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Article *in* Cellular Oncology · October 2015

DOI: 10.1007/s13402-015-0252-6

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ORIGINAL PAPER

Lamin A/C deficiency is an independent risk factor for cervical cancer

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Accepted: 22 October 2015

Abstract
Background In the past, cervical cancer has been linked to Human Papilloma Virus (HPV) infection. Previously, we found that pre-neoplastic breast and ovarian lesions may be associated with lamin A/C deficiency, resulting in abnormal nuclear morphologies and chromosomal instability. Ultimately, these phenomena are thought to lead to cancer. Here, we assessed lamin A/C deficiency as an indicator for the risk to develop cervical cancer.
Methods The expression of lamin A/C was assessed by Western blotting in cervical uterine smears (CUS) of 76 adult women from Benin concomitant with nuclear morphology assessment and HPV genotyping using microscopy and PCR-based assays, respectively. *In vitro* analyses were performed to uncover the mechanism underlying lamin A/C expression alterations observed *in vivo*. The presence of cervical intra-epithelial neoplasia (CIN) was assessed by colposcopy.
Results Normal lamin A/C expression (group A) was observed in 39 % of the CUS, weak lamin A/C expression (group B) was observed in 28 % of the CUS and no lamin A/C expression (group C) was observed in 33 % of the CUS tested. Infection with oncogenic HPV was found to be significantly higher in group C (36 %) than in groups A (17 %) and B (14 %). Two years after our first assessment, CIN was observed in 20 % of the women in group C. The in vitro application of either a histone deacetylase inhibitor (trichostatin) or a protein kinase inhibitor (staurosporine) was found to restore lamin A/C expression in cervical cancer-derived cells.
Conclusion Lamin A/C deficiency may serve as an independent risk factor for CIN development and as an indicator for preventive therapy in cervical cancer.

Keywords Lamin A/C deficiency · Oncogenic HPV · Cervical neoplasia · Cervical cancer prevention

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Published online: 04 November 2015

1 Introduction

Cervical cancer is one of the main causes of mortality among women worldwide and the risk to develop cervical neoplasia is often associated with HPV infection. A late detection of cervical cancer may be lethal [1, 2]. Previously, we found that the nuclear envelope proteins lamin A/C, which are involved in the differentiation of epithelial cells and the function and morphology of their nuclei, may be absent in premalignant breast and ovarian lesions [3, 4]. Changes in cellular and nuclear morphologies, as well as aneuploidies, are often employed by pathologists to identify precancerous lesions [3–6]. Changes in nuclear morphology usually result from irregularities of the nuclear lamina, which is the scaffold of the nuclear envelope (NE) and is composed of lamins, i.e., A-type and B-type lamins [5, 6]. The A-type lamins (lamin A and C) are transcribed from a single gene, *LMNA*, and are primarily expressed in endoderm cells during embryogenesis and differentiated epithelial cells after birth [5, 6]. Lamins B1 and B2 are the major B-type lamins and are expressed in various mammalian cells [7, 8]. In association with other proteins of the nuclear envelope, the lamin A/C proteins form a scaffold that maintains the nuclear structure, regulates DNA synthesis, repairs DNA damage, organizes chromatin, regulates gene transcription and, in addition to maintenance of the differentiated state of the cell, assures proper cell cycle progression and migration [7–13]. Several studies have shown that loss of lamin A/C expression represents one of the molecular alterations that may generate some of the the main hallmarks of neoplasia and carcinoma [3, 4, 14–18]. Cells lacking lamin A/C expression have been found to display abnormal nuclear morphologies, defective cell cycle kinetics, polyploidies and structural chromosomal aberrations [19–21]. In the past, lamin A/C deficiency has been used as a biomarker for skin cancers, colon cancers, ovarian cancers, breast cancers and neuroblastoma but, as yet, not for cervical cancers [3, 4, 20–22]. Interestingly, lamin A/C deficiency has been noted when oncogenic HPV proteins are expressed during the development of cervical neoplasia and cancer [23–25]. Here, we used the absence of lamin A/C expression in cervical epithelial cells as a biomarker to pre-symptomatically screen women with a risk to develop cervical neoplasia, along with their oncogenic HPV status.

2 Materials and methods

2.1 Study population

We included 76 sexually active women of 20 to 60 years old, who attended the hospital of Mènontin in Cotonou (Benin) for regular gynecological examination. A signed informed consent was obtained from all women before cervical uterine smears (CUS) were collected. The sample collection was carried out under supervision of a gynecologist. Women with vaginal bleeding were excluded from the collection. This study was approved by the Research Ethic Committee of the Institute of Biomedical Sciences and Applications (CER-ISBA), and by the Ministry of Health in Benin. The procedures followed for this sample collection and the research protocol were in accordance with the ethical standards of our institutional and national committees on human subjects and the Helsinki Declaration.

2.2 Cervical cell collection and processing

A disposable sterile speculum was introduced in the vagina and a sterile disposable cytobrush was introduced in the speculum to reach the cervical-uterine junction where cells were collected by rotating the cytobrush clockwise twice. Next, the cytobrush was placed in a 50 ml collection tube containing 5 ml sterile ice-cold phosphate buffered saline (PBS), which was kept on ice and delivered within an hour to the Laboratory of Biochemistry and Molecular Biology (LBBM), Cotonou, Benin, for processing. All collection tubes were centrifuged to harvest the cells which were washed once with ice-cold PBS before division into samples for (i) protein extraction for Western blotting, (ii) DNA extraction for oncogenic HPV genotyping and (iii) cell smears to assess nuclear morphologies.

