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BRCA1 Protein Deficiency in Breast Cancer Microbiopsy Lysate Delineates Patient Survival Time

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Abstract **Introduction:** Breast-Cancer gene 1(BRCA1) encodes for protein which has many cellular functions including DNA damage repair and maintenance of genome integrity. Malfunction or deficiency of BRCA1 protein, due to mutations or epigenetic inactivation, may provoke breast epithelial cell dedifferentiation and initiate cancer. In fact, mutants of BRCA1 predispose to breast and ovarian cancers. In the past this biomarker was not investigated for breast cancer prevention and care in Benin. The aim of our work is to assess the expression pattern of BRCA1 gene and protein in precancerous and cancerous breast tissue microbiopsies to determine the molecular mechanisms underlying BRCA1 protein suppression and potentiate prognosis and targeted personalized therapy in Benin. **Method**: This study obtains the institutional ethical approval. Microbiopsy tissues (n = 54) were collected in the Visceral surgery department of the National University Hospital Center HKM (CNHU-HKM) located in the city of Cotonou (Benin) for diagnosis and prognosis purposes. Polymerase chain reaction (PCR) technic with primers targeting Exon 1 and Exon 2 of BRCA1 was used to assess gene transcription capability. Immunoblotting was used to determine BRCA1 protein profile in breast cancer tissue microbiopsy lysates. Ethical approval was obtained for this study. Kaplan–Meier curves analysis was performed to determine the median survival time according to BRCA1 gene and protein patterns. **Results**: We observed that 87% of samples had lost the expression of BRCA1 protein. Among them, 78% of the loss was not associated to gene deletion. The disparity between the presence of the BRCA1 gene and the lack of protein expression suggested that the silencing of BRCA1 may be due to epigenetic inactivation in most patients. Ultimately, Kaplan Meyer's survival curve analysis showed that the lack of BRCA1 amplification at Exon 1 or Exon 2 diminished the median survival time of cancer patients to 20 months. **Conclusion**: BRCA1 protein translation is impaired by Exon 1 or Exon 2 mutation /deletion along with epigenetic inactivation in breast cancer; all together influences median survival time. The delineation of the molecular mechanism underlying BRCA1 gene inactivation leading to protein deficiency will be an excellent molecular tool for African breast cancer prognostic and personalized targeted therapy in the future.

Keywords: *breast cancer, microbiopsy, BRCA1 silencing, median survival time, prognosis*

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1. Introduction

Breast cancer is a harmful disease mainly encountered in women worldwide [1]. In the twenty-first century, it was observed that the incidence of breast cancer is rising annually worldwide [2,3]. In the United States of America (USA) there is a big disparity for breast cancer incidence between White women (130.8/100.000) and black women (126.7/100.000), though the mortality is lower in white women (20.3 death/100.00) compared to black women (28.4 death/100.000) as previously reported [4]. In 2017, the incidence of breast cancer among Black women in the United States (USA) was 29,274 of which 6,427 (21%) have died [5]. Although the incidence of Breast cancer ranks number one among women cancer in USA, the incidence of mortality due to this disease is lower and ranks number two among Black American women [5]. In contrast, the morbidity and mortality linked to this affection in Sub-Saharan African countries are impressive. Indeed, the number of new cases was estimated at 1.06

million in 2018 and the number of deaths was 693,000 (69%) as reported [2].

In Africa, disparity is also noticed when comparing North African women to Sub-Saharan African women [6,7]. Breast cancer has higher incidence in North African women that in Sub-Saharan African women, but the mortality rate is higher for black women in sub-Saharan Africa [6,7]. All this disparity may be linked to some inequities in accessibility to early diagnosis and treatment for most women in sub-Saharan Africa [8]

In Benin, breast cancer ranked number one among women cancers followed by cervical cancer [9,10]. The incidence of women breast cancer in Benin (22.6/100.000) is lower than the one of Black American but the mortality is higher in Benin [5,10]. Biological parameters assessed in developed countries for breast cancer prevention and treatment include breast cancer gene 1 and 2 (BRCA 1 and 2) which harbor differential numerous mutations or epigenetic modifications according to the race and family history of cancer [11,12,13,14,15]. It was reported in developed countries that 30% of breast cancer are linked to Breast-cancer gene 1 and 2 (BRCA1 and BRCA2) mutations [13]. In developed countries, BRCA1 or BRCA2 mutations are preferentially investigated in patients with variety of breast cancer types including precancerous stage and triple-negative [13]. Beside breast cancer, mutation of BRCA1 or BRCA2 gene or their epigenetic inactivation was also linked to other cancers including ovarian carcinogenesis [16,17,18]. BRCA1 and BRCA2 analyses are routine procedures for cancer prevention and care among black women in America but not in Africa including Benin.

Indeed, in the past, no study has associated the incidence of breast cancer in Benin to BRCA1 or BRCA2 mutations or epigenetic silencing. BRCA1 gene composed of 22 exons is present on the chromosome 17 locus q21.2 and can harbor more than 200 mutations linked to breast or ovarian cancer [11,12,15,19,20,21]. BRCA2 is another breast cancer gene located on the chromosome 13 locus q12.3 and is also incriminated in breast carcinogenesis [22]. BRCA1 and BRCA2 play several roles in maintaining the integrity of the genome including crucial role in DNA damage repair and tumor suppression throughout different mechanisms [15,22,23]. Overall, there was a higher rate of cancers in women with BRCA1 mutations than with BRCA2 [24]. It is known that familial breast cancers with BRCA1 mutation are different from BRCA2 tumors and sporadic cancers [25]. Besides, it was shown that women diagnosed with an inherited mutation in the BRCA1 or BRCA2 genes have a significant increased risk of developing epithelial ovarian cancer during their lifetime [17].

