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Dynamism of Ribosomal Protein RPL28 as Biomarker for Monitoring Epigenetic-initiated-breast-cancer Therapy

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Abstract Background: Many oncology biomarkers were reported for breast cancer diagnosis and targeted therapy. Most biomarkers were hormonal receptors, but few studies integrated GATA transcription factors, nuclear envelop protein lamin A and ribosomal proteins, all of which were disrupted in cancer to facilitate cell proliferation, migration and metastasis. The overexpression of GATA6 in breast cancer cells is involved in epithelial-mesenchymal transition (EMT) while the overexpression of ribosomal large subunit (60S) protein RPL28 is involved in protein synthesis and cell proliferation. The objective of this study is to investigate in breast cancer cell line MCF7 the expression profile of GATA6 and RPL28 as biomarkers to evaluate the efficiency of breast cancer therapy with histone deacetylase inhibitor Suberoyl-Bis-Hydroxamic Acid (SBHA) in case that epigenetic modifications influence protein expression. Methods: Immuno-blot was used to assess GATA6 and RPL28 expression profile in MCF7 cell lysate before and after treatment with SBHA for 12h. GATA6 and RPL28 were also assessed in presence and absence of nuclear envelop protein lamin A in MCF7 stably transfected with exogenous wild type lamin A conjugated to red fluorescent protein (LA-RFP). Results: Our principal findings were that GATA6 and RPL28 were overexpressed in MCF7 lacking lamin A. GATA6 expression is higher in MCF7 control lacking lamin A. Exogenous transfection of LA-RFP in MCF7 was enough to reduce GATA6 and RPL28 at a background level like the one obtained with SBHA treatment. Conclusions: The significance of our study is that breast cancer therapy can be achieved with histone deacetylase inhibitor in case that epigenetic modifications underlie and initiate cancer mechanisms. The dynamism of biomarkers GATA6 and RPL28 can be used as bioindicators to evaluate anti-cancer drug effectiveness.

Keywords: *breast cancer, epigenetic modifications, GATA6 transcription factor, ribosomal protein RPL28, Histone deacetylase inhibitor, Suberoyl-Bis-Hydroxamic Acid, lamin A*

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1. Introduction

Breast cancer is the first leading cause of women death linked to cancer in sub-Saharan Africa including Benin [1]. The death toll is due to the absence of early breast cancer screening with potent molecular biomarkers [2]. Many biomarkers have been proposed to diagnose and survey breast cancer progression after first clinical suspicion [3]. Among the biomarkers most known were GATA3 transcription factors, the estrogen receptor alpha (Erα), the progesterone receptors (PR), the Human Epidermal Growth Factor Receptor2 (HER2/ NEU) the breast cancer protein BRCA1 and BRCA2 [3,4]. Gene mutations are often looked as the main cause of biomarker alterations [4,5]. In the last two decades it was reported that most breast cancers was linked to protein abnormal expression due to epigenetic modifications or post-translational degradation even though the gene is lacking mutations [6]. In recent study we have demonstrated that nuclear envelop protein lamin A, usually deficient prior to breast cancer initiation, could be restored with histone deacetylase inhibitor Suberoyl-Bis-Hydroxamic-Acid (SBHA) associated to the reduction of abnormally expressed GATA3 and Erα [7]. Breast cancer treatment with SBHA had significantly induced cell death alongside with restoring cell cycle regulation protein p21 [7]. During cell proliferation, breast cancer cells are entitled to increase the potential of ribosomal activities to produce enough proteins for nascent cells. Hence, our study focused on the profile of ribosomal RPL28 also named RL28 (ribosomal protein 28 of 60S large ribosomal subunit) in MCF7 lacking lamin A and in MCF7 transfected with exogenous lamin A tagged with red fluorescence protein (MCF7-LARFP) as previously reported [7]. RPL28 peptide sequence includes