2.3 Reagents

Tris-base, glycine, sodium dodecyl sulfate, bis-acrylamide and nitrocellulose membranes were purchased from Bio-Rad Inc. (USA). NaCl, KCl, Tween-20, protease inhibitor PMSF, 2-mercaptoethanol, methanol, glycerol, sodium azide, hematoxylin, all-trans retinoic acid, acetic acid, Lugol's iodine, trichostatin and staurosporine were purchased from Sigma-Aldrich (USA). The primary antibodies used were rabbit anti-lamin A/C (Transduction Lab, KY, USA) and mouse anti-ß-tubulin (Santa Cruz Biotechnology, CA, USA). The HRP-conjugated secondary antibodies, i.e., anti-rabbit and anti-mouse, were purchased from Bio-Rad Inc. (USA). For protein detection on Western blots, the chemiluminescence reagent "Super Signal West Dura Extended Duration Substrate" produced by PIERCE was purchased from Thermo Scientific (Rockford, IL, USA).

2.4 Protein extraction and Western blotting

Cells were placed in lysis buffer A [50 mM Tris-HCl (pH 7.9), 150 mM NaCl, 0.1 mM EDTA, 1 % NP-40, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 30 mM NaF and 0.5 % protease inhibitor cocktail] and kept on ice for 30 min with vigorous agitation every 5 min. The resulting

supernatants were collected by centrifugation (12,000 g for 5 min at 4 °C) and an aliquot of each supernatant was used to measure the protein concentration with a Bio-Rad Dc protein assay kit before boiling in SDS sample buffer for 5 min. Next, the samples were loaded on 7.5 % SDS-polyacrylamide gels and run at 100 V for 2 h in tris-glycine buffer after which the proteins were transferred from the gels to nitrocellulose membranes using transfer buffer containing tris-glycine and 20 % methanol. The membranes were blocked with 5 % milk in 1× TBS pH 7.6 containing 50 mM Tris-HCl, 150 mM NaCl and 0.1 % Tween-20 (TBST) for 30 min at room temperature before incubation in primary rabbit-anti-lamin A/C antibodies in 1 % milk/TBST for 1 h at room temperature. After this incubation, the blots were washed 4 times for 10 min with TBST and incubated with HRP-conjugated secondary anti-rabbit antibody in 1 % milk/TBST. Finally, the blots were washed 4 times for 15 min with TBST, incubated for 3 min in Super Signal West Dura Extended Duration Substrate and exposed to X-ray film. Essentially the same protocol was used for the detection of ß-tubulin as a loading control.

2.5 Cell morphology assessment by microscopy

Cells were washed twice with ice-cold PBS and fixed in 85 % methanol/PBS overnight at −20 °C. The fixed cells were spread onto glass slides and dried for 2 h in an incubator with a temperature preset at 37 °C. The dried slides were stained with hematoxylin for 40 s and rinsed three times for 10 min with distilled water after which they were air-dried overnight and covered with mounting medium and coverslips. Stained cell nuclei were visualized under a Nikon Eclipse TE 300 microscope with a 20× objective lens and images were taken and processed using Adobe Photoshop software.

2.6 Immunohistochemistry on tissue microarrays

Archived cervical cancer tissues (n = 10) were derived from the Fox Chase Cancer Center Tissue Bank (PA, USA). The method used for immunohistochemistry (IHC) was as previously described [6–9]. Lamin A/C staining was performed using rabbit anti-lamin A/C antibodies according to the protocol previously described [6–9]. The presence of lamin A/C was assessed using a 20× objective lens on a Nikon Eclipse TE 300 microscope equipped with a Roper Scientific photometrics 12-bit range Camera. Image acquisition was carried out using Meta imaging series (MetaVue) software.

2.7 DNA extraction

DNA was extracted from cells using a standard phenol-chloroform method. Briefly, cells were treated with lysis buffer containing proteinase K (20 mg/ml) and RNase. Next, phenol was added to the cell lysate (v/v), mixed and centrifuged at 10,000 rpm at 4 °C for 10 min. The upper aqueous phase was transferred to a new Eppendorf tube and chloroform was added (v/v), mixed and centrifuged at 10,000 rpm at 4 °C for 10 min. The supernatant was collected in a new Eppendorf tube and the DNA was precipitated with ice-cold ethanol (96 %) at −20 °C for 4 h and recovered after centrifugation at 12,000 rpm at 4 °C for 10 min. The resulting DNA pellet was washed with ice-cold ethanol (70 %) and recovered after centrifugation at 12,000 rpm at 4 °C for 5 min. Finally, the DNA pellet was air-dried at 55 °C for 20 min and dissolved in Tris-EDTA (TE) buffer. The DNA concentration was measured using a spectrophotometer (260 nm). The DNA was kept at −20 °C until HPV genotyping.

2.8 HPV genotyping

First, the quality of the isolated DNA was assessed by PCR amplification of ß-actin before PCR amplification of HPV using multiplex primers (Sigma-Aldrich) for genotyping. A nested multiplex PCR (NMPCR) that combines degenerate E6/E7 consensus primers with type-specific primers was carried out as reported previously [25]. In each PCR reaction positive and negative controls were included. First a consensus PCR was performed (GPEG from Sigma-Aldrich) to confirm the presence of HPV DNA. After this, the amplified product was used again for a second amplification with the set of multiplex primers (nested PCR) to determine the HPV genotype.

