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The Inverse Correlation of Nuclear Protein Lamin A and Ribosomal Proteins L28 Determines Molecular Initiation of Cervical Carcinogenesis in the Context of HR-HPV Infection

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Abstract **Background**: High risk HPV (HR-HPV) infections associated to cervical cancers are among breaking cancer research interest all other the world. Nevertheless, few studies addressed about the molecular alteration initiator of cervical cancer. The objective of our study is to highlight the variation of known cancer biomarkers such as nuclear envelope protein (lamin A) and ribosomal protein L28 (RPL28) in function of HR-HPV status to use them as screening biomarkers for cervical cancer prevention. **Materiel and Methods:** Upon research ethic approval and signed informed consent from each participant**,** Uterine Cervix Swab (UCS) were collected from 32 seemingly healthy women to isolate epithelial cells for biochemical analyses which include a polymerase chain reaction (PCR) for HR-HPV genotypes and an immunoblotting for nuclear and ribosomal proteins (lamin A and RPL28) status determination. **Results and discussion:** The data were classified in function of HR-HPV status. In absence of HR-HPV all samples displayed lamin A expression but low or undetectable RPL28 expression. In presence of HR-HPV most samples have low or undetectable lamin A expression but display mild or high expression of RPL28. This profile was more prominent for HR-HPV 56, 58 and 66. Overexpression of RPL28 correlated with the one of cancer biomarker cleaved caspase6. **Conclusion**: Although the determination of HR-HPV genotypes is fascinating, the screening should always be associated to known cancer biomarkers (RPL28 or cleaved caspase6) to properly manage the care of patients who are on the verge of developing cervical cancer.

Keywords: *Lamin A, RPL28, HPV, cleaved caspase6, cancer prescreening*

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1. Introduction

It was established that the suppression of nuclear envelope protein lamin A was a hallmark for ovarian, breast and cervical cancer cells [1,2,3]. High-risk Human papilloma virus (HR-HPV) initiated cervical cancer subsequently to lamin A phosphorylation and degradation [4]. Suppression of lamin A associated to polyploidy and chromosomal numerical instability was perceived as a stress signal that induces the overexpression of tumor suppressor protein p53 [1,2]. The protein p53 is a transcription factor involved in DNA damage repair and apoptosis all of which preventing damaged cells from becoming malignant [5,6]. In normal physiological state p53 is regulated by an association to MDM2 which is a proto-oncogene [5,6]. As matter of fact, in response to DNA damages, some ribosomal proteins including RPL37 undergo proteasomal degradation in the nucleoplasm and subsequent activation of p53 occurs [7,8,9]. The loss of nuclear membrane protein lamin A altered nuclear structure, nucleolar organization and chromosomal stability; all of which is first perceived as nuclear stress allowing the overexpression of p53 and p21 involved in cell cycle arrest in G2 phase [1,2]. Consequently, the activated p53 will impair cell cycle progression allowing then the formation of polyploid cells that will lead to proliferating aneuploid cells which will progress to cancer cells in the absence of p53 in a long run [10]. It was also established that stress signals can induce ribosome biogenesis and binding of certain ribosomal proteins to MDM2, leading subsequently to an inhibition of MDM2 function with activation of p53. [5,6,7,11]. Succeeding inactivation of p53 may allow aneuploid cells to proceed through aberrant cell division and participate to cervical