134 aa which can be mutated sometime [8]. Studies had reported that RPL28 increased in some cancers including esophagus squamous cell carcinoma and colorectal cancer [8]. Up to present, no data was published on the profile of RPL28 as biomarker for breast cancer. RPL28 dynamic throughout the nuclear membrane to reach the nucleoplasm or the cytoplasm could be affected by the deficiency of lamin A protein that perturbs nuclear membrane integrity and biomolecule trafficking [9]. RPL28 profile could be a potent candidate for breast cancer diagnosis and indicator for personalized anti-cancer therapy effectiveness. Concomitantly to RPL28 evaluation, we have also evaluated the GATA6 transcription factor profile, previously reported to be overexpressed in breast cancer and to induce epithelial-mesenchymal transition (EMT) of breast epithelial cells [10]. EMT provided mobility to breast cancer cells to migrate to distance organs during metastasis process [10]. This makes GATA6 a potential therapeutic target to inhibit breast cancer cell metastasis [10]. In our current investigation we evaluated the effect of SBHA on cell proliferation biomarker (RPL28) and on cell migration biomarker (GATA6) to delineate suitable biomarkers for monitoring anti-cancer drug effectiveness and breast cancer patient survivor as it was done for BRCA1 [11]. BRCA1 suppression or reduction subsequently to promoter hypermethylation has been observed in most sporadic breast cancers (ductal and lobular infiltrating tumors) and could be restored with drugs such as aromatase inhibitors and resveratrol [10,11].

2. Materials and Methods

2.1. Materials and Reagents

Some of materials and reagents were previously reported [7,9]. Briefly, reagents needed for cell lysis such as protease inhibitor phenyl-methyl-sulfonyl fluoride (PMSF), 2-mercaptoethanol, methanol, glycerol, 1,4-Dithiothreitol (DTT), sodium fluoride (NaF), Tris-Hydrochloride (tris-HCl) and sodium dodecyl sulfate (SDS) and Bromophenol blue were purchased from Sigma-Aldrich, USA.

Reagent needed for protein electrophoresis and immunoblot, such as: Tris-Base, glycine, sodium dodecyl sulfate, Sodium chloride (NaCl), potassium chloride (KCl), Tween-20, sodium azide (NaN3), were purchased from Sigma-Aldrich (USA). Bis-acrylamide, nitrocellulose membrane, MES buffer and protein ladders were purchased from Bio-Rad Inc (USA).

Invitrogen™ Novex™ NuPAGE™ MES SDS Running Buffer (20X) and protein ladder were purchased from Invitrogen, USA. NuPAGE MES SDS Running Buffer used for electrophoresis to migrate small and medium size proteins was purchased from Invitrogen, USA.

The primary antibodies against human GATA6 transcription factor and ribosomal protein RL28 were from Santa Cruz Biotechnology (CA, USA). Primary antibody against ß-actin (mouse IgG) was from transduction lab BD Bioscience (USA). The peroxidase conjugated secondary antibodies targeting primary antibody produced in mouse or rabbit were from Bio-Rad Inc (USA).

Protein detection reagent “Super Signal West Dura Extended Duration Substrate” made by PIERCE was from Thermo Scientific (Rockford, IL USA). X-ray films were from Kodak (USA).

Cell culture materials including dishes, DMEM media, antibiotics penicillin/streptomycin, fetal bovine serum-albumin (FBS) and Dimethyl Sulfoxyde (DMSO) used as control or diluent for some powder reagents, were purchased from Fisher Scientific (USA). Histone deacetylase inhibitor Suberoyl-Bis-Hydroxamic-Acid (SBHA) powder was purchased from BIOMOL (USA).

2.2. Cell Culture

Breast cancer cell line (MCF7) as well as recombinant plasmids stably transfected with histone H2B gene fused to green fluorescent protein (PCDNA3-H2BGFP) and MCF7 cell stably transfected with lamin A gene fused to red fluorescent protein (PCDNA3-LA-RFP), were previously reported [7]. Breast cancer cell lines were seeded in 6 well cell culture dishes and incubated in DMEM medium containing 10% FBS and antibiotic [7]. Drug experiment was performed as previously reported with DMSO (mock) or with histone deacetylase inhibitor SBHA (1 or 2 µM) [7].