The multiplex primers used allow the detection of both high grade and low grade oncogenic HPV. The genotypes were divided into 4 groups according to the set of primers used. Set 1 with 5 primer pairs detects HPV-16, −18, −31, −59 and −45 and yields PCR products of 457, 322, 263, 215 and151 bp, respectively. Set 2 with 5 primer pairs detects HPV-33, −6/11, −58, −52 and −56 and yields PCR products of 398, 333, 274, 229 and 181 bp, respectively. Set 3 with 4 primer pairs detects HPV-35, −42, −43 and −44 and yields PCR products of 358, 277, 219 and 163 bp, respectively. Set 4 with 4 primer pairs detects HPV-68, −39, −51 and −66 and yields PCR products of 333, 280, 223 and 172 bp, respectively. All PCR products were run through 1.5 % agarose gels, after which amplified bands were stained with ethidium bromide and detected under UV light. The HPV genotype was determined according to the size of the band, as specified above.

2.9 Colposcopy

Two years after CUS collection, a subset of the women was invited to the clinic for video colposcopic examination. A SONY Video Colposcopy Digital Imaging System driven by Multiline preview pic software was used for viewing and documenting. Colpophotographs of the uterine cervix were taken before and after staining with diluted acetic acid followed by Lugol's iodine solution to reveal cervical lesions. The

lesions appeared white when stained with acetic acid (visual inspection with acetic acid, VIAA+) and yellow when stained with Lugol's iodine solution (visual inspection with Lugol's solution, VILI+). Colpophotographs were captured using a 26× zoom magnification.

2.10 Lamin A/C expression induction

For this purpose an established endometrial cancer cell line (ECC-1), which has lost lamin A/C expression, was used (a gift from Dr. Xu's laboratory at the University of Miami, FL, USA). The cells were cultured in DMEM containing 10 % FBS and penincillin-streptomycin at 37 °C in the presence of 5 % CO_2. The cells were seeded in 6-well dishes and cultured till they reached 80 % confluence. Then the medium was replaced by fresh medium containing all-trans retinoic acid (ATRA, 1 μM), a histone deacelylase inhibitor (trichostatin, TSA, 0.5 μM) or a Ser/Thr protein kinase inhibitor (staurosporine, 0.3 μM). All solutions were prepared from stocks previously dissolved in Dimethyl Sulfoxide (DMSO). 10 μl of DMSO was used as a vehicle control. The cells were incubated with the respective drugs for 4 h and processed for Western blot analysis as described above.

2.11 Statistical analyses

The F-test function of Excel was used to assess whether the CUS tested exhibit different levels of HPV infection. CUS with normal lamin A/C expression were classified in group A, CUS with low lamin A/C expression were classified in group B and CUS without lamin A/C expression were classified in group C. The HPV status was compared between the groups. CUS with HPV were classified as 0 if they were not infected with oncogenic HPV, as 1 if they were infected with one type of oncogenic HPV, as 2 if they were co-infected with two types of oncogenic HPV and as 3 if they were co-infected with three types of oncogenic HPV. Differences between groups were considered significant if $p < 0.05$.

3 Results

3.1 Lamin A/C expression and HPV infection in cervical uterine smear samples

Cervical uterine smear (CUS) samples were assessed for lamin A/C expression by Western blotting**.** The cells collected were mostly surface epithelial cells that normally express lamin A/C in their differentiated state and may lose lamin A/C expression during malignant transformation [3, 4]. An example of a Western blot showing lamin A/C expression in parallel with HPV genotyping is shown in Fig. 1. Samples with a normal lamin A/C expression are marked as belonging to group A, samples with a low lamin A/C expression as belonging to group B and samples with no lamin A/C expression as belonging to group C.

A normal lamin A/C expression was observed in 6 samples (A101, A117, A125, A128, A118 and A119), a low expression in sample B132 and absence of expression in samples C152 and C155. The latter was associated with HPV-66/18 infection (Fig. 1). The examples shown in Fig. 2 indicate that cells with no lamin A/C expression (i.e., C155) exhibit atypical nuclear morphologies and multiple nuclei compared to cells with a normal lamin A/C expression (i.e., A117). Similar analyses were carried out on all 76 samples included in this study. A normal lamin A/C expression was observed in 30/76 (39 %) of the CUS, a weak expression in 21/76 (28 %) of the CUS and no expression in 25/76 (33 %) of the CUS.

3.2 Lamin A/C negative cervix uterine smear samples exhibit abnormal nuclear morphologies

The nuclear morphology of the CUS cells was assessed by microscopy after hematoxylin staining. The photographs in Fig. 2 show cells from CUS with a normal lamin A/C expression (A117) and from CUS with no lamin A/C expression (C155). In the A117 cells the nuclear morphology was found to be normal (Fig. 2a). In the C155 cells, however, the nuclear morphology was found to be abnormal (Fig. 2b). Most of the cells lacking lamin A/C expression exhibited an enlarged and irregular nuclear shape, which suggests that they were already transformed (Fig. 2b). Therefore, women with CUS lacking lamin A/C expression could be at risk of developing cervical cancer.