cancer initiation following cell cycle arrest and aneuploidy formation [10]. Little or no information is openly available linking the overexpression of RPL28 to cancer hallmarks and especially to uterine cervical cancer initiation in the context of HR-HPV infection. In previous works we have reported the association between the loss of lamin A in surface epithelial cells of uterine cervix and pre-neoplastic lesions [3]. Altogether, our data suggested that prior to cancer initiation, the loss of lamin A induced p53 to arrest cell cycle progression which later progressed to unregulated proliferation subsequently to p53 impounding [11]. In cervical cancers, HPV protein E6 specifically binds to and destroys p53 which leads to failure of cell cycle checkpoint and progression of damaged-DNA throughout cell cycle favoring tumor formation similarly to the one encountered with mutated p53 [13]. It was shown that E6 oncoprotein and its interacting partner E6-associated protein (E6-AP) form a complex that binds to p53 and triggers its proteolytic degradation [13]. Furthermore, epigenetic regulation in HR-HPV-infected cells can silence tumor suppressor genes while upregulating oncogenes all of which are relevant elements to carcinogenesis [14,15]. The epigenetic abnormalities in cancer involve aberrant aspect of chromatin structure, including histone deacetylation, DNA methylation, chromatin remodeling, and non-coding RNAs (ncRNAs) or microRNA (mir) synthesis [14,15,16]. The overexpression of RPL28 was also compared to the overexpression of cancer biomarker cleaved-caspase6. The relationship between the deficit of lamin A and the overexpression of RPL28 was already determined in breast cancer cells and could be further considered [12]. Also, the relationship between the deficit of lamin A and the overexpression of cleaved-caspase6 was already demonstrated in ovarian cancer cells and tissues [14]. In this research article we investigated the association between lamin A silencing and overexpression of RPL28 in presence and absence of HR-HPV to delineate which combination of biomarkers is suitable for cervical cancer screening, prevention and treatment.

2. Materials and Methods

2.1. Facilities and Ethical Approval

The study was carried out in the Unit of Biochemistry and Molecular Biology (UBBM), division of Molecular Biomarkers in Cancer and Nutrition (BMCN) located within the Institute of Biomedical Sciences and Applications (ISBA) in Cotonou (Benin Republic). Sample collection took place among outpatients and staff of zonal hospitals in the city of Cotonou. For this study ethical approval was obtained from ISBA ethical and research committee (CER-ISBA) as well as from the Ministry of Health in Benin. Signed informed consent was obtained from each participant before sample collection.

2.2. Reagents

Polymerase chain reaction reagents (PCR) was from sigma-Aldrich. Chemical reagents for electrophoresis and sample processing including Tris-base, glycine, NaCl, KCl, Tween-20, protease inhibitors cocktail, 2-mercaptoethanol, glycerol, sodium azide and phosphate buffered saline were from sigma Aldrich. The primary antibodies made in rabbit against lamin A and cleaved-caspase6 were from Transduction Lab (USA). Polyacrylamide reagents, nitrocellulose membrane and HRP-conjugated secondary antibody (anti-rabbit) made in goat were from Bio-Rad Inc. (USA). A Super Signal West Dura Extended Duration Substrate made by PIERCE was purchased from Thermo Scientific (Rockford, IL USA).

2.3. Sample Collection and Processing

For participant selection, we considered the following inclusion criteria: they should be over 20 years, seemingly healthy, sexually active. Most of them came for routine gynecological exam, birth control planning. Some of them were hospital employees including nurses. We have also considered the following exclusion criteria: they should not be pregnant, nursing mothers, should not have menstrual flow, and should not be within the first trimester post-childbirth. Among the sample collected according to the guidelines of the Declaration of Helsinki, 32 samples were paired analyzed for nuclear envelop and ribosomal proteins (lamin A and RPL28). Sample identification was done with scramble numbers and could not be traced to the individual.

Prior to sample collection, disposable speculum was inserted into the participant vagina to facilitate the access to the uterine cervix with sterile disposable cytobrush. Epithelial cells were collected following uterine cervix swabs (UCS) by moderate scrapping uterine cervix surface epithelium with cytobrush (two clockwise rotations of a disposable sterile soft plastic cytobrush). The uterine fluid trapped on top of the brush contains the surface epithelial cells taken from the surface of the endocervical and exocervical junction. The brush with UCS was stored in a sterile tube (Urine Cytobacteriology Examination tubes UCBE) containing 1 ml of ice-cold phosphate buffered saline (PBS) mixed with protease inhibitor cocktails solution, and stored at 4°C in an ice cooler until delivery in the laboratory for processing which includes cell lysis, protein denaturation with sodium dodecyl sulfate (SDS), β-mercaptoethanol and glycerol solution and boiling at 90°C before storage at -20°C until used for analysis with western blot technique as previously published [3].