2.3. Cell Transfection and Selection

The procedure for cell transfection and selection follows the one previously described [7,9]. In brief MCF7 cells were seeded in 6 wells cell culture dish and transfected with PCDNA3-H2BGFP or PCDNA3-LARFP in 1 ml serum free medium for 8 h. Subsequent achievement of stable transfection was possible with cell selection in DMEM medium containing 1nM of neomycin “G418” [7,9].

2.4. Immunofluorescence

MCF7 cells stably expressing histone H2B-GFP (MCF7 H2B-GFP) or lamin A-RFP (MCf7-LA-RFP) were cultured in 6 well-dishes in dye free DMEM medium and visualized directly under Zeiss fluorescence microscope linked to Zeiss AxioCam camera [7,9]. Pictures were taken with AxioVision Rel.4.8 software [7,9].

2.5. Cell Lysate Processing

Cell pellet was washed twice with PBS and lysed on ice for 30 min with 200 µl of cell lysate buffer containing 50 mM Tris-HCl (pH 7.9), 150 mM NaCl, 0.1 mM EDTA, 1 % NP-40, 0.5 mM DTT, 0.5 mM PMSF, 30 mM NaF and 0.5 % protease inhibitor cocktail. Protein denaturation was achieved with 50 µl of (4X stock) SDS sample buffer containing Tris-HCl, β-mercapto-ethanol, glycerol and bromophenol blue, followed by boiling at 95°C for 5 min. Boiled samples were stored in freezer (-20° C) until needed for analysis [7,9].

2.6 Cell Processing for Western Blot

Before western blotting, protein samples were boiled once more and loaded on 10 % SDS-polyacrylamide gels for GATA6 and β-actin; on 12 % SDS-polyacrylamide gels for RPL28. Protein migration was achieved with electrophoresis at 100 volts for 1h 30 min, in NuPAGE™ MES SDS Running Buffer (1X). After migration, proteins are transferred from the gels onto the nitrocellulose membranes with a transfer buffer containing Tris-glycine and 20 % methanol. Before applying primary antibody, membranes were saturated with 5 % milk previously diluted in 1X Tris-buffered-saline containing 0.1% Tween-20 (TBST) for 30 min at room temperature. Subsequently incubation was carried out with primary antibody anti-GATA6, anti-RPL28 or mouse anti β-actin at room temperature. All antibodies were previously diluted in 1% milk/TBST before use. After binding of primary antibody to targeted protein, membranes were washed 4 times for 10 min with TBST and incubated with HRP-conjugated-secondary antibody anti-rabbit (GATA6 and RL28 detection) or anti-mouse (β-actin detection) for 1h followed by 4 washes with TBST. Then membranes were briefly incubated with Super Signal West Dura Extended Duration Substrate (3 min). Membranes were then wrapped in saran wrap, placed inside a radiography cassette in a darkroom and covered with premium blue x-ray films (Phenix, research products, USA). The chemiluminescence brightness emitted by the complex "protein<primary antibody< HRP-conjugated-secondary antibody", stamped marks on the X-ray film. These marks were revealed as dark bands on the X-ray film by the film processor and specified protein presence and size on the membrane [7,9].

3. Results

3.1. Transfection of Exogenous Lamin A-RFP in MCF7 Localized around the Nuclear Envelop

The presence of endogenous lamin A around the nuclear membrane is a phenotype of Breast Normal Surface Epithelial (BNSE) cell as shown in Figure 1-A. Immunofluorescence was used to assess the localization of lamin A in BNSE cells (Figure 1-A) and in MCF7-LA-RFP cells (Figure 1-B) as previously reported [7,9].