3.3 Loss of lamin A/C expression in cervical epithelia with neoplastic lesions

Immunhistoochemical (IHC) staining for lamin A/C was carried out on 10 independent archived tissues of cervical squamous epithelium. The results obtained indicate that cells in normal cervical squamous epithelium express lamin A/C while transformed cells in neoplastic cervical lesions appear to have lost lamin A/C expression (Fig. 3, red arrows). We found that all normal cervical surface epithelial cells were well organized within the cervical epithelium (Fig. 3a), whereas all cervical neoplastic cells were found to be disorganized and intraepithelial, with atypical nuclei (Fig. 3b). Cervical intraepithelial neoplasia (CIN) lesions, known as precancerous lesions, are usually asymptomatic. Our data indicate that lamin A/C may be deficient in CIN.

3.4 Lamin A/C deficiency and oncogenic HPV status in CUS samples of seemingly healthy women

All 76 seemingly healthy women attending the Mènontin Hospital (Benin) were tested negative for HIV-1 and −2. In

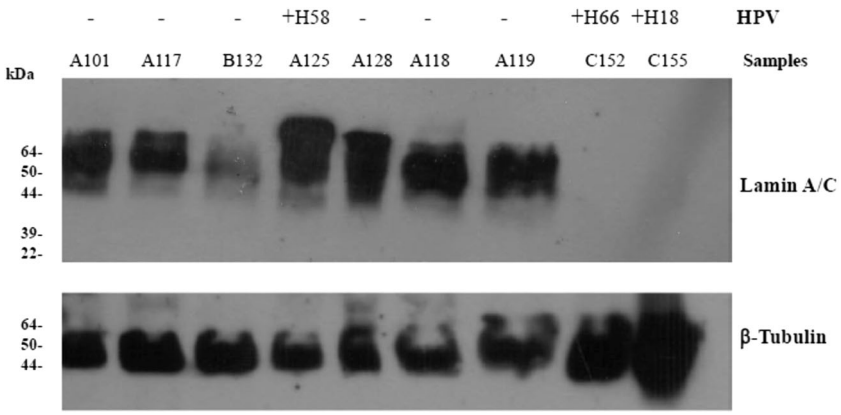

Fig. 1 Western blot showing lamin A/C expression in conjunction with HPV status. Sample A125 is positive for HPV-58 and lamin A/C, whereas samples C152 and C155 are positive for HPV-66/18 and negative for lamin A/C. ß-Tubulin was used as an internal loading control

order to investigate the implications of HPV infection on the risk of developing cervical neoplasia, we set out to test all CUS samples for the presence of oncogenic HPV. The results are listed in Table 1 along with the lamin A/C expression results. We found that oncogenic HPV was present in CUS samples positive for lamin A/C expression, as well as in CUS samples exhibiting low or no lamin A/C expression. In group A we observed 5/30 (17 %) CUS cases positive for oncogenic HPV (type 16, 45/56, 66, 58, 66), in group B we observed 3/21 (14 %) CUS cases positive for oncogenic HPV (type 51, 33, 33) and in group C we observed 9/25 (36 %) CUS cases positive for oncogenic HPV (type 66, 18, 51, 33, 58, 56, 43/44, 42/59/68, 56).

3.5 Combined lamin A/C deficiency and oncogenic HPV infection increases the risk to develop cervical neoplasia

No strong association was observed between lamin A/C deficiency and the presence of oncogenic HPV (Table 1). The incidence of oncogenic HPV was, however, significantly higher in group C with lamin A/C deficiency (36 %) compared to group A with a normal lamin A/C expression (16 %; $p = 0.018$) and group B with a low lamin A/C expression (14 %; $p = 0.001$). This difference was not significant between group A and B ($p = 0.12$). Women with lamin A/C deficiency and oncogenic HPV infection have a cumulative risk to develop cervical neoplasia, as shown in Fig. 4 (red arrow).

3.6 Colposcopic follow-up of women with deficient lamin A/C expression reveals CIN lesions

Two years after the detection of lamin A/C deficiency, we invited all 25 women at a putative risk to develop cervical neoplasia. Fifteen women agreed to undergo a second screen with colposcopy. Visualization of the lower vaginal tract including the uterine cervix was carried out by a gynecologist to determine whether the uterine-cervix junction was positive or negative for possible precancerous or cancerous lesions. To this end, a SONY Video Colposcopy Digital Imaging System driven by Multiline preview pic software associated with illumination and magnification was used. The visualization was performed before and after the application of diluted acetic acid (visual inspection with acetic acid, VIAA+) followed

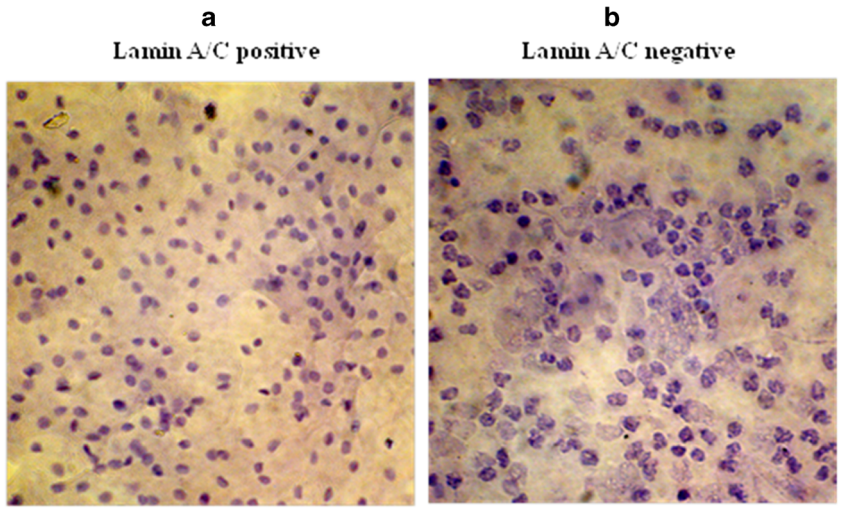

Fig. 2 Nuclear morphologies of CUS cells after hematoxylin staining. (**a**) CUS with normal lamin A/C expression [A117] contain cells with normal nuclei. (**b**) CUS with no lamin A/C expression [C155] contain cells with abnormal and enlarged nuclei. Images were taken with a 20× objective