2.4. Polymerase Reaction Reagents (PCR) for HPV Genotyping

DNA was extracted with standard phenol-chloroform method after cell disintegration with lysis buffer containing proteinase K (20 mg/ml) and RNase as previously reported [3]. In brief, after adding phenol to cell lysate (v/v), the solution mix was centrifuged at 10,000 rpm at 4°C for 10 min. The upper aqueous phase was isolated in a new Eppendorf tubes and chloroform was added (v/v), mixed and centrifuged at 10,000rpm at 4°C for 10 min. The supernatant was collected in a new Eppendorf tube and DNA pellet was obtained after precipitation with ice-cold ethanol (96 %) at −20°C for 4 h and recovered following centrifugation at 12,000 rpm at

4°C for 10 min. The DNA pellet was washed once with ice-cold ethanol (70 %) and recovered after centrifugation at 12,000rpm at 4°C for 5 min. In the last step, the DNA pellet was air-dried at 55 °C for 20 min and dissolved in Tris-EDTA (TE) buffer. The DNA concentration was measured with a spectrophotometer wavelength set at 260 nm. The DNA solution was kept at −20°C until used for HR-HPV genotyping as previously reported [3].

2.5. Western Blot Technics

Protein migration was performed with bisacrylamide gel electrophoresis in denaturing condition. The migration buffer was composed of Tris-base, glycine and SDS as previously reported [1,3,10]. The choice of the gel percentage to be prepared depends on the size of target protein. In our case a 12% bisacrylamide gel was used to be able to analyze the RPL28 with a molecular weight of 15 kDa. Prior to analysis, frozen samples were heated to 95 ° C for 10 min before loading in gel-wells along with standard protein molecular weight markers [3,10]. The gel was mounted in the migration tank filled with running buffer (tris-glycine-SDS) under an electric field set at 100 volts and a migration time set at 90 min; proteins were separated according to their molecular weight.

After migration protein were transferred from the gel to a nitrocellulose membrane to be accessible for detection with specific antibodies. The transfer process from the gel to the membrane was done at 35 volts for 1 h 30 min. After the transfer, the membrane was incubated in a ponceau red solution for approximately 5 min at room temperature to verify that the proteins have been properly transferred to the nitrocellulose membrane surface.

Prior to the immunodetection, membranes were saturated with a blocking buffer (5% skim milk or BSA in PBS and 0.01%tween-20, PBST). The blocking was done for 1 hour at room temperature and with moderate stirring followed by two washes for 5 min with PBST. After membrane saturation, we applied the specific primary antibody that will attach to the binding site of the target protein on the membrane surface.

To reveal target proteins and evaluate their expression level in UCS cell lysates, the membranes after saturation were incubated in the presence of primary antibodies diluted in a solution of 1% BSA (1g of BSA in 100 ml PBST). Here we incubate one membrane with primary antibody targeting RPL28 and a second membrane with primary antibody targeting caspase6. The data regarding lamin A and β-actin used as loading control was already published and is not shown in this manuscript [3,10].

The incubations were carried out at room temperature for 2h or at 4°C overnight with gentle agitation. The membranes were then rinsed in PBST three times followed by an incubation with the secondary antibody for 1 hour at room temperature under gentle rocking. Secondary antibody peroxidase-conjugated anti rabbit or peroxidase-conjugated anti-mouse was applied according to the primary antibody species production. After incubation, the membranes were then rinsed in PBST four times 5 min. Membranes were then incubated in a Chemiluminescence reagents according to the manufacturer protocol [3,10]. The membrane was wrapped in a transparent saranwrap film and placed in an auto radiographic cassette and then covered with an auto radiographic film in the dark room. The revelation was done with an automated developer (HQ-350XT, hu.q).

2.6. Statistical Analysis

To perform non-parametric analysis, protein expression was classified as no expression (0.1); low expression (1), normal expression (2) or over-expression (3) for lamin A and RPL28. Data from participant group without HPV infection was compared to participant group with High-Risk HPV (HR-HPV) infection. Excel software was used to present the data as histograms and to calculate covariation coefficients of the two biomarkers among participant group without HPV vs participant group with HPV.

