (2.99) |
| SCC, stage IIA | Without parametral invasion, but involving upper 2/3 of vagina | 1 (1.49)  |
| SCC, stage IIB | With parametral invasion | 1 (1.49) |
| SCC, stage III | Extends to pelvic wall or lower third of vagina | 3 (4.48) |
| Total          |                |             | 66                  |

(B)

| Protein | Antibody | Origin | Clone | Epitope detection | Dilution factor | Tissues for positive controls | Manufacturer |
|---------|----------|--------|-------|-------------------|----------------|-------------------------------|--------------|
| ANXA6   | Sc-1931  | Goat polyclonal | N-19  | N-terminus       | 400            | Liver hepatocytes              | SantaCruz    |
| HSP27   | Sc-13132 | Mouse monoclonal | F-4   | Amino acids 32–108 | 400            | Tonsilla                      | SantaCruz    |
| PRDX2   | WH007001 | Mouse monoclonal | 4E 10-2D2 | Amino acids 1–199 | 100           | Tonsilla                      | Sigma-Aldrich|
| NCF2    | HP006040 | Rabbit polyclonal | PrEST | Full-length        | 150            | Glandular epithelium of colon | Sigma-Aldrich|
| TPM4    | WH000717 | Mouse monoclonal | 4E4-1D2 | Full-length       | 200            | Placenta, decidual tissue     | Sigma-Aldrich|

Abbreviations: CIN = cervical intraepithelial neoplasia; FIGO = International Federation of Gynecologic Oncology; SCC = squamous cervical cancer; SCE = squamous cervical epithelial.
histoscore, that is, as a sum of the scores of the intensity and of the cell number counts (Cheng et al, 2008).

DNA cytometry

DNA cytometry was performed on tissue sections (6 µm) of CIN2/3 and SCC. Slides were stained with Feulgen and the DNA content in nuclei of atypical cells was measured as described (Steinbeck et al, 1999). DNA values were determined in relation to a corresponding control, which denoted the normal DNA (diploid) content at 2c region. Histograms with a narrow stem line in the 2c region represented diploid genomically stable tumours, whereas those with a broad stem line in the 2c region expanding towards the 4c region were classified as diploid genomically unstable. Histograms with a narrow peak outside the 2c region were considered to be aneuploid genomically stable, whereas with a broad peak outside the 2c region and additional peaks exceeding the 4c region were classified as aneuploid genomically unstable. Approximately 100 cells were analysed for each tumour specimen.

Statistical analysis

Software Statistica 6.0 (StatSoft Inc., Tulsa, OK, USA) was used for the $\chi^2$ test, t-test, P-value, software MedCalc 11.2.1.0 (MedCalc Software bvba, Mariakerke, Belgium) for ROC analysis, evaluation of sensitivity and specificity. Relapse-free survival (RFS) and overall survival (OS) were evaluated using univariate Kaplan–Meier analysis with log-rank test. A difference of $P<0.05$ was considered statistically significant (Glantz, 1998).

RESULTS

Intensity of expression of ANXA6, HSP27, PRDX2, NCF2, and TPM4 in normal uterine cervix and during development of SCC

In squamous cervical epithelial (SCE), expression of ANXA6 was localised to the epithelial cell membrane with moderately intense expression in the submucosal stroma. Positive expression of ANXA6, HSP27, PRDX2, NCF2, and TPM4 was observed in normal uterine cervix (SCE) and during development of SCC.

Figure 1 Six selected cases (1–6) showing immunohistochemical expression of ANXA6 (A), HSP27 (B), PRDX2 (C), NCF2 (D), and TPM4 (E), magnification: × 400 (D1, D2, E2, C6 have magnification × 200), noncropped images with inserts. Observed expression of ANXA6 was membranous in SCE (A1), cytoplasmic and sporadic in CIN2/3, MicSCC and InvSCC (A2–A6). Expression of HSP27 in the nuclei was observed in CIN2/3, MicSCC (B2–B4), and less in InvSCC (B5–B6). Expression of PRDX2 was cytoplasmic in positive cases. Expression of NCF2 was observed in the cytoplasm (D2–D6), and expression of TPM4 in the cytoplasm was observed mainly in SCE (D1). Histopathological description. SCE (1): stratified layers of epithelial cells with a thin layer of parabasal cells. CIN2/3 (2–3): dysplastic cells with hyperchromatic irregular nuclei and koilocytic atypia with perinuclear clear vacuolisation as characteristic of HPV infection (marked with black arrows – C2, A4, B4) that gradually substitute epithelial layers from basal membrane throughout the epithelium forming carcinoma in situ. MicSCC (4): invasion of dysplastic cells into underlying stroma for <3 mm in depth and <7 mm horizontally. InvSCC (5–6): irregular infiltrates of tumor cells in connective tissue with inflammatory reaction.
Table 2  Breakdown of studied cases by intensity of expression of ANXA6, HSP27, PRDX2, NCF2, and TPM4 (A) and histoscore (B)