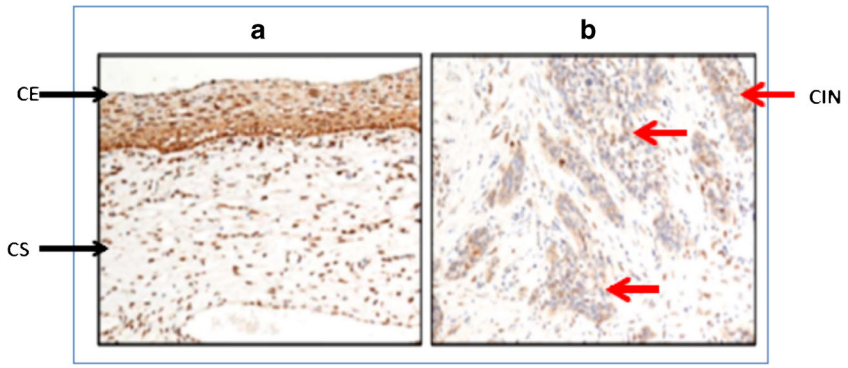

Fig. 3 (**a**) Immunohistochemistry showing that lamin A is mostly expressed in normal cervical squamous epithelium (CE) and sporadically in cervical stroma (CS) cells, whereas its expression is lost in CIN lesions (**b**) associated with a disorganization of neoplastic cells with abnormal nuclear morphologies that invade the cervical stroma (marked by red arrows)

by Lugol's iodine solution (visual inspection with Lugol's solution, VILI+), also known as Schiller's test. Three out of the fifteen women (20 %) was found to have developed CIN lesions (displayed in Fig. 5). Normal squamous epithelium stains brown by iodine due to the presence of glycogen, whereas precancerous lesions and invasive cancer do not stain with iodine and appear yellow due to low levels or complete absence of glycogen.

Cervical lesions appeared white when stained with acetic acid (VIAA+) as shown in Fig. 5c, e and g, and yellow when stained with Lugol's iodine solution (VILI+) as shown in Fig. 5d, f and h (indicated by black arrows). One woman with HPV-56 did not develop CIN but displayed leucorrhoea (Fig. 5a and b), while another woman who tested positive for triple oncogenic HPV-42/59/68 developed CIN-2 (Fig. 5c, d). Two of the women who tested negative for oncogenic HPV developed CIN-1 (Fig. 5e and f) and CIN-3 (Fig. 5g and h), respectively.

Taken together, we found that 3/15 (20 %) women with lamin A/C deficiency developed CIN-1, CIN-2 or CIN-3. Overall, none of the women showed any clinical sign indicating that their uterine cervix was developing a malignant lesion. Our putative prognosis two years ahead based on lamin A/C deficiency was instrumental to distinguish these women and to advise them to undergo cryotherapy for the CIN-1 and CIN-2 lesions, or hysterectomy for the CIN-3 lesion. As such, lamin A/C deficiency may serve as a bio-indicator for the early detection and prevention of cervical cancer and as an independent risk factor next to viral infection (HPV and HIV).

Table 1 Incidence of women infected with oncogenic HPV according to lamin A/C expression status

	%HPV-	%HPV+
L A/C positive (n = 30)	83	17
L A/C weak (n = 21)	86	14
L A/C negative (n = 25)	64	36

The incidence of oncogenic HPV in group C (lamin A/C negative; 36 %) is higher than in groups A (lamin A/C positive, 17 %) and B (lamin A/C weak, 14 %)

3.7 *In vitro* mechanisms underlying loss of lamin A/C expression

Besides mutations in the *LMNA* gene that can lead to laminopathies, lamin A/C deficiency associated with cellular transformation and neoplasia has been linked to epigenetic events such as histone deacetylation of the *LMNA* gene promotor, lamin A/C degradation after Ser/Thr phosphorylation, excessive cleavage of lamin A by activated caspase-6 and inhibition of lamin A synthesis by anti-retroviral drugs or vitamin deficiency [24–30]. The underlying causes of lamin A/C deficiency are depicted in Fig. 6 and are non-exhaustive. Due to