Tumors linked to *BRCA1* mutations tend to be of the basal-like phenotype, have a high histologic grade, and lake the expression of three hormonal receptors including estrogen receptor alpha (ERα), progesterone receptor (PR), and human epidermal growth factor receptor (Her2); all of which are hallmark for triple-negative breast cancer or TNBC [14,26]. *BRCA2*-related tumors resemble more closely to sporadic tumors [27]. According to several population-based studies, TNBC is more frequent in Black women than in White (Caucasian) women [28,29]. TNBC linked to epigenetic modifications of BRCA1 is more frequent in young women compared to old women [14,30]. The TNBC is associated to a high recurrence risk and poor five-year survival rates relatively to other breast cancers [31]. The prevalence of BRCA1/2 pathogenic variants has been predominantly studied in European-ancestry descendants than African ancestry descendants [24]. Furthermore, the mutation on BRCA1 gene were extensively investigated unlike the protein profile [15,21,22,32].

In Africa, few studies have investigated BRCA1 or BRCA2 protein expression profile in breast cancer tissues. Additionally, BRCA1 or BRCA2 gene mutations have never been investigated as biomarker for breast cancer prevention and care in Benin. We focused our study on the integrity of the first 2 exons (Exon1 and Exon2) involved in the transcription and translation initiation site of BRCA1 gene. Thus, our studies investigated BRCA1 genes and protein profiles in breast cancer tissue microbiopsy DNA extracts and cell lysates. We focus only on BRCA1 Exon1 and Exon2 along with BRCA1 protein to differentiate breast cancers linked to epigenetic silencing from the ones linked to gene mutation and their relation to median survival time.

2. Materials and Methods

2.1. Materials and Reagents

2.1.1. Protein Analysis

The materials and methods used in this study were previously reported [9,33,34,35]. In brief, gel running buffer was composed of Tris-Base, glycine, Sodium chloride (NaCl), potassium chloride (KCl) purchased from Sigma-Aldrich (USA). Cell lysate reagent was composed of sodium dodecyl sulfate (SDS), sodium fluoride (NaF), Tris-base, 2-mercaptoethanol, glycerol, sodium azide (NaN3), purchased from Sigma-Aldrich (USA). The methanol used in the transfer buffer, the Tween-20 used in the antibody solution and the membrane washing solution were purchased from Sigma-Aldrich (USA). Bis-acrylamide, nitrocellulose membrane, protein ladders and peroxidase conjugated secondary antibodies anti-rabbit and anti-mouse were purchased from Bio-Rad Inc (USA). The primary antibodies against BRCA1 and BRCA2 (rabbit IgG) were from Santa Cruz Biotechnology (CA, USA). Primary antibody against ß-actin (mouse IgG) was from transduction lab BD Bioscience (USA). The protein detection luminol reagents were from Bio-Rad Inc (USA). The X-ray films were from Kodak (USA).

2.1.2. DNA Extraction and PCR Reagents

Pure grade Tris-Base, Boric Acid, Agarose powder, Ethylene-di-amine-tetraacetic acid (EDTA), 2-deoxyribonucleic acid (DNA) ladder and Gel-Red were from Sigma-Aldrich (France) as previously reported [Capo-chichi 2016a,b]. Primer sequences targeting Exon 1 and Exon 2 of BRCA1 were ordered from IDT inc. (USA). The primer sequences were previously reported [31].

2.2. Population Study

2.2.1. Ethical Considerations

The Local Ethics Committee for Biomedical Research at the University of Parakou (CLERB-UP) and the Administration committee of CNHU-HKM have approved this study. Participation in this study was strictly voluntary. The collection of breast cancer tissues subsequently to microbiopsy procedures, was conducted according to the guidelines of the Declaration of Helsinki. Participants have been informed and signed consent was obtained prior to the procedures and analyses [35]. All participants provided information regarding social status and nutritional diet. After sample collection anonymous identifying numbers were given to the samples sent to the laboratory for BRCA1 analysis.

2.2.2. Procedure for Breast Cancerous Tissue Sample Collections by Microbiopsies

Micro fragments of breast cancerous tissues were collected by the oncologist surgeon by microbiopsies [35]. For the present study, fifty-four (54) women with suspicion of breast cancer underwent microbiopsy procedure to collect microtissue for cancer type and grade confirmation by pathology. Patients diagnosed with breast cancer were treated at the National University Hospital Center-HKM (CNHU-HKM) of Cotonou (Benin) in the department of Visceral Surgery and Oncology. Collected samples were placed in a 5 ml sterile collection tube containing 1 ml of sterile ice-cold phosphate buffer saline (PBS) to preserve tissue fragments and cells. Samples were kept in ice-cooler and delivered within an hour to the laboratory of Molecular Biomarkers in Cancer and Nutrition (BMCN) located in the Institute of Applied Biomedical Sciences (ISBA), for processing as previously reported for cervical and breast tissue samples [33,35].

2.3. Microbiopsy Tissues Processing

Tubes containing the collected samples were centrifuged to eliminate debris before cutting microtissues into two pieces and stored in two Eppendorf tubes. Each piece will be used for: (i) DNA extraction and PCR to analyze BRCA1 gene status; (ii) protein extraction and immunoblotting to analyze BRCA1 protein profile according to previously published methods [34,35].

2.4. DNA Extraction Method

Briefly, the phenol-chloroform method was used to extract DNA from microtissue cells. Lysis buffer containing proteinase K (20 mg/ml) and RNAse was added to the sample for cell membrane disruption as previously reported [34]. Then, equal volume of phenol was added to the cell lysate, followed by centrifugation at 10,000 rpm (4 °C) for 10 min to collect the upper aqueous phase into a new Eppendorf tube. Equal volume of chloroform was added to the collected upper aqueous phase, followed by another centrifugation (10,000 rpm at 4°C for 10 min). The supernatant was collected in a new Eppendorf tube and DNA was pulled-down as pellet after addition of 96 % ice-cold ethanol and incubation at -20°C for 4h. The DNA pellet was recovered after centrifugation (12,000 rpm at 4°C for 10 min) and washed with 70 % ice-cold ethanol followed by centrifugation at 12,000 rpm at 4°C for 5 min [34].