3. Results

3.1. Frequency of the Loss of lamin A and RPL28 Overexpression

To determine the association between the loss of lamin A and the overexpression of RPL28, we used UCS of known lamin A status which were collected in seemingly normal individual in Zonal hospitals in the city of Cotonou (Benin republic) as previously reported [3]. Here in, 16 samples without HPV were compared to 16 samples with HR-HPV for the analysis to point out the oncogenesis effect of HPV in some samples.

In the subgroup without HR-HPV infection (n=16) lamin A was normal in all participants 16/16 while RPL28 expression was undetectable in 7/16 and low in 9/16 participants. No overexpression of RPL28 was reported in this subgroup (Table 1).

In the subgroup with HR-HPV infection (n=16) lamin A was normal in 7/16, low expression in 7/16 and absent in 2/16 participants. RPL28 was undetectable or low in 7/16, detectable in 6/16 and overexpressed in 3/16 participant (Table 1).

Altogether, we observed an inverse association between the frequency of lamin A deficiency and RPL28 overexpression in the participant samples.

Table 1. Frequency of lamin A and RPL28 expression among group without HPV and with HPV

Total n =32	Subgroup without HPV HPV-; n= 16	Subgroup with HPV+ HPV+; n=16
No lamin A	0	2
Low lamin A	0	7
Normal lamin A	16	7
N =	16	16
No RPL28	7	7
Low RPL28	9	6
High RPL28	0	3
N =	16	16
Lamin A vs RPL28 Covariation coefficients	0	-0.2548

3.2. Lamin A, RPL28 and HPV Status

Controls samples without high-risk HPV (HR-HPV) infection show inverse covariation of lamin A versus RPL28 expression (Table 1 and Figure 1). Among all HPV genotypes identified in UCS samples, the infections with HPV56, HPV58 and HPV66 were associated to the loss of lamin A and upregulation of RPL28. The inverse prominent expression of lamin A and RPL28 are linked to HPV 56, HPV58 and HPV 55 infection (Figure 2).