(A) Breakdown of cases by the intensity of expression of protein, number of patients (%)

| Diagnosis      | ANXA6, cytoplasmic expression | ANXA6, sporadic cells | HSP27, cytoplasmic expression | HSP27, nuclear expression | HSP27, stroma |
|----------------|-------------------------------|-----------------------|-------------------------------|--------------------------|--------------|
|                | 0+                           | 1+                    | 2+                           | 3+                       |              |
| SCE            | 0                            | 0                     | 0                            | 0                        |              |
| CIN2           | 3 (42.8)                     | 2 (28.6)              | 2 (28.6)                     | 0                        |              |
| CIN3           | 7 (28.0)                     | 9 (36.0)              | 9 (36.0)                     | 0                        |              |
| MvSCC          | 2 (22.2)                     | 3 (133)               | 4 (44.4)                     | 0                        |              |
| InvSCC         | 18 (76)                      | 4 (167)               | 2 (8.3)                      | 0                        |              |
|                | 0                            | 2 (12.5)              | 19 (79.2)                    | 0                        |              |
|                | 0                            | 0                     | 0                            | 0                        |              |
|                | 0                            | 0                     | 0                            | 0                        |              |
|                | 0                            | 0                     | 0                            | 0                        |              |
|                | 0                            | 0                     | 0                            | 0                        |              |
|                | 0                            | 0                     | 0                            | 0                        |              |
|                | 0                            | 0                     | 0                            | 0                        |              |
|                | 0                            | 0                     | 0                            | 0                        |              |

(B) ANXA6* HSP27* PRDX2* NCF2* TPM4* 

| Protein marker patterns of cervical cancer precursors | ANXA6* | HSP27* | PRDX2* | NCF2* | TPM4* |
|-----------------------------------------------------|--------|--------|--------|-------|-------|
| Diagnosis                                           | Cytoplasm | Cytoplasm | Nuclear | Stromal | Cytoplasm | Cytoplasm | Stromal | Cytoplasm |
| SCE                                                 | 0.5 ± 0.0 | 1.3 ± 0.0 | 3.2 ± 1.7 | 1.63 ± 1.7 | 0.0019 ± 0.0 | 0.00191 | 0.00119 | 0.00120 |
| CIN2                                                 | 2.25 ± 1.2 | 1.25 ± 1.5 | 2.00 ± 1.7 | 1.50 ± 1.9 | 1.63 ± 1.7 | 0.00119 | 0.00120 | 0.00120 |
| CIN3                                                 | 3.00 ± 1.0 | 2.00 ± 1.4 | 3.20 ± 1.7 | 1.70 ± 1.0 | 1.63 ± 1.7 | 0.00119 | 0.00120 | 0.00120 |
| MvSCC                                                | 3.00 ± 1.0 | 2.00 ± 1.4 | 3.20 ± 1.7 | 1.70 ± 1.0 | 1.63 ± 1.7 | 0.00119 | 0.00120 | 0.00120 |
| InvSCC                                               | 0.50 ± 0.0 | 1.3 ± 0.0 | 3.2 ± 1.7 | 1.63 ± 1.7 | 0.0019 ± 0.0 | 0.00191 | 0.00119 | 0.00120 |
| |
| Abbreviations: CIN = cervical intraepithelial neoplasia; InvSCC = invasion SCC; MvSCC = microinvasion SCC; SCC = squamous cervical cancer; SCE = squamous cervical epithelium. * Histoscore: score of the intensity of expression plus the score of the number of cells that express the protein. Bold values stand for statistically significant differences.
expression of TPM4 was found in six (25%) cases. Expression of PRDX2 and NCF2 was observed. Weak cytoplasmic and nuclear expression in half the cases (11–43.8%). Weak cytoplasmic expression of HSP27 was found in almost all cases and moderate protein was uncommon. Moderate/strong cytoplasmic expression was detected in 19 (79.2%) cases, although diffuse cytoplasmic expression of the protein was uncommon. Moderate/strong cytoplasmic and stromal expression of ANXA6 in 18 cases (72%) with a presence of sporadic ANXA6 cells. Expression of PRDX2 in the cytoplasm of dysplastic and cancer cells gradually decreased from SCE, which was similar to expression in SCE, which was higher in MicSCC than in InvSCC. Expression of PRDX2 in MicSCC was similar to expression in SCE, which was higher than in InvSCC. Expression of TPM4 was found in six (25%) cases.