3.2. Cancer Biomarker Expression Dynamic in Breast Cancer Cell Line MCF7

MCF7 is a breast cancer cell line with total absence of lamin A [7,9]. Treatment with SBHA was shown to restore lamin A [7]. Particularly, treatment of MCF7 cell line stably transfected with lamin A-RFP (MCF7-LA-RFP) has induced cell apoptosis, cell cycle regulation protein P21 while reducing GATA3 and ERα [7]. The matching MCF7 lysates used to assay P21, GATA3 and ERα were used in this study to evaluate the expression of GATA6 and RLP28 in presence and absence of SBHA for 12h. Protein expression was revealed on film and displayed in Figure 2. GATA3 is reused as control for the efficiency for SBHA inhibition activity [7]. Due to the MES buffer used for migration, GATA3 appears as compact bands not differentiating the two GATA isoforms. The overexpression of GATA6 is more intense in MCF7 cell line lacking lamin A than in MCF7 transfected with lamin A. Thus, subsequently to the absence of lamin A, GATA6 is tremendously overexpressed in breast cancer cells. In MCF7 control transfected with histone H2B-GFP (MCF7-H2B-GFP), SBHA treatment for 12 h reduced slightly GATA6 and GATA3. The transfection of exogenous lamin A-RFP in MCF7 (MCF7-LA-RFP) was enough to reduce significantly GATA6 and GATA3. Additional, treatment with SBHA did not reduce GATA6 or GATA3 further, suggesting that there was a threshold of GATA6 and GATA3 expression which was regulated by lamin A expression.

Ribosomal large subunit protein RPL28 was overexpressed in breast cancer cell line MCF7. Transfection of lamin A in MCF7 was enough to reduce RPL28 expression to a background level. Treatment with SBHA did not reduce further RPL28 (RL28) in presence of lamin A (MCF7-LARFP) as shown in Figure 2.

Histone deacetylase inhibitor SBHA action on GATA6 and GATA3 expression regulation impacted also cell growth (Figure 3).

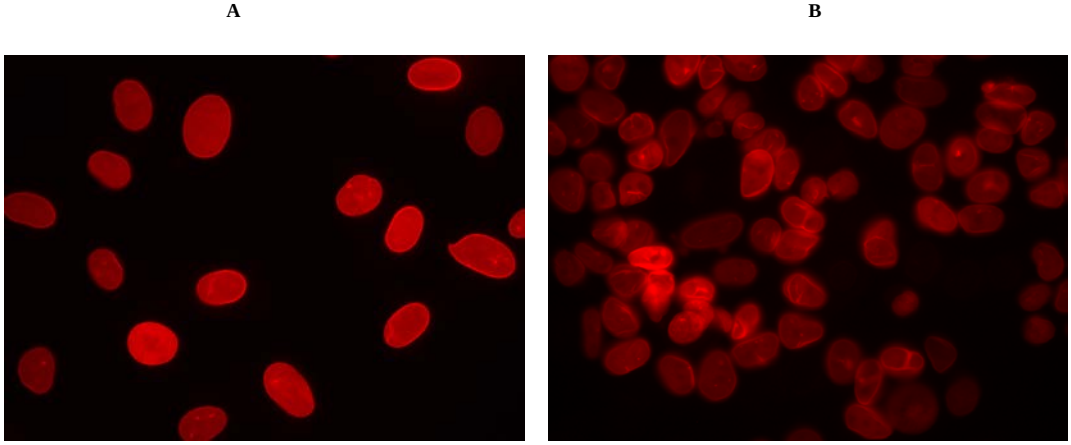

Figure 1. Nuclear envelope protein lamin A expression in breast normal surface epithelial (BNSE) cells and MCF7-LARFP. All BNSE cells express lamin A as shown in panel A. Breast cancer cells from MCF7 cell line transfected with lamin A-RFP display also lamin A-RFP localization around the nuclear envelope as shown in panel B