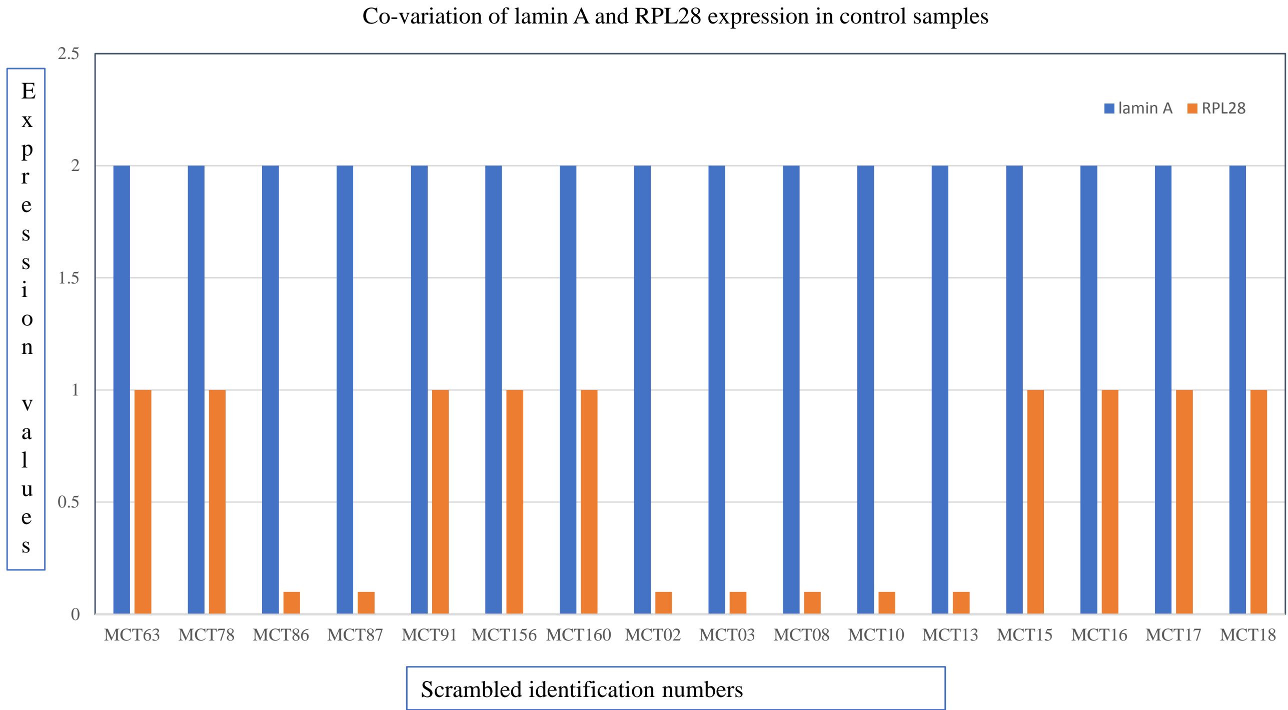

Figure 1. Histogram of control samples based on lamin A expression, showing the profile of lamin A and RPL28 in absence of HR-HPV. Sample numbers were scrambled (n=16). MCMT86, MCT187, MCT02, MCT10 and MCT13 show trace level of RPL28. For all of them lamin A level is normal

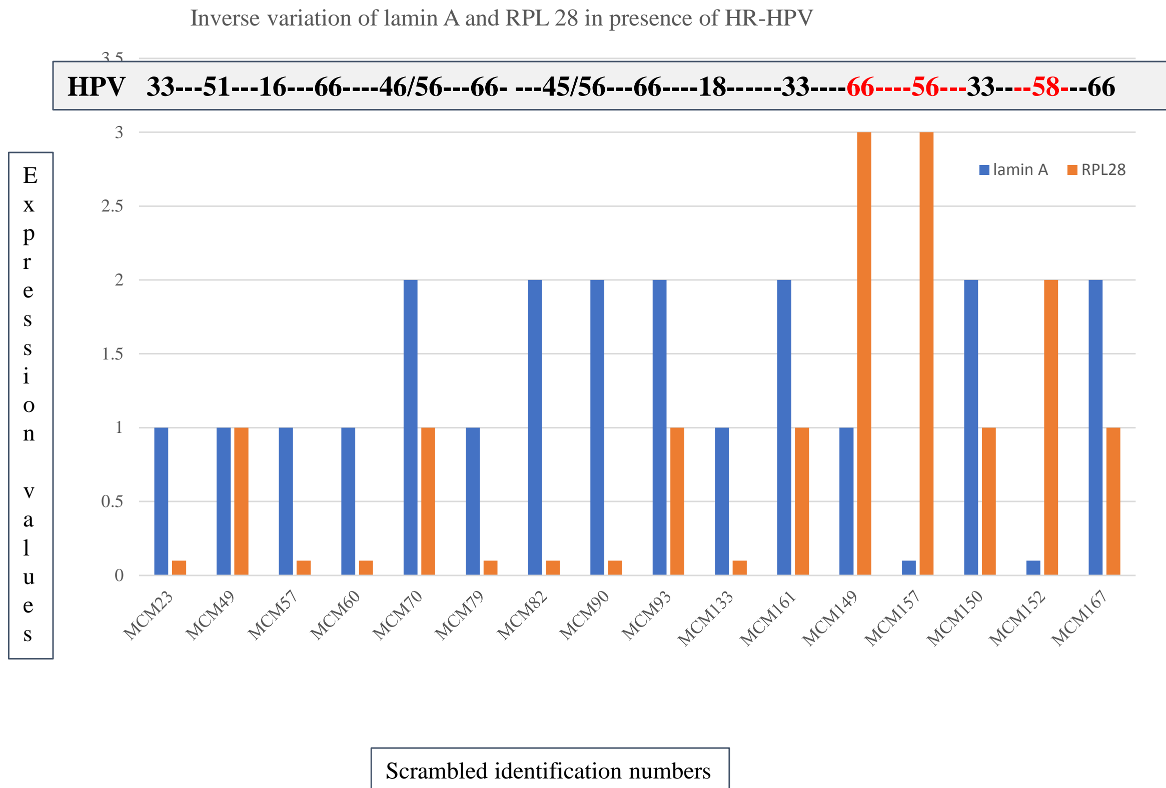

Figure 2. Inverse variation of lamin A and RPL28 expression in participants infected with HR-HPV. In absence of lamin A, RPL28 expression is very high as displayed for participant MCM149, MCM 157 and MCM152.