**Histoscore of expression of ANXA6, HSP27, PRDX2, NCF2, and TPM4 in SCC carcinogenesis**

The histoscore (a sum of scores representing number of cells expressing the protein and intensity of staining) is another parameter used to evaluate proteins. Diffuse cytoplasmic expression of ANXA6 was lower in InvSCC than in MicSCC and CIN3. The number of sporadic ANXA6 + cells increased towards InvSCC (Figure 2; Table 2B). Expression of HSP27 in the cytoplasm of dysplastic cells was higher in CIN2/3 than in SCE. A diffuse cytoplasmic expression of NCF2 was similar in the dysplastic cells of CIN2/3, but higher in MicSCC than in InvSCC. Expression of PRDX2 in MicSCC was similar to expression in SCE, which was higher than in InvSCC. Tropomyosin 4 expression in the cytoplasm of dysplastic and cancer cells gradually decreased from SCE to InvSCC, with a significant difference between CIN3 and InvSCC.

**Sensitivity and specificity**

For distinguishing between normal SCE and CIN2/3, expression of HSP27 in the cytoplasm had the best sensitivity and specificity (Figure 3A). Expression of PRDX2 and TPM4 in the cytoplasm was associated with both the highest sensitivity and specificity for differentiation between SCE and InvSCC, and expression of PRDX2 and ANXA6 in the cytoplasm was the most sensitive and specific for differentiation between MicSCC and InvSCC (Figure 3B). Finally, expression of ANXA6 and TPM4 in the cytoplasm provided the best specificity and sensitivity for distinguishing between CIN2/3 and InvSCC.

**Coexpression of ANXA6, HSP27, PRDX2, NCF2, and TPM4 in CIN3 and SCC**

ANXA6 was coexpressed with PRDX2 in the cytoplasm of dysplastic cells in 3/4 of cases (P = 0.057; Table 3) of CIN3 and MicSCC. A correlation between genomic instability and positive nuclear expression of HSP27 in dysplastic cells was observed. Between genomically unstable CIN2/3 and MicSCC, 11 (91.7%) cases with positive nuclear expression of HSP27 (P = 0.061) were found.

**Prognostic value of cytoplasmic HSP27 expression in InvSCC cancer cells**

Relapse-freeRFS and OS were evaluated in 14 patients with InvSCC, who were followed for 60–72 months (5–6 years) after primary diagnosis (Figure 4C). Patients with weak HSP27 cytoplasmic expression (two cases, 14.3%) had the worst RFS (P = 0.019) and OS (P = 0.014), compared with other patients.
(Figure 4A and B). Patients with strong HSP27 cytoplasmic expression (four cases, 28.6%) had worse RFS ($P = 0.062$) and OS ($P = 0.048$) than patients with moderate expression of that protein (eight cases, 57.1%). Among the nine patients who were diagnosed at stages IB1-2, three patients showed strong HSP27 expression, whereas six patients showed moderate expression of the protein.

**Figure 3** ROC curves displaying sensitivity and specificity for detection of CIN2/3 by expression of HSP27 (A), differentiation of MicSCC from InvSCC (B), and of CIN2/3 from MicSCC and InvSCC by expression of ANXA6, PRDX2, TPM4, HSP27, and NCF2 in the cytoplasm (C). Cutoff for histoscores is presented in corresponding tables.
Within a 3–4 year period, relapse with lethal outcome was observed in two (66.7%) out of three cases with strong HSP27 expression and in one (16.7%) out of six cases with moderate protein expression ($P<0.001$).

**DISCUSSION**

Mutations that occur during progression from dysplasia to invasive SCC are reflected by changes in expression of tissue proteins. We previously identified the differential protein marker pattern that characterises SCC (Hellman et al., 2009; Lomnytska et al., 2010), and in current study, we analysed expression of proteins ANXA6, HSP27, PRDX2, NCF2, and TPM4 during SCC carcinogenesis.