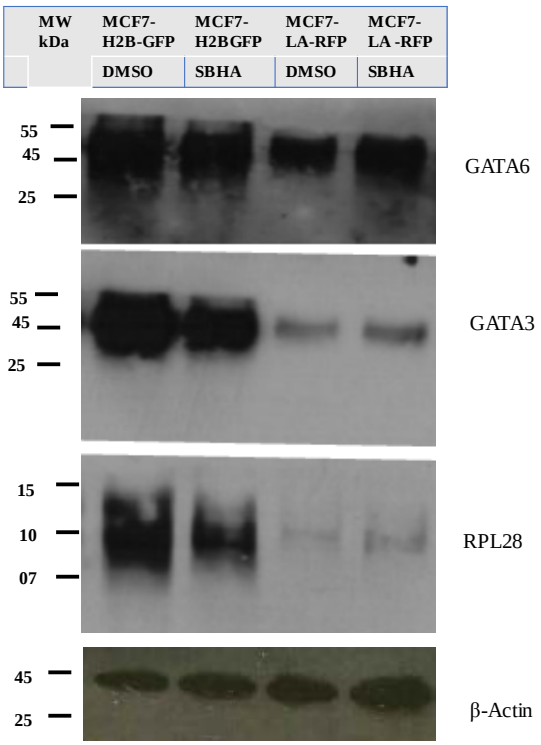

Figure 2. Decrease in RPL28 as biomarker for an efficient follow-up of anticancer therapy with Suberoyl-Bis-Hydroxamic Acid (SBHA). GATA6 is overexpressed in breast cancer cells. More remarkably MCF7 has higher expression of GATA6 which is slightly reduced after stable transfection of lamin A-RFP or treatment with SBHA for 12h. The reduction of RPL28 was significant after transfection of lamin A-RFP or treatment with SBHA.

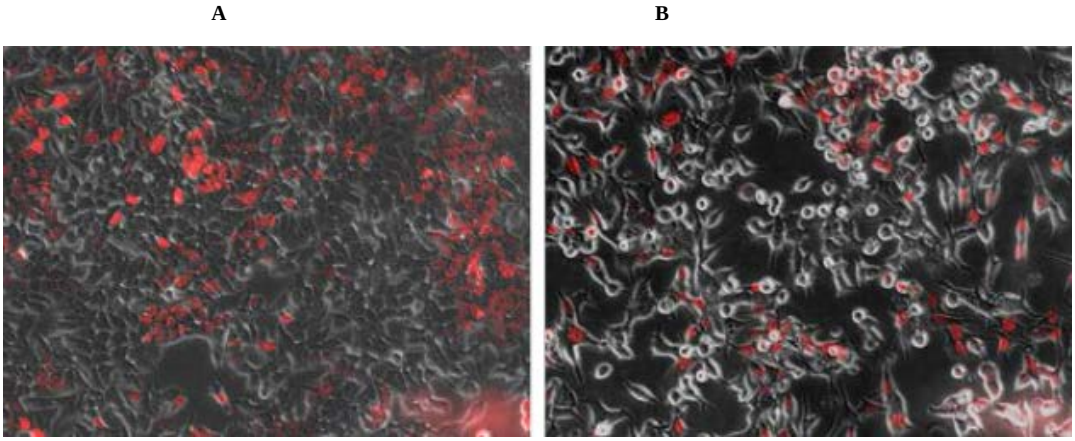

Figure 3. Cell apoptosis is induced in MCF7 in presence of LA and SBHA. MCF7 transfected with lamin A-RFP (MCF7-LARFP) was treated with DMSO (A) and with Suberoyl-bis-hydroxamic acid "SBHA" (B) for 12 h. DMSO did not induce cell death in MCF7-LARFP (A) in contrast to SBHA induced apoptotic cell death (B).

3.3. SBHA Induces Cell Death in Presence of Lamin A

MCF7 transfected with lamin A RFP (MCF7-LARF) and treated with DMSO or SBHA were displayed in Figure 3. Treatment of MCF7-LARFP with DMSO (mock) did not induce cell apoptosis (Figure 3A) in contrast to cell treated for 12h with 2 µM of SBHA diluted in DMSO (Figure 3B). Apoptotic cells appeared rounded as displayed in Figure 3B.