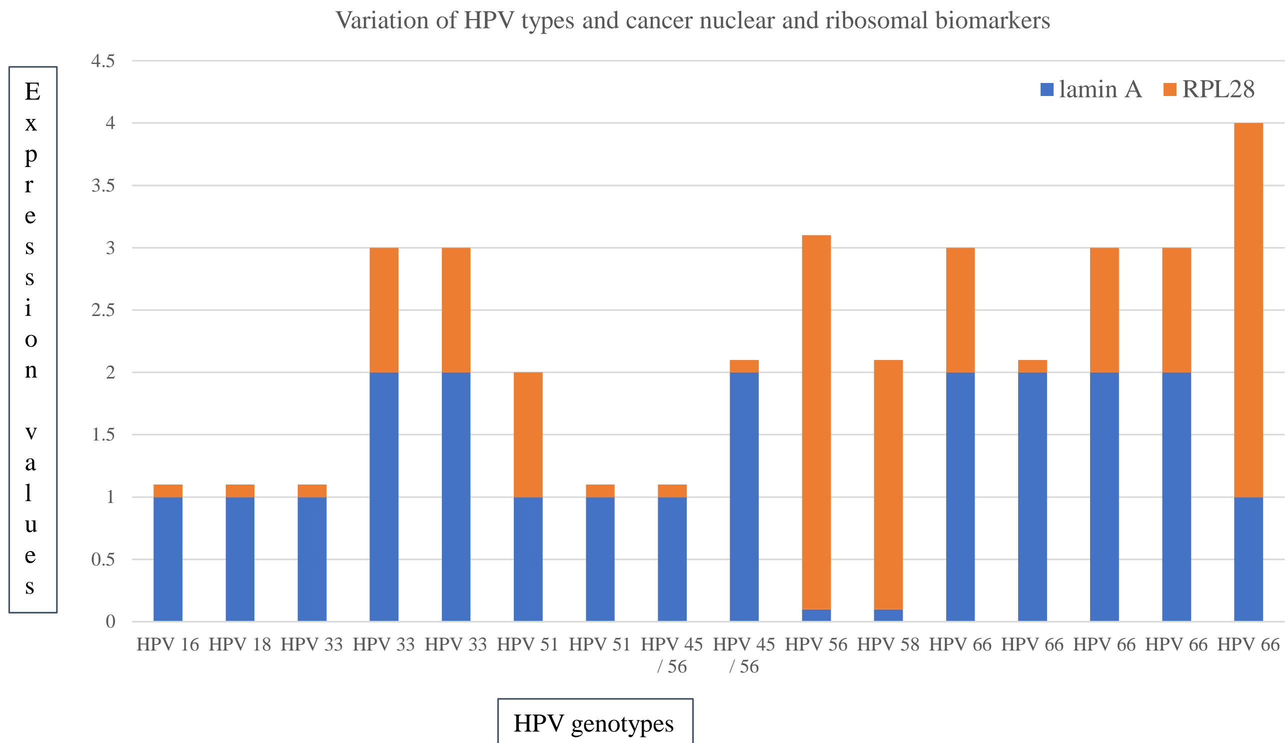

Figure 3. Expression of nuclear lamin A and ribosomal protein RL28 in function of HR-HPV genotypes. The inverse expression of lamin A and RPL28 was observed in all HPV infected samples but was more prominent with HPV56, HPV 58 and HPV66 genotypes

In absence of lamin A, the over expression of RPL28 is strongly noticeable for participant MCM149, MCM 157 and MCM152 (Figure 2). The coefficient of covariance of lamin A and RPL28 is -0.2548 in presence of HPV while the coefficient of covariance is null (0) in the group without HPV (Table 1).