The DNA pellet was air dried for 20 min and solubilized in tris-EDTA (TE) buffer. The concentration of DNA was measured with a spectrophotometer (260 nm). DNA extract was then stored at -20°C in a freezer until needed for BRCA1 genotyping by Polymerase chain reaction "PCR [20,31]. PCR primer sequences used to amplify BRCA1 gene (Exon 1 and Exon 2) was previously reported [20,31].

Primer sequences for Exon 1 and Exon 2 (5' – 3') are as displayed [20,31]

- BRCA1-Exon 1, forward primer sequence: 5' TAG CCC CTT GGT TTC CGT G 3'
- BRCA1-Exon 1, reverse primer sequence: 5' TCA CAA CGC CTT ACG CCT C 3' [Friedman 1994]
- BRCA1-Exon 2, forward primer sequence: 5' GAA GTTG TCA TTT TAT AAA CCT TT 3'
- BRCA1-Exon 2, reverse primer sequence: 5' TGT CTT TTC CCT AGT ATG T 3' [20]

2.5. Polymerase Chain Reaction (PCR)

To verify DNA purity, GAPDH primer was used for PCR control before setting up the PCR for BRCA1 amplification [Capo-chichi 2016 a, 2016b]. The primers used in this study were targeting Exon 1 and Exon 2 of BRCA1 gene. Amplification reaction was performed with a PCR master mix reagent in a final volume of 20 µl containing a mixture of 2X master mix (10 µl), DNA (1 µl), primer (1 µl) and ddH2O (8 µl). After PCR, the amplified products were migrated on 1 % agarose gel immersed in tris-borate-EDTA buffer (TBE) containing Gel-Red dye to visualize amplified BRCA1 Exon after exposure to 300 nm transilluminator followed by photography [33,34]. Our method did not focus on single nucleotide polymorphism but rather on the integrity of BRCA1 gene transcription initiation site by amplification of Exon 1 and Exon 2. These two exons are used as our molecular biology parameters for the evaluation of the transcriptional veracity of BRCA1 [20,31].

2.6. Western Blot Procedure

Lysates from breast cancer micro-tissue cells were run on 7.5% polyacrylamide electrophoresis gels for 2 h 30 min under electric power of 100 volts. After electrophoresis, gels harboring migrated proteins were put in contact with nitrocellulose membranes to transfer proteins under 35V for 2h. Nitrocellulose membranes covered with proteins were individually blocked with 5% skim milk diluted in Tris-buffered-saline-tween-20 (TBST) for 30 min. Nitrocellulose membranes were then incubated for 1 h at room temperature with a primary antibody produced in rabbit and directed against BRCA1 or BRCA2. The primary antibodies were pre-diluted in 1% skim-milk and TBST before use. Membranes were rinsed three times with TBST and incubated for 1h at room temperature with HPR-conjugated secondary antibody anti-rabbit diluted in 1% skim milk-TBS. The membranes were then washed four times (10 min each). For protein

detection, the membranes were incubated with luminol reagents for 3 min, wrapped with plastic sheets, placed in autoradiography cassette then, overplayed with autoradiography films for 30 minutes in dark room. Then, films were processed with a developer to show the presence of proteins as dark bands. As loading control, β-Actin was used [33,34,35].

2.7. Statistical Analysis

Kaplan –Meier method was used to determine median survival rate according to BRCA1 gene Exon 1 or Exon2 amplification pattern and BRCA1 protein profiles [36]. For our study, the comparison of median survival rate according to BRCA1 Exon 1 or Exon 2 amplification or absence of amplification, were considered significant for $p<0.05$.

3. Results

This study focus is mainly BRCA1 gene integrity, protein expression, and Kaplan-Meier median survival time.

3.1. Characteristics of the Women Participating in This Study

The participants in this study are relatively young (20-40 years) as shown in Table 1. They all have different ethnic and societal background. Most of them were married with at least one child. Their diets were mostly corn based meals, vegetables, and fruits. We encountered few married women with higher level of education, adequate income and with health insurance who followed physician's advice for early breast cancer screening (Table 1). Overall, 25% of women had history of breast cancer in their father's family while 20% of them had history of breast cancer in their mother's family. Women with low level of education have limited financial resources to pay for medical expenses or are afraid of societal judgment and hide their disease at early stage. They consult oncologist when the disease is an advanced stage and could no longer be hidden.

3.2. BRCA1 Protein Expression Dynamic in Breast Cancer

3.2.1. Western Blot Showing BRCA1 Protein Expression in Breast Cancer Cells

To verify the expression profile of the BRCA1 proteins in our samples, we used anti-BRCA1 antibody for immunoblotting. The immunoblotting has the advantage to differentiate samples with impaired translation (absence of protein) from samples with protein isoforms delineating mutations. Thus, the expression of BRCA2 protein was detected with anti-BRCA2 antibody to show the differential expression pattern of BRCA1 and BRCA2 in breast cancer. BRCA2 was shown in this western blot for comparison. Overall, most of the samples had lost both BRCA1 and BRCA2 expression. Some of them had lost BRCA1 but not BRCA2 protein (Figure 1). The loss of BRCA1 protein was observed in 87% of patient microbiopsy samples (Table 2). The loss of BRCA2 protein was observed in 67% of microbiopsy samples while 33% express BRCA2 protein isoforms related to undetermined mutations. The causes of the loss of BRCA1 or BRCA2 could be divers including mutations or epigenetic modifications (silencing). However, PCR reaction targeting Exon1 and Exon2 of BRCA1 showed the presence of Exon1 and Exon2 in most cases, suggesting an epigenetic regulation that suppresses protein translation (Figure 1).