4. Discussion

For decades, African background women had a lower incidence of breast cancer and yet, higher mortality rate compared to Caucasian women [12]. In the context of breast cancer treatment in low income countries' clinical set, we propose that the molecular mechanism underlying individual cancer should be thoughtfully scrutinized to tailor suitable anti-cancer treatment. Cancer biomarker alterations subsequently to epigenetic modifications could be totally restored with appropriate drugs such as histone deacetylase inhibitors and DNA methyltransferase inhibitors [10].

It is well known that histone deacetylation directed DNA methylation and vice versa [10]. Regarding previous reports and our present report, we proposed that triple negative breast cancers (TNBC) which had loss ERα, PR, HER2/Neu and BRCA1 expression due to promoter hypermethylation should be treated with resveratrol to restore DNA repair protein BRCA1, then with SBHA (or analogues) to restore cell differentiation marker lamin

A alongside with cell cycle regulation protein P21 expression [7]. Furthermore, treatment with SBHA will be reducing tumor markers GATA6, GATA3 and RPL28; while reversing epithelial-mesenchymal transition (EMT). All together will inhibit cancer cell proliferation and metastasis.

Overall, the presence of lamin A will restore breast epithelial cell differentiation hallmark with reduction of GATA6, GATA3 and RPL28 while achieving cell growth regulation. It was reported that disruption of ribosomal biogenesis by extracellular or intracellular stimuli induces ribosomal stress, leading to an accumulation of unincorporated RPs [13]. Henceforward, extracellular toxic reagents or environmental pollution, similarly to intracellular oncogenic molecular alteration may induce RPL28 overexpression in the pathway of tumorigenesis. In this study, we report that dynamic expression of RPL28 could be used to evaluate breast cancer sensitivity to histone deacetylase inhibitor drugs (SBHA) and analogues. Correspondingly it was reported that breast cancers with overexpression of RPL19 were more sensitive to apoptosis-promoting drugs that induce endoplasmic reticulum stress [14].

Inclusive breast cancer treatment should use multidisciplinary approaches and multiple molecular biomarkers to personalize targeted breast cancer therapies. This new view on breast cancer treatment will be tremendous when put in application to benefit medical doctors and patients

Thus, targeted personalized therapy is a new method of choice to treat breast cancer faster with affordable medications reducing patient death related to poor financial income and aggressive chemotherapy in low income countries.

Overall, the current treatment method should be put in application in all hospital in low income countries to reduce the consequences linked to inappropriate treatment including financial hardship, uncontrolled morbidity and most importantly, patient death.

5. Conclusion

Multidisciplinary approach is becoming available for cancer treatment in low income countries including Benin. Focusing on oncology biomarker dynamism will help to delineate better treatment for all cancers. As for breast cancer, our study is the first one to uncover the dynamism of RPL28 subsequently to the treatment with histone deacetylase inhibitor SBHA. This treatment could save most patients barring breast cancer initiated by epigenetic modifications.

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Statement of Competing Interests

This study is included in UAC student graduate research program and is not funded by an organization. Therefore, there is no competing interest to report.

Author Contribution

- Ms. Sara Houngue is a PhD student who performed bench work on breast cancer.
- Ms. Blanche Aguida is a PhD student who performed bench work on cervical cancer
- Dr. Callinice D. Capo-chichi, PhD, MPH is the thesis director who designed and directed this study along with editing the current manuscript.

List of Abbreviations

BNSE: Breast Normal Surface Epithelial
BRCA1: Breast Cancer 1
EMT: Epithelial-Mesenchymal Transition
ERα: Estrogen Receptor alpha
H2B: Histone H2B
HER2: Human Epidermal growth factor Receptor 2
GFP: Green Fluorescent Protein
PR: Progesterone Receptors
RFP: Red Fluorescent Protein
RPL: Ribosomal Protein of Large subunit
SBHA: Suberoyl-Bis-Hydroxamic Acid
TBST: Tris Buffered Saline Tween-20
TNBC: Triple Negative Breast Cancer

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