We observed that ANXA6 expression gradually increased during the progression from CIN2 to MicSCC, whereas it decreased in InvSCC. Cleavage of the NH$_2$-domain severely impairs the function of ANXA6 (Gerke and Moss, 2002), while full-length ANXA6 is generally described to be under expressed in cancer and during carcinogenesis (Francia et al., 1996). Previously, we found that only the NH$_2$-terminal domain of ANXA6 was overexpressed in SCC (Lomnytska et al., 2010), which may indicate dysfunction of ANXA6 in SCC and explain the decrease in expression of full-length protein, as detected by IHC in cases of InvSCC. ANXA6 localises to the endoplasmic reticulum and plasma membrane (Barwise and Walker, 1996), and relocation of the protein from the cytosol to the plasma membrane is dependent on the elevation of Ca$^{2+}$ influx (Buzhynskyy et al., 2009; Sztolsztener et al., 2009). We did not observe ANXA6 expression on plasma membranes of atypical cells, where expression was mainly cytoplasmic. One regulator of ANXA6-dependent plasma membrane dynamics is EGF, through the EGF-dependent influx of Ca$^{2+}$ (Strzelecka-Kiliszek et al., 2008), and development of CC is also determined by EGF activity (Bellone et al., 2007). We observed that the number of strongly ANXA6$^+$ cells increased during the progression from CIN2 to InvSCC. We previously suggested that this observation represents nuclear expression of ANXA6 in cancer cells (Lomnytska et al., 2010), however, because of the variable histopathological appearance we could not exclude the possibility that these were infiltrating immune cells. Further analysis showed that these cells were negative for vimentin and CD68 and thus could not be identified as fibroblasts, endothelial cells, lymphoid cells, monocytes, or macrophages. Appearance of ANXA6$^+$ cells

### Table 3

| Protein 2, localisation | Positive | Negative | $P$-value |
|-------------------------|----------|----------|-----------|
| ANXA6, cyt              |          |          |           |
| CIN 3 – MicSCC          | 12/18 (66.7%) | 0/2 | 0.057     |
| (n = 20)                | 5/18 (27.8%) | 2/2 (100%) | 0.272     |
| TPM4, cyt (n = 29)      | 16/24 (66.7%) | 2/5 (40%) | 0.304     |
| NCF2, cyt (n = 26)      | 8/23 (34.7%) | 3/5 (60%) | 0.879     |
| InvSCC                  | 2/6 (33.3%) | 6/20 (30%) |           |
| (n = 26)                | 4/6 (66.7%) | 14/20 (70%) |           |
| NCF2, cyt               | 9/11 (81.8%) | 6/10 (60%) | 0.281     |
| CIN 3 – MicSCC          | 2/11 (18.2%) | 4/10 (40%) |           |
| (n = 21)                | 7/10 (70%) | 5/10 (50%) |           |
| HSP27, nuc (n = 20)     | 2/10 (20%) | 5/10 (50%) |           |
| InvSCC                  | 2/8 (25%) | 6/18 (33.3%) | 0.677     |
| (n = 26)                | 6/8 (75%) | 12/18 (66.7%) |           |
| HSP27, nuc (n = 26)     | 6/8 (75%) | 7/18 (38.9%) | 0.096     |
| InvSCC                  | 2/8 (25%) | 11/18 (61.1%) |           |
| HSP27, nuc              | 9/12 (75%) | 5/8 (62.5%) | 0.560     |
| CIN 3 – MicSCC          | 3/12 (25%) | 3/8 (37.5%) |           |
| (n = 21)                | 11/12 (91.7%) | 5/9 (55.6%) | 0.061     |
| Ploidy                  | 1/12 (8.3%) | 4/9 (43.4%) |           |
| TPM4, cyt (n = 24)      | 4/12 (33.3%) | 3/12 (25%) | 0.66      |
| Ploidy                  | 8/12 (66.7%) | 9/12 (75%) |           |
| NCF2, sporadic          | 2/12 (16.7%) | 0/13 | 0.133     |
| InvSCC                  | 14/15 (93.3%) | 16/18 (88.9%) | 0.663     |
| ANXA6, sporadic (n = 33) | 1/15 (6.7%) | 2/18 (11.1%) |           |

**Abbreviations:** CIN = cervical intraepithelial neoplasia; InvSCC = invasion SCC; MicSCC = microinvasion SCC; PRDX = peroxiredoxin; SCC = squamous cervical cancer; TPM = tropomyosin. *DAU* = diploid and aneuploid unstable; *DAS-diploid and aneuploid stable.

With a 3–4-year period, relapse with lethal outcome was observed in two (66.7%) out of three cases with strong HSP27 expression and in one (16.7%) out of six cases with moderate protein expression ($P<0.001$).
only between dysplastic and cancer cells was an interesting observation, which may improve diagnosis.