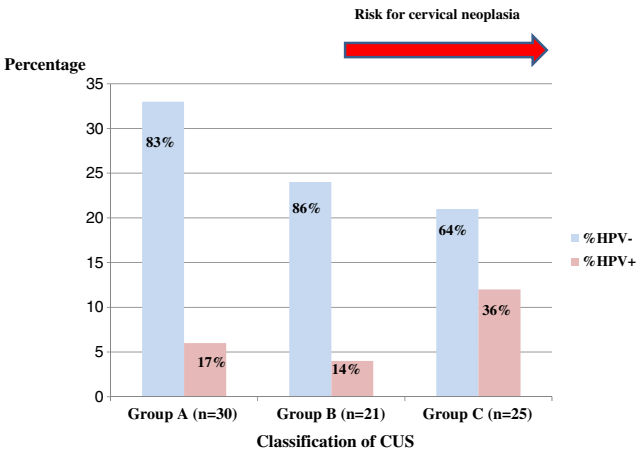

Fig. 4 Histogram representing the incidence of oncogenic HPV infection in association with the level of lamin A/C expression. Group A specifies CUS with a normal lamin A/C expression; group B specifies CUS with a weak lamin A/C expression; Group C specifies CUS deficient of lamin A/C expression. The incidence of oncogenic HPV infection is significantly higher in group C (36 %) than in group A (17 %) and group B (14 %). No significant difference is observed between group A and group B. The women in group C have higher risk to develop cervical neoplasia in case they are infected with oncogenic HPV (red arrow)

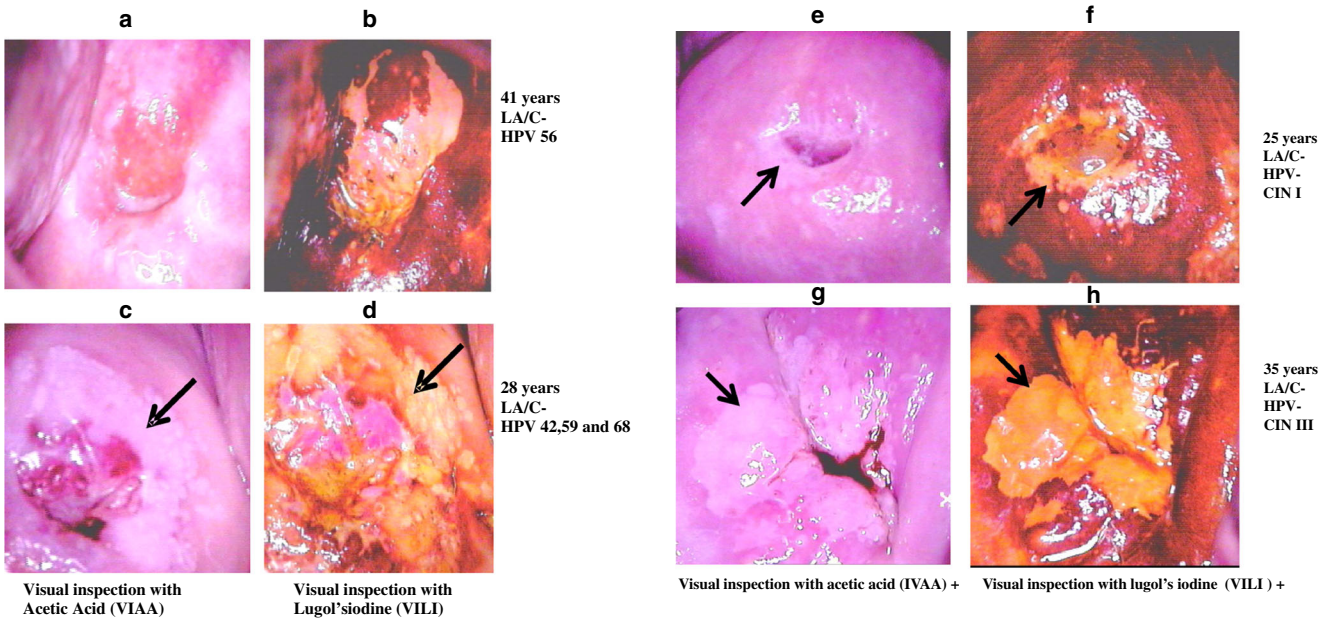

Fig. 5 Colposcopy images of the uterine cervix of four women suspected two years earlier for a risk to develop cervical neoplasia based on lamin A/C deficiency. Cervical intraepithelial lesions (CIN) appear white when stained with acetic acid (VIAA+) in panels a, c, e and g and yellow when stained with Lugol's iodine solution (VILI+) in panels b, d, f and h (marked by black arrows). Two women tested positive for oncogenic HPV infections (a-b and c-d), one of whom developed CIN-2 (c-d) and the other one leucorrhea (a-b). A 25 year-old female developed a CIN-1lesion (e-f) and a 35 year-old (asymptomatic) female developed a CIN-3 lesion (g-h)

these various causes, more extensive investigations should be carried out per case to determine the specific cause of the deficiency before a recommendation can be made for personalized care to prevent cervical neoplasia and/or cancer. Certain drugs such as histone deacetylase inhibitors (HDACI), (viral) Ser/Thr protein kinase inhibitors or caspase-6 inhibitors may restore the expression of laminA/C or induce apopotosis in neoplastic cells, thereby reducing the risk of progression to cancer.