In summary, most samples had lost BRCA1; few of them had preserved BRCA2 while BRCA1 was lost (A1, H8 and I9). The western blot image (Figure 1) showed two samples (A1 and I9) with BRCA2 isoforms. Identification numbers were scrambled to not trace to patients. In this manuscript we will only focus on data related to BRCA1 protein and Exon1 and Exon2 as molecular indicators of gene transcription and translation potencies. The frequencies and percentages of BRCA1 protein expression in 54 microbiopsy tissue lysates were reported in Table 2. Overall, 87% of our samples were negative for BRCA1 protein.

Table 1. Societal characteristics of women with breast cancer in Benin

Societal characteristics and nutritional parameters	Percentage in breast cancer cases
Patients number	**N = 54**
Patients age range [20 - 40 years]	**87%**
Marital status	
Single	**15%**
Married	**85%**
Divorcé	**5%**
Windows	**5%**
Nutritional survey	
Fruits	**90%**
Vegetables	**90%**
Cereals	**80%**
Green tea	**60%**
Alcohol	**15%**
Profession	
Higher education	**40%**
Uneducated	**60%**
Family history of cancer	
Father's family	**25%**
Mother's family	**20%**

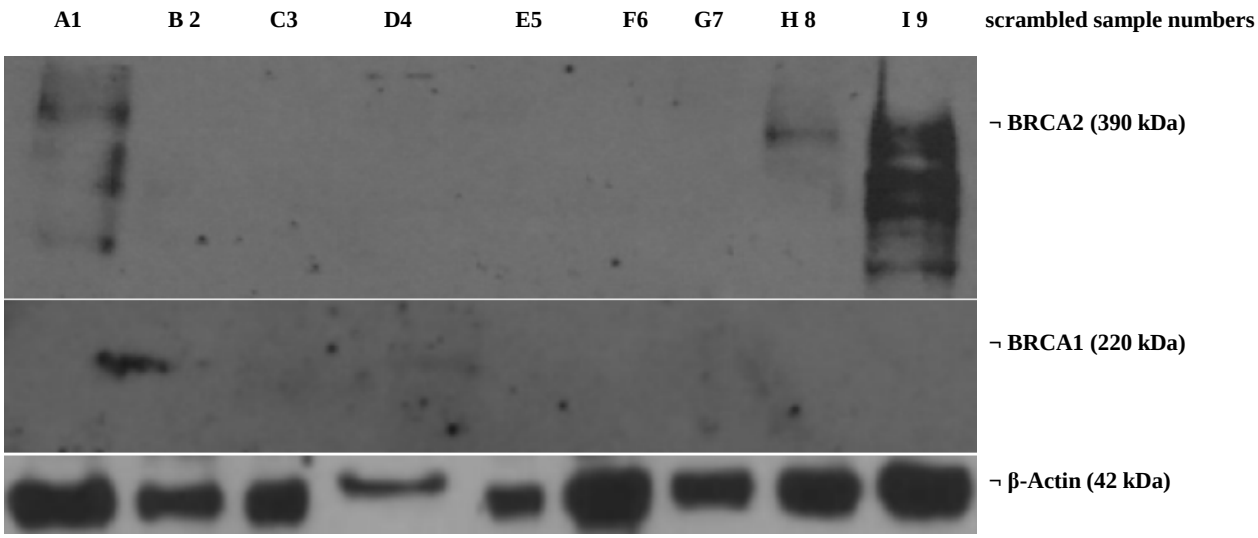

Figure 1. Example of western blot showing the expression of BRCA2 (390 kDa) and BRCA1 (220 kDa) in breast cancer micro-biopsies cell lysates (n=54); as loading control β-actin is used. Most samples have lost BRCA1. Few samples have preserved BRCA2 while BRCA1 was lost (A1, H8 and I9). On this blot, two samples (A1 and I9) displayed isoforms of BRCA2. Number were scrambled to not trace patients.

Table 2. BRCA1 protein expression frequency and percentage in breast cancer microbiopsy cell lysates

BRCA1 Protein	Frequency	Percentage
Positive (+)	7	12.96
Negative (-)	47	87.04
Total	54	100.00

3.2.2. Diagram Showing BRCA1 Protein Expression in Breast Cancer Cells

Some patient diagnosed with cancer are in advanced stage of invasive ductal carcinoma. Triple negative breast cancer patients are also negative for BRCA1 protein. The percentage of patient negative and positive for BRCA1 is displayed in Figure 2.

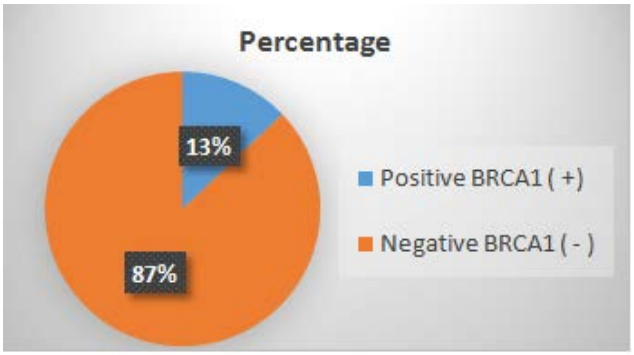

Figure 2. Diagram showing the percentage of patient with no expression of BRCA1 versus expression of BRCA1 protein

Figure 3. Histogram showing the trend of tumor grade spotted by microbiopsy intervention. Among the 45 women samples analyzed by histology, most of them were at early stage of the disease (grade 1 and grade 2).