3.3. Correlation between the Overexpression of RPL28 and Cancer Biomarker Cleaved Caspase6

To verified that the overexpression of RPL28 is associated with other cancer hallmarks, we have also investigated simultaneously the overexpression of cancer biomarker cleaved-caspase6 as shown in Figure 4. Indeed, the overexpression of RPL28 follows the same pattern as the overexpression of cleaved-caspase6 (Figure 4). Few samples were run simultaneously in parallel on two bisacrylamide gels in the same electrophoresis gel tank and transferred simultaneously on two nitrocellulose membranes. The protein revelation pictures for cleaved-caspas6 and RPL28 are shown (Figure 4).

As such, Samples A and C did not show expression of cleaved caspase6 nor RPL28. Samples E, F, H and I displayed middle to low expression of cleaved-caspase6 and RPL28. Samples B, D, G displayed high expression of both cleaved caspase6 and RPL28 suggesting the presence of cancer hallmark (Figure 4).

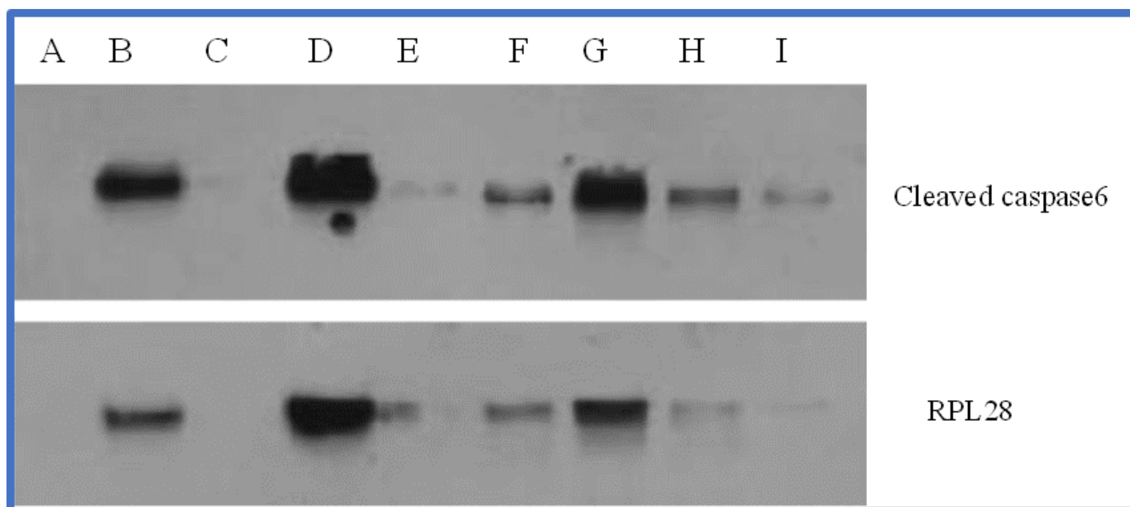

Figure 4. Western blot showing the association between overexpression of cleaved-caspase6 and RPL28. Sample A,C, E,F,H and I have no or low expression of both cleaved caspase6 and RPL28 while samples B, D and G display overexpression of both caspase6 and RPL28

4. Discussion

Our previous studies had shown that the loss of nuclear protein lamin A in ovarian cancer was associated to the activated cleaved caspase6 [14]. Also, the overexpression of RPL 28 was reported in breast cancer along with the loss of lamin A [12]. The present study shows diversity patterns for lamin A and RPL28; the dominant hallmark is that there is an inverse association between lamin A expression and RPL28. The expression of RPL28 correlates perfectly with cleaved caspase6 in this study and suggests a molecular communication between lamin A disappearance along with the overexpression of RPL28 and cleaved caspase6 in the pathway of cancer initiation and progression [12,15]. From our data it appears that the relationship linking the silencing of lamin A to the upregulation of RPL28 is complex and should be further investigated. The overexpressed ribosomal proteins in cancer could also be a target for anticancer drugs such as cisplatin (CIS) and doxorubicin (DOX) which had proven to decreased ectopically expressed ribosomal proteins [7,8]. There are many drugs that can inhibit ribosome biogenesis and should also be considered for efficient cancer cell growth control [16].