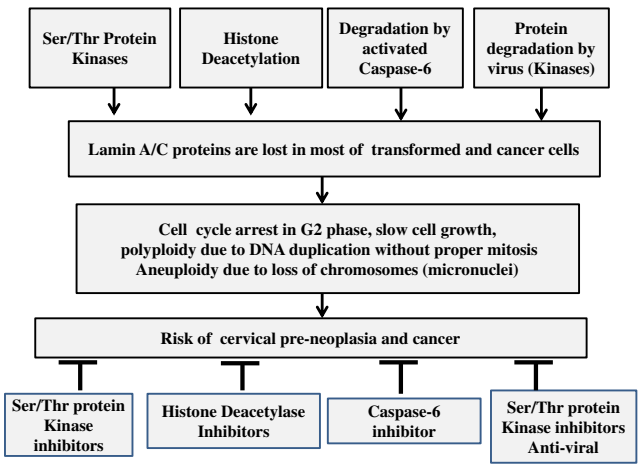

Fig. 6 Summary of various factors underlying lamin A/C deficiency in cervical epithelial cells and putative personalized treatment options before the onset of neoplasia. The use of drugs like Ser/Thr protein kinase inhibitors, histone deacetylase inhibitors or caspase-6 inhibitors may restore the expression of lamin A/C or induce apopotosis of neoplastic cells and, thereby, reduce the risk of progression to cancer

3.8 Lamin A/C expression restoration in human uterine cancer cells using drugs

The human endometrial cancer-derived cell line ECC-1 was treated with DMSO (control), the differentiation inducer all-trans retinoic acid (ATRA), the HDACI trichostatin (TSA) and the Ser/Thr kinase inhibitor staurosporine. Restoration of lamin A/C expression was achieved after treatment of the ECC-1 cells for 4 h with TSA or staurosporine. A Western blot example is shown in Fig. 7. These results indicate that histone deacetylation of the *LMNA* gene promoter and lamin A/C phosphorylation may explain the occurrence of lamin A/C deficiency in neoplastic or cancer cells. Treatment with ATRA was not found to restore lamin A/C expression within 4 h. Usually ATRA treatment is effective after 4 days in cancer cells still expressing RA receptors. We found that the ECC-1 cells started to go into apoptosis after 4 h and that the majority of the cells (98 %) died after a 24 h incubation period in medium containing TSA or staurosporine (data not shown). Further work is required to fully elucidate the mechanisms underlying the restoration of lamin A/C expression.

4 Discussion

Nuclear envelope structural lamin A/C proteins are expressed in differentiated epithelial cells, but are absent in undifferentiated cells and transformed or neoplastic cells prior to carcinogenesis [3, 4, 26]. Cells that have lost lamin A/C protein

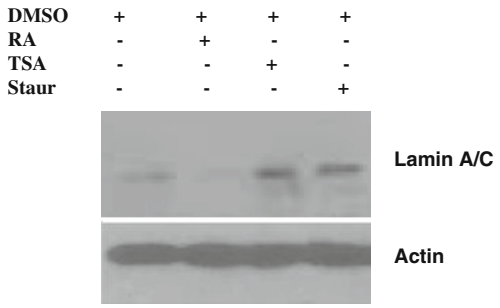

Fig. 7 Western blot showing the expression of lamin A/C after a 4 h treatment of human uterine endometrial cancer-derived cell line (ECC-1) with the histone deacetylase inhibitor Trichostatin (TSA) or the Ser/Thr protein kinase inhibitor staurospaurine ("staur"). ATRA did not restore lamin A/C expression within 4 h while TSA and staurosporine restored lamin A/Cexpression within 4 h

expression usually exhibit hallmarks of cancer cells, including an abnormal nuclear shape and chromosomal instability [3, 4]. Several factors are thought to be involved in lamin A/C deficiency, including mutations in the *LMNA* gene, epigenetic *LMNA* promoter modifications, lamin A/C protein degradations, Ser/Thr protein kinase inhibitions, deficiency in vitamins and viral infections, all of which may contribute to carcinogenesis [24–30]. Indeed, treatment with protease inhibitors has been found to impair the activity of a protease (Ste24p), which is involved in the maturation of pre-lamin A into functional lamin A [27–29], whereas viral infections (HPV and/or HIV) have been found to induce lamin A/C degradation [23, 24, 27]. These factors may contribute to the molecular alterations that occur prior to cervical neoplasia and carcinogenesis [25]. Other factors that may incite cervical neoplasia include improper nutrition or unbalanced diet and smoking [33–37].

In pathology/cytology, cancer cells are usually diagnosed according to anomalies in nuclear structure and size [31]. In our previous studies we were able to reproduce the hallmarks of cancer cells (abnormal nuclear morphology and polyploidy) by suppressing lamin A/C expression in normal human surface ovarian epithelial (HOSE) cells [3, 4]. Here, lamin A/C deficiency was found to be associated with atypical nuclear morphologies in several primary cervical uterine smear (CUS) samples that progressed to cervical neoplasia within two years.

The observed lack of lamin A/C expression in 33 % of the samples tested is striking and may serve as a bio-indicator to identify women who have an increased risk to develop cervical cancer. Some studies have reported that repeated exposure to malaria infection, HIV infection or HIV/HPV co-infection may induce cervical cancer [2, 30, 32]. It has also been reported that activated HPV has a strong potential to degrade lamin A/C [30]. We found that in five CUS samples with HPV infection lamin A/C protein was present, suggesting that at the time of analysis HPV was not yet actively degrading lamin A/C. The CUS cases in group C with lamin A/C deficiency and HPV infection (36 %) appeared to have an increased risk to develop cervical cancer. Altogether, lamin A/C deficiency appears to be a risk factor and a biomarker for the development of CIN prior to the visual appearance of cervical lesions and, thus, may be employed to prevent cervical cancer.