Table 3. Histological grade of breast cancer

Cancer histological grade	Frequency	Percentage
1	14	31.11
2	15	33.33
3	6	13.33
4	4	8.90
5	6	13.33
Total	45	100

3.3. Histological Grades of Breast Microbiopsy Samples Used in This Study

Histological analyses were performed by pathologists for 45 patients out of 54. Cancer type were mostly invasive ductal carcinoma with various grades (1-5) as shown in Table 3. Most of the microbiopsy procedures were done at early stage of tumor development grade 1 and grade 2 as shown by the histogram in Figure 3. The trend of tumor grade spotted by microbiopsy procedure among the 45 samples of women who agreed for histological analysis, showed effectively that most of them were at an early stage of the disease (grade 1 and grade 2) as displayed in Figure 3. Immunohistostaining for BRCA1 protein was not done by pathologists as BRCA1 was not among the list of biomarkers used in our hospital setting for breast cancer treatment. Unlike immunoblotting (western blot) immunohistochemistry is unable to identify or differentiate protein isoforms.

3.4 The BRCA1 Gene Transcription and Translation Veracity in Microbiopsy Lysates

BRCA1 gene veracity was evaluated with Exon 1 and Exon 2 amplification. The Figure 4 displayed some of our DNA electrophoresis gels showing the PCR amplification products of Exon 1 and Exon 2 of BRCA1 gene. Amplification of Exon 1 and Exon 2 was our molecular biology parameters to verify the integrity of mRNA transcription starting site that will further permit translation. However, our molecular biology parameters do not give us information about other mutations that may exist along all exons of BRCA1 gene (not needed here). The amplification of GAPDH gene was used as a control for DNA loading control. According to our PCR results, we find out that there is variability in the amplification of Exons 1 and 2 of the BRCA1 gene (Figure 4). Some samples had loss Exon 1 or Exon 2 or both, all of which could impair mRNA transcription and protein translation. In cases where Exon 1 was lost this could be due to a deletion, however the loss of both Exons 1 and 2 for the same sample could be due to an extensive deletion at the transcriptional starting site of BRCA1 gene. In these cases, the loss of expression of the BRCA1 protein could be attributed to a gene deletion. Samples with amplification of Exon 1 and Exon 2 could transcribe mRNA and translate BRCA1 protein. Other mutations along the gene may impair protein function. On the gels presented in Figure 4, amplification of Exon 1 was not observed for samples 15, 17, 19 and 21. Amplification of Exon 2 was not observed for samples 15, 17, 19, 21 and 26. The PCR were repeated three times.

The amplification of GAPDH gene was used as a control for DNA amplification accuracy and gel loading

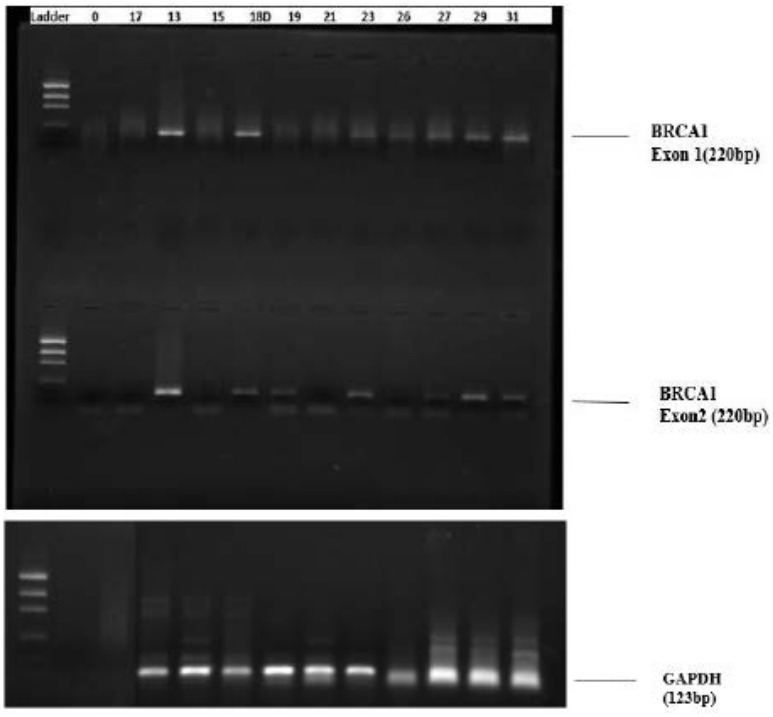

Figure 4. Electrophoresis gel showing the amplification of Exon 1 and Exon 2 of BRCA1 by PCR. The amplification of Exon 1 and Exon 2 verifies the integrity of mRNA transcription starting site that will permit translation further. The amplification of GAPDH gene was used as a control for DNA amplification accuracy and gel loading