HSP27 is a member of the HSP family that maintains protein structure, restores denaturated, aggregated, and damaged proteins, and are therefore activates when subjected to different types of stress, such as oxidative stress, inflammation, and malignant transformation (Ciocca and Calderwood, 2005). It was recently reported that HSP27 expression in cervical precursor lesions is higher than in SCE, and that it is higher in invasive SCC than in precursor lesions (Ono et al., 2009). We have also observed increase of the cytoplasmic expression of HSP27 in cervical precursor lesions. However, cytoplasmic expression of HSP27 in microinvasive and invasive SCC was lower than in precursor lesions. Previously noted low sporadic expression or no expression of HSP27 in InvSSC was not explained (Ono et al., 2009). We observed that RFS and OS for patients with weak or strong HSP27 expression in cancer cells were lower than for patients with moderate and essentially normal (as in SCE) protein expression. In uterine cervix, HSP27 has a role in the maturation of squamous cells and is associated with more differentiated tumours, whereas its decreased expression associates dedifferentiation and transformation to adenocarcinoma. At the same time, highly expressed HSPs, including HSP27, may facilitate cancer progression by repairing cells that were damaged by chemo- and radiotherapy and by protecting them from apoptosis (Gauss et al., 2001). When overexpressed, PRDX2 protects cancer cells from oxidative stress and thus mediates resistance to chemo- and radiotherapy (Soini et al., 2006; Smith-Pearson et al., 2008). NCF2 is an NADPH oxidase cytosolic component and the gene that encodes this protein is upregulated by TNF-α (Gauss et al., 2005; Ammons et al., 2007). TNF-α and the encoding gene impact development of CC by increasing susceptibility to infection with HR-HPV (Deshpande et al., 2005). In our study, NCF2 was overexpressed in MicSSC. In cervical precursor lesions, we observed a tendency for coexpression of ANXA6 and NCF2.

Tropomyosins (TPMs) are actin-interacting protein components of the cytoskeleton that have been implicated in neoplastic-specific alterations of actin-based organisation. Rearrangement of microfilament bundles, morphological alterations, and increased cell motility are major features of a transformed phenotype and are usually associated with decreased expression of nonmuscle isofoms of TPMs (Helfman et al., 2008). Decreased expression of TPMs can be caused by hypermethylation of the encoding gene (Bharadwaj and Prasad, 2002) or dysfunction of Rho-kinase (Bharadwaj et al., 2005). In our study, expression of the TPM4 protein was significantly lower in MicSSC and InvSSC than in precancerous lesions and SCE. In precursor lesions we observed coexpression of TPM4 and NCF2, usually characterised by overexpression of NCF2 in atypical cells and of TPM4 in differentiated cells.

Our study is the first to assess a marker proteins pattern previously identified by proteomics-based analysis of SCC for the changes in expression during the sequential steps of SCC carcinogenesis. In particular, cytoplasmic expression of ANXA6 gradually increased in CIN and MicSSC and decreased in InvSSC. Sporadic ANXA6 + cells were observed between dysplastic cells. Cytoplasmic expression of HSP27 increased towards CIN and was more intense in dysplastic lesions than in MicSSC and InvSSC. Cervical precursor lesions and invasive cancer were characterised by nuclear expression of HSP27. MicSSC was characterised by overexpression of PRDX2 and NCF2, whereas expression of TPM4 was observed mostly in SCE and partially in precursor lesions. Our

**Figure 4** Relapse-free (A) and overall (B) survival of patients with SCC (C) with various expression of HSP27 in cytoplasm of cancer cells (univariate method by Kaplan–Meier, log-rank test).
findings describing the differences in expression of marker proteins during SCC carcinogenesis may be useful for developing more objective methods for early diagnosis of precursor SCC lesions and for monitoring patients.

ACKNOWLEDGEMENTS

This study was supported by grant from Swedish Cancer Foundation (070623, CAN 2007/1044), Ki Cancer Strategic Grants (5888/05-722), Swedish Research Council (521-2008-2899), Medical Research Council, and Cancer Society in Stockholm, Stockholm County Council, Swedish Labor Market Insurance and EU grant FLUODIAMON. We thank Carmen Flores-Stiano for technical assistance and Susan Larsson (Connection LLC, Swedish to English Translations) for language assistance.

Conflict of interest

The authors declare no conflict of interest.

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British Journal of Cancer (2011) 104(1), 110 – 119
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