Although lamin A/C deficiency has already been reported in cancers originating from the ovary, breast, skin, neuronal and gastrointestinal tract [1, 2, 21–23, 41], lamin A/C deficiency prior to the initiation of cervical neoplasia has so far not been reported. Lamin A/C deficiency may occur concomitantly with GATA6 suppression [18, 43]. Therefore, low or absent lamin A/C expression could be used in association with other biomarkers such as GATA6 to identify women at risk of developing cervical cancer [44]. Precancerous lesions may have a latency period of 2 to 10 years before they progress into invasive cervical cancer. These precancerous lesions, also known as cervical intraepithelial neoplasia (CIN), may vary from grade 1 to grade 3 (CIN1-CIN3) and can be predicted, as shown here, with biomarkers (i.e., lamin A/C deficiency) independent of HPV infection. It has been reported that lamin A/C deficiency may be due to epigenetic modification, which may be restored by treatment with a caspase-6 inhibitor [17] or green tea [38, 39]. Lengthy treatment (4 days) of undifferentiated cells with ATRA may also induce lamin A/C expression in some carcinoma cells [40]. We found that restoration of lamin A/C expression can be achieved with a HDAC inhibitor (trichostatin, TSA) or a Ser/Thr protein kinase inhibitor (staurosporine). This restoration suggests that lamin A/C deficiency during the initiation phase of cell transformation may result from epigenetic modification and protein phosphorylation rather than gene mutation.

Overall, routine pap-smear tests should be combined with lamin A/C expression analysis in order to identify women seemingly healthy but at risk to develop cervical cancer [42]. In the female population that we investigated in Benin, 25/76 (33 %) were at risk to develop cervical cancer based on lamin A/C expression, regardless HPV status. Clearly, not all cervical lesions are associated with HPV infection [45] and screening solely for HPV infection is not sufficient to spot women at risk of developing cervical cancer. Lamin A/C deficiency seems to be a potent molecular bio-indicator to identify such women, irrespective of infectious HPV status, especially in middle and low income countries.

The efficacy of trichostatin and staurosporine as anti-cancer drugs has been reported for some cervical cancer-derived cell lines such as Hela and Caski, but the exact underlying mechanisms have not yet been elucidated [46, 47]. We found that nuclear lamin A/C expression modulation may, at least in part, explain the anticancer potency of HDAC and Ser/Thr protein kinase inhibitors. It has been shown that AKT1 can phosphorylate lamin A/C at S404 in a canonical AKT consensus motif [48, 49]. Another study has reported that p27(Kip1) was down-regulated while AKT1 was

up-regulated in both primary cervical cancer samples and cervical cancer-derived cell lines [50]. Therefore, drugs that can inhibit AKT activity (and known to phosphorylate lamin A/C) may also be used to control the growth of cervical cancer cells [50]. Thus, the effectiveness of anti-cancer therapy may be evaluated through its efficiency to restore lamin A/C expression. Lamin A/C deficiency in CUS samples may serve as a prognostic factor for the risk to develop cervical neoplastic lesions and as a bio-indicator to start personalized anti-cancer therapy.

ATRA all-trans retinoic acid, *CUS* cervical uterine smear, *ECC* endometrial carcinoma cell, *HPV* human papilloma virus, *LA/C* lamin A/C, *PCR* polymerase chain reaction, *RA* retinoic acid, *TSA* trichostatin.

Acknowledgments We thank Dr. Toussain Anagbla and nurse Wanou Sylvia, Gynecology/obstetric service, Hospital Mènontin, Benin for sample collection and Sossah Hiffo Jonas, master student, for assistance in HPV genotyping. The rector of the University of Abomey-Calavi, Pr. Sinsin A. Brice (Benin) and all the members of his office are acknowledged for their constant encouragement and support for all our innovative projects towards cancer research in Benin. We also thank Toni Yeasky, research assistant at the Sylvester Cancer Center, Miller Medical School, University of Miami, Miami, USA for being in charge of studying the mouse model of lamin A/C deficiency. Dr. Elizabeth Smith, Dr. Robert Moore and Dr. Wensi Tao, Sylvester Cancer Center, Miller Medical School, University of Miami, Miami, FL, USA are acknowledged for their support. Special thanks go to Dr. Elizabeth Smith for editing this manuscript. We are especially grateful to Dr. Véronique Tognisode, obstetrician, gynecologist and Director of Clinic Houéyiho, for her excellent work during the colposcopic examination in her medical center (Cotonou, Benin).

Authors' contributions

1. CDC: Principal investigator who has designed and carried out the study on lamin A/C as bio-indicator to assess the risk of developing cervical cancer among African women.
2. AB: PhD student who is involved in sample collection and lamin A/C analysis by Western blotting.
3. CNW: Specialist of HPV genotyping who supervised the HPV genotyping in this study.
4. QKC: Pathologist who has performed the lamin A/C immunostaining on the tissue microarray.
5. GO: Director of Hospital Mènontin who has supervised the sample collection by gynecologist and nurses.
6. VKA: Obstetrician and gynecologist who has performed the colposcopic examinations, the cervical neoplastic diagnoses and treatment (Cotonou, Benin).
7. XX: Adviser in the study.
8. SA: Director of the laboratory of Biochemistry and Molecular Biology within the University of Abomey Calavi (UAC) in Benin, where the analyses were carried out. He has established the protocol for HPV genotyping.

Compliance with ethical standards

Competing interest The authors have no competing interests. This is exclusively a collaborative academic research project between researchers from the University of Abomey Calavi (UAC), Benin, the Fox Chase Cancer Center, Philadelphia, USA, the University of Miami, Miller school of Medicine, USA, and the medical doctors and gynecologists of the Hospital Mènontin, Benin.

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