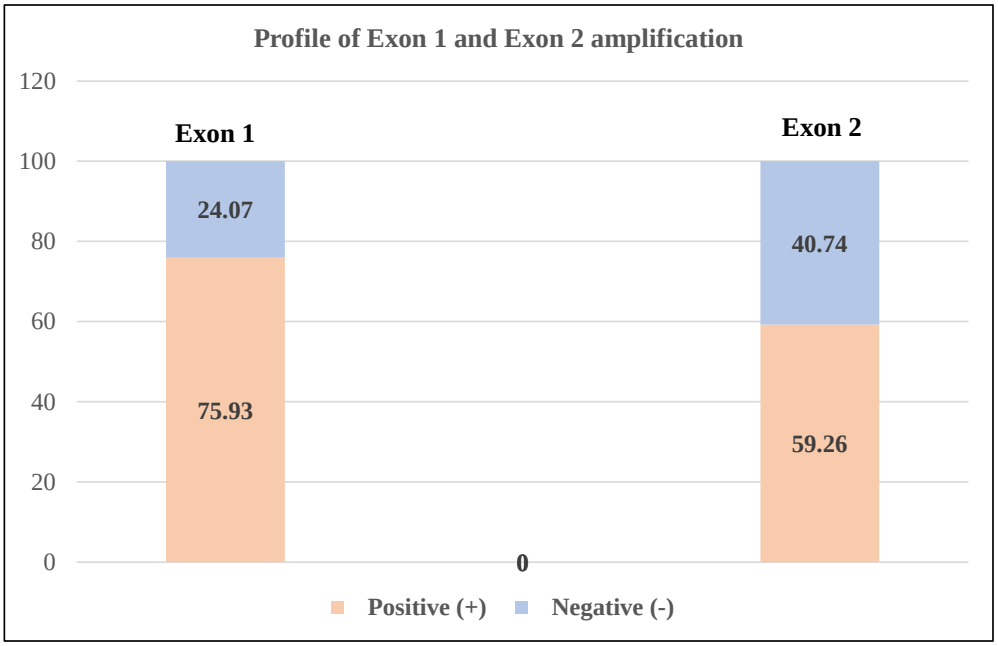

Figure 5. Histogram showing amplification of BRCA1 gene at exon1 and 2. Exon 1 is deleted in 24% of breast cancer tissues while exon 2 is deleted in nearly 41% of them. Absence of Exon 2 impairs gene transcription and further protein translation

3.5. Profile of BRCA1 Coding Potency in Breast Cancer Tissues

Histogram was used to stamp BRCA1 coding potency among breast cancer patients according to the amplification of Exon1 and Exon 2. As such, percentage of BRCA1 Exon 1 and Exon 2 amplification is reported in Figure 5. Less than 25% had Exon1 deletion but nearly 41% had Exon 2 deletion which will impair transcription initiation and further more translation.

According to our Western Blot and PCR results, we found out that BRCA1 Exon 2 deletion also conditioned the loss of the protein as Exon 2 is the site of translation initiation. Some samples had BRCA1 Exon 1 and Exon 2 amplification but no protein expression. In this case, epigenetic event may be involved in the loss of protein expression. The specific epigenetic modification will be determined in different study.

3.6. Relation between BRCA1 Protein Expression and the Median Survival Time in Women Harboring Breast Cancer

The Kaplan-Meier method was used to evaluate the median survival time in patients with BRCA1 protein expression and with patient lacking BRCA1 protein. The absence of BRCA1 protein shorten the median survival time of breast cancer patient in our study population. As such, the median survival time is reduced to 20 months in most of them as displayed in Figure 6 (red curve). Looking through the Kaplan-Meier curve of BRCA1 protein expression, we can notice that few samples have BRCA1 protein expression. The difference of median survival time rate was not statistically significant ($P= 0.86$) between patients expressing BRCA1 protein (blue curve) or not (red curve) as the protein in place might not be functional due to others underlying mutations.

3.7. Relation between BRCA1 Gene Translation Potency and the Median Survival Time in Women Harboring Breast Cancer (grade 1-5)

Amplification of Exon1 and Exon 2 of BRCA1 gene was used as molecular biology parameter to evaluate gene transcription and translation potencies. In case that BRCA1 Exon 1 or Exon 2 failed to be amplified, the median survival time is less than 20 months for most of them as displayed in Figure 7. Patients with impaired Exon 1 and/or Exon 2 have shorter lifetime bringing the median survival time to 20 months, the difference is statistically significant $p<0.005$ in both cases. Kaplan-Meier curve was established according to BRCA1 Exon1 amplification potency (Figure 7-A) and Exon 2 amplification potency (Figure 7-B). Indeed, the difference is statistically significant between patients with Exon 1 amplification (blue curve) and patient without Exon 1 represented by red curve ($P = 0.0198$). The difference is statistically significant between patients with Exon 2 amplification (blue curve) and patient without Exon 2 represented by red curve ($P=0.0043$). There is a disparity between the presence of the gene and the protein expression profile. Some samples with amplification of Exon 1 and Exon 2 of BRCA1 do not have protein expression. All of which suggested that the suppression of BRCA1 may also involve epigenetic regulation in these patients.

3.8. Hormonal Receptors Anomalies

Numerous biological aberrations are involved in breast cancers. We summarized in Table 4 the status of the three hormonal receptors ERα, PR and HER2 for 46 samples.

Triple negative receptors (ERα-, PR- and HER2-) were observed in 50% of our samples (Table 4).

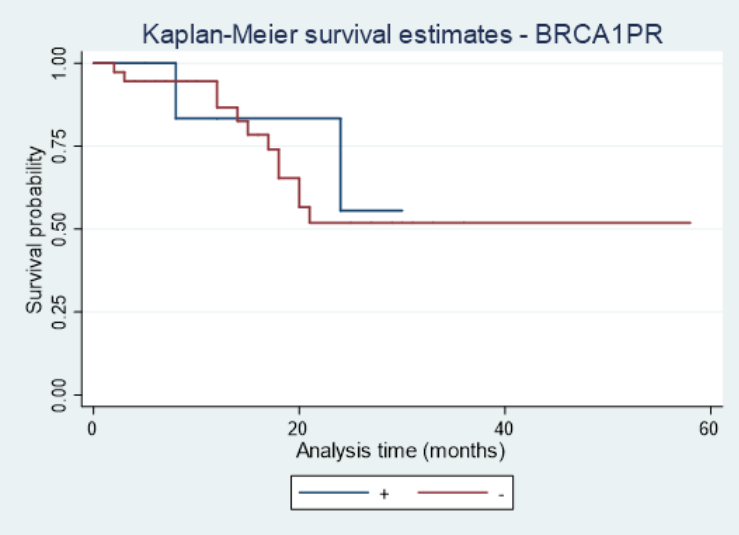

Figure 6. Kaplan-Meier median survival time in patient expressing BRCA1 protein (blue curve) versus patient lacking BRCA1 protein (red curve). The Kaplan-Meier curve shows that the median survival time is reduced to 20 months in women who have totally lost BRCA1 protein expression, few samples have BRCA1 protein expression. The difference of median survival time rate was not statistically significant between patients expressing BRCA1 protein (blue curve) or not (red curve); *p=0.86.*