As far as HPV genotype associated to carcinogenesis is concerned, HPV 56, HPV 58 and HPV66 infections are associated to this pattern (low lamin A & high RPL28) and were already reported to be the most carcinogenic HPV genotypes in Benin [12,17]. Our previous work on HIV and HPV impacts on cervical cancer development has shown that antiretroviral (ARV) drugs can inhibit cancer development [17]. Surprisingly we have noticed that ARV protected from lamin A degradation and cervical cancer initiation [17]. Chemotherapeutic drugs inhibiting ribosomal protein biogenesis at various levels may also contribute to cancer therapy [17,18]. The implication of epigenetic modifications including DNA methylation, histone deacetylation and micro-RNA expression should also be considered before the implementation of efficient treatment cervical cancer treatment [18-22].

In another previous study of HPV infectious status in women in Benin, we have reported that HPV56 and HPV 58 were more linked to cervical lesions in our population [23]. In this study we observed that these two types of HR-HPV are also linked to the molecular dysregulation leading to cervical cancer. Women with HPV infection but expressing lamin A and low or no RPL28, have less risk to develop cervical lesions in contrast to women with HR-HPV associated to the loss of lamin A and overexpression of RPL28.

Other all, combining HPV genotypes to nuclear and ribosomal cancer biomarkers is a best way to control cervical cancer development and progression. The phenotype of low lamin A expression and high RPL28 expression predisposes to cervical cancer. Similarly, samples that displayed high expression of both cleaved caspase6 and RPL28 suggested the presence of cancer hallmarks.

Thus, participant with such biomarker profile (simultaneous overexpression of RPL28 and cleaved-caspase6) should be closely monitor for cervical lesions. All these molecular biomarkers variation discovery will contribute to cervical lesions care before clinical symptoms.

5. Conclusion

Screening for cervical cancer development is very challenging for gynecologists in absence of clinical symptoms or visual embossing in the uterine cervix. Abnormal expression of cancer molecular biomarkers is ahead of the appearance of clinical symptoms and can be used to analyze cervical uterine swab to start treatments to reinstate normal expression before diverting to the appearance of clinical symptoms. Our study is the first one to propose this cervical cancer screening that will be very helpful to prevent cervical cancer worldwide. Overall, the downregulation of lamin A and upregulation of RPL28 may best predict people at risk of developing cervical cancer in contrast to sole HPV genotype determination.

Acknowledgements

Special thanks to all the students who were trained in my laboratory during their graduate research and have participated in sample collections or other tasks in the laboratory. We are also grateful to the staff managing the university of Abomey Calavi (UAC) and all medical personnel that have participated indirectly to this study.

Statement of Competing Interest

This study is the continuation of former works already published on cervical cancer biomarkers and HPV genotypes within UAC. This line of research falls into graduate and PhD student training in molecular biomarkers for cancer prevention and treatment. This study was funded by our laboratory working on molecular biomarker in cancer and nutrition and there is no competing interest to report.

Consent for Publication

Not Applicable.

Ethical Approval

The protocol of this study was formerly approved by the Research Ethic Comity of the Institute of Biomedical Sciences and Applications and the Ministry of health of Benin Republic. Signed informed consent was obtained willingly from each volunteer participant before sample collection.

Author Contribution

- Professor (Ph.D., MPH) Callinice D. Capo-chichi had designed and funded this project. She had participated in

sample collection; bench works and data analysis. She wrote and edited this publication.

- Ms. Cherita Agbangbatin is a former graduate student involved in bench works and sample processing.
- Blanche Aguida is a former PhD. student involved in bench works and sample processing.
- Dr. Sara Houngue (Ph.D.) is a postdoctoral involved in bench works and sample collection.
- Professor Ambaliou Sanni was the former Director of the Unit of Biochemistry and Molecular Biology (UBBM) who had set up HPV analysis in the university.

List of Abbreviations

CUS: cervical uterine swab
HPV: Human papilloma virus
PBS: Phosphate buffered saline
PCR: Polymerase chain reaction
RPL: ribosomal protein

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