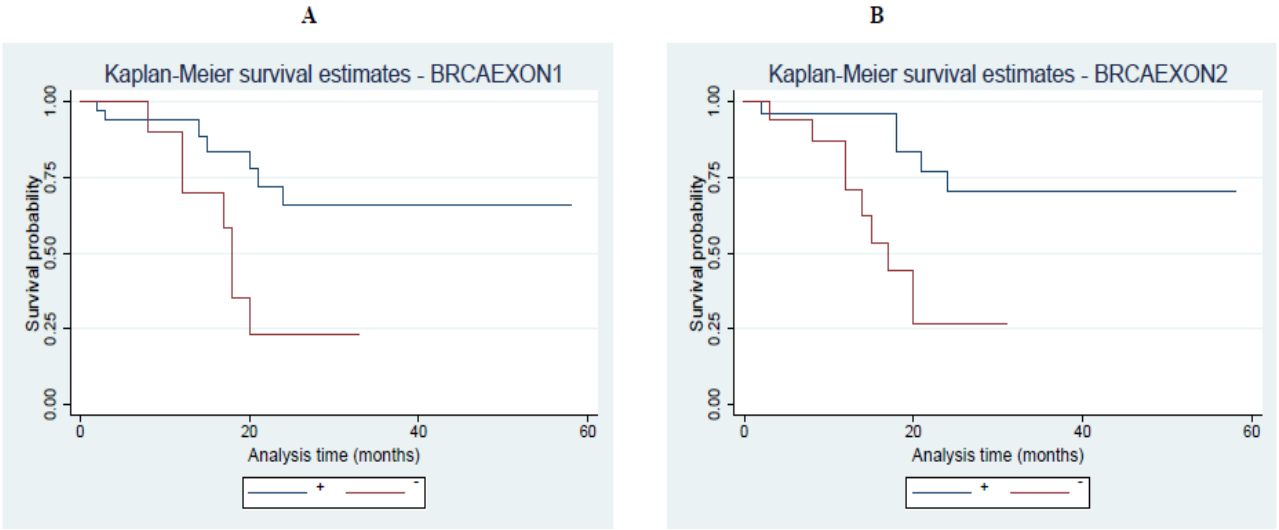

Figure 7. The Kaplan-Meier curve is established according to the amplification potency of BRCA1 Exon 1 or BRCA1 Exon 2 in samples DNA extract. A: represents the Kaplan-Meier curve according to Exon 1 amplification; the median survival time is lower than 20 months for patients with deletion on BRCA1 Exon 1. The difference is statistically significant between patients with Exon1 amplification (blue curve) and patient without exon 1 represented by the red curve ($p = 0.0198$). B: represents the Kaplan-Meier curve according to Exon 2 amplification; the median survival time is lower than 20 months for patients with deletion on BRCA1 Exon 2. The difference is statistically significant between patients with Exon 2 amplification (blue curve) and patient without exon 2 represented by the red curve ($p = 0.0043$)

Table 4. Hormonal receptors status in the samples

Summarized biological parameters	Numbers (n=46)	
	Frequency	Percentage
Estrogen receptor (ER)		
Positive	20	43%
Negative	26	57%
Progesterone receptor (PR)		
Positive	20	43%
Negative	26	57%
Human Epidermal Receptor 2 (HER-2/Neu)		
Positive	22	48%
Negative	24	52%
TNBC: (ER-, PR- and HER2-)	23	50%

4. Discussion

Several factors are involved in breast carcinogenesis which is the result of a complex interaction between exogenous factors (environmental, lifestyle, diet, physical activities), endogenous factors (age, number of pregnancies, genetic mutations, epigenetic modifications) and the transmission of breast cancer susceptibility genes [36,37,38,39]. Familial history of cancers in general, may also influence the risk of developing breast cancer in all women including African descendant [29].

BRCA1 protein is a tumor suppressor which has roles in the maintenance of genome stability through variety of mechanisms including DNA-damage repair, control of cell-cycle progression, regulation of gene transcription processes and apoptosis [13,15,16,21,40]. Women with highly penetrant BRCA mutations have a 55-60 % risk to develop breast cancer [26,27].

Our study investigated the expression profile of the BRCA1 gene expression potency and protein status in breast cancer microtissues in Benin. We observed that young women (30 - 45 years) were the most affected by this pathology. It was shown that breast cancers among young women are aggressive with rapid progression leading to a higher mortality rate compared to older women [12]. Since most breast cancer patients are young in our population, their disease is mostly aggressive with a high mortality rate [10,29,38].

Among exogenous factors inducing molecular alterations leading to breast cancer, poor nutritional status was reported [39]. In this study many women diets included fruits, fresh vegetables, meat and herbal infusions (tea), all of which suggested that they had seemingly healthy good diet. Dietary guidelines advice healthy diet based on high consumption of fruits, vegetables, whole grains, poultry and fish, and low consumption of red meat and high-fat dairy products, to improve the overall prognosis and survival of women diagnosed with early-stage of breast cancer [39]. Experimental study on cancer cell culture suggested that the consumption of diet containing lycopene (Tomatoes) may also help to restore BRCA1 protein expression not related to gene mutation [22]. As matter of fact, treatment with retinoic acid (lycopene) was able to restore BRCA1 and BRCA2 protein expression in ER-positive breast cancer cell lines MCF7 [22].

Our study verified the expression of BRCA1 and BRCA2 proteins in breast cancer micro tissues for prognostic purposes. The frequency of the loss in BRCA1 and BRCA2 protein expression is inequivalent and may be due to a mixture of genetic mutations and epigenetic modifications [14,37]. Somatic mutations of BRCA1 gene are not associated with sporadic breast tumors but rather BRCA1 promotor methylation which reduce gene expression [14,30,37]. The absence of BRCA1 or BRCA2 protein will cause damage at cellular levels which can lead to breast cancer [36]. Retrospectively, the absence of BRCA1 protein in our study, could be due to many mechanisms including, germline mutations causing the absence of BRCA1 transcript or its premature termination, non-sense microRNA (miR) mediating RNA degradation, hypermethylation at the gene promoter silencing transcription and furthermore translation [14,15,30,37].

According to our data BRCA1 Exon1 and Exon2 are differentially affected in the tissue samples analyzed with respectively 25% deletion of Exon1 and 50% deletion of Exon 2. The absence of protein while gene transcription potency is not affected could be due to epigenetic silencing by histone deacetylation or microARN expression [14,30]. BRCA1 protein expressed in cancer tissues samples may not be functional due to other mutations along BRCA1 gene giving non functional isoformes of BRCA1 protein. Wild type BRCA1 has contless physical interactions with many proteins to carry out its multiple cellular functions [15,21].

Thus, the absence of BRCA1 normal function will lead to genomic instability, development of breast, ovarian or other cancers [14,15,37]. The sequence of Exon2 constitutes the site of initiation of protein translation which could justify the loss of protein observed in some cases. We have reported earlier in different manuscript the status of ERα and PR with other cancer biomarkers [35]. The absence of three receptors such as ERα, PR and HER-2, is a marker for TNBC type and is observed in half of our breast cancer patients. This subtype of breast cancers is the most common histological subtype observed in BRCA1 carriers [31]. The prevalence of TNBC in *BRCA1* carriers is considerably higher than the general population with a prevalence of 11-20%. Germline mutations in the BRCA1 and BRCA2 genes account for 20-25 % of inherited breast cancers and about 10 % of all breast cancer cases [15,40].

The variability in biological parameters observed in our study (Table 4) makes breast cancer treatment challenging and complex. Personalized targeted therapy should always prevail after meticulous analysis and careful consideration of therapy that will reverse the biological anomalies. Many studies conducted on breast cancer reported higher grade of ER negative tumor type in African women compared to white Americans [10,38]. Triple negative breast cancer (TNBC) is a general molecular characteristic in breast tumor associated to inherited BRCA1 and BRCA2 mutations [38]. TNBC and high-grade pathology are frequent in young women along with BRCA1 promoter methylation [14,30].

The search for the expression profile of BRCA1 along with the three hormonal receptors in mammary tumors will help tremendously to treat each patient in sub-Saharan Africa, specifically according to the molecular mechanism underlying the initiation of their cancer. Our study method exposed 22% of deficiency in BRCA1 protein linked to gene deletion (Exon1 or 2) in our population bearing breast cancer. All of which is in range with the frequency of BRCA1 germline mutation related to breast cancer previously reported [15,40].

In African women and particularly in Benin, our study demonstrated that BRCA Exon1 and/or Exon2 deletion carriers had shorter median survival time. Then BRCA1 Exon1 and/or Exon 2 deletion carriers have a higher risk of early mortality in comparison to non-carriers independently of other cancer biomarkers reported earlier [35,42].

Indeed, the Kaplan-Meier survival curve suggested that patients with loss of BRCA1 expression have a reduced lifespan whereas patients who have expressed BRCA1 have a longer survival time. In addition, Kaplan Meier's

analysis shows a correlation between the absence of BRCA1 and a decrease in the median survival time of patients (20 months). This suggested that women with BRCA1-deficiency breast cancer should have a different therapy than those with intact BRCA1 to extend their lifetime. The absence of BRCA1, which is part of genetic factors, should be considered as determining factors in the diagnosis, screening, and treatment of breast cancer in all cancer patient cases. This will allow them to understand the molecular mechanism underlying the initiation of cancer and will also contribute to extend their lifespan. Epigenetic downregulation of BRCA1 and other biomarkers associated to breast cancer could be restored with specific drugs [23,35]. Regarding the molecular mechanism displayed by cancer cells to silence BRCA1 protein expression or alter its protein function, the outcome will be genomic instability and carcinogenesis in breast, ovarian or other tissues [14,15,17,25].

By focusing our study on BRCA1 status and breast cancer we discovered that the consideration of Exon1 and Exon2 amplification as molecular biomedical diagnosis tools, could contribute efficiently to breast cancer prognosis and patient personalized therapy to alleviate disparity in treatment and mortality.

The effective application of relevant biomarkers analysis based on the quest for BRCA1 Exon1 and BRCA1 Exon2 integrities for the purposes of breast cancer diagnosis, prognosis and treatment should be integrated in all African Cancer Centers protocols. This will contribute to treat efficiently cancer patient and have a better follow-up and outcome. Overall, the inclusion of investigation to determine the molecular mechanism underlying BRCA1 deficiency or malfunction will be an excellent asset to better personalize targeted breast cancer therapy in African Countries.

Abbreviation

BRCA1: Breast cancer type 1 susceptibility protein
BRCA2: Breast cancer type 2 susceptibility protein
ERα: Estrogen receptor alpha
EGFR: Epidermal Growth Factor Receptor
HER2: Human Epidermal Growth Factor Receptor 2
PR: Progesterone receptor
PBS: Phosphate buffered Saline
SDS, sodium dodecyl sulfate.
TBS, Tris Buffer Saline
TBST, Tris Buffer Saline plus Tween20
TNBC: Triple Negative Breast Cancer
Tris-HCl, Tris-Hydrochloride

Ethical Approval

Ethical Approval for this study was obtained from the Local Ethics Committee for Biomedical Research of Parakou University (CLERB-UP). The study was also approved by the administration committee of the National University Hospital Center-HKM (CNHU-HKM).

Competing Interest

This is exclusively an academic investigation and a collaboration between researchers from the Faculty of Sciences and Technics (FAST) and the Faculty of Health Sciences of the University of Abomey Calavi (UAC) and the National University Hospital Center HKM (CNHU-HKM) in Benin. The authors have no competing interests to declare.

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Availability of Data

All data obtained are transcribed in this manuscript. For ethical and legal reasons individual data collected could not be made publicly available.

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