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Comparison Between two Markers in the Identification of Free Plasma DNA Fractions in Pacients with Breast Cancer

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***Abstract*— Cancer is one of the most cause of death in the world, with increased incidence each year. About 1.7 million women died In 2012, 1.7 million women died in the world with breast cancer. This study aimed to quantify the FDNAF by two markers, the gene expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and ß-actin, and evaluate which is the best to detect women with breast cancer.50 women were recruited to dose the FDNAF, 30 with breast cancer and 20 healthy controls. DNA was extracted from plasma, and Real time PCR reaction using the GAPDH and β-actin were performed. The GAPDH was the most adequate marker to identify the patients with breast cancer, because they presented higher levels of fragments of free DNA, when compared to healthy controls.**

***Index Terms*— Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) , ß-Actin.**

I. Introduction

Cancer is one of the leading causes of death, mainly in developed countries. In 2012, the World Health Organization estimated over 14 million new cases of cancer and almost 8.2 million deaths worldwide. Breast cancer were diagnosed in 1.7million with 521 000 deaths (1, 2). Among women, breast carcinoma appears as the most common type of cancer, with about 1.7 million new cases and nearly 530 thousands deaths annually (3). In addition, incidence rate of breast cancer has increased due to growth in life expectancy, urbanization and adoption of occidental lifestyle.

Non-invasive methods of breast cancer detection have been the major focus of experts. Usually, these methods are easy to perform, which allow them to be used in large scale among the population. Consequently, they might produce a positive impact on both early diagnosis and mortality rate.

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Determination of the free DNA fraction (FDNAF) correlates with the diagnosis of several types of cancers (4-8). Furthermore, such correlation was also described in lupus, preeclampsia and in patients with multiple organ dysfunction (9-11).

In plasma, the presence of DNA fragments was first described in 1948 (12). Later on, it was observed that patients with cancer or under treatment also exhibited FDNAF in serum. Higher FDNAF levels were associated with presence of metastases and after radiotherapy, with prognostic correlation (4). Nevertheless, the mechanism by which the FDNAF gets into the blood circulation is not yet clear. It is hypothesized that could be due to cellular necrosis, with irregular fragments pattern, or apoptosis (13, 14). Furthermore, it has not been established normal reference values for cases and healthy controls.

Thus, this study aimed to quantify the FDNAF by two markers, the gene expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and ß-actin, and evaluate which is the best to detect women with breast cancer.

II. Material and Methods

A. Study Design

This was a population-based case-control study. Women were examined at a local mastology center (Odete Valadares Maternity, Belo Horizonte, Minas Gerais, Brazil) from January until July, 2006.

The adopted inclusion criteria were to be female, over eighteen-years old, and to have been referred to a mammography screening at the center. On the other hand, women under the age of eighteen, with a previous personal history of any etiology of cancer and with no mammography request or negative biopsy exam for malignancy were excluded from the study.

In total, 50 women were recruited to dose the FDNAF. The control group was composed of 20 women without benign or malignant mammary pathology, with routine mammography screenings considered normal (BIRADS 1 or 2)(15). The cases were consisted of 30 women whose mammograms were highly suspicious of malignancy (BIRADS 4 or 5)(15). Thereafter, malignant breast neoplasia (invasive carcinoma) was confirmed by biopsy.

The study was approved by the local ethical committee (The study followed the Declaration of Helsinki and was approved by the National Committee of Ethics in research

(protocol number: 1889/2005) and written informed consent was obtained from all participants.

B. DNA Extraction

From each patient, whole blood was collected in a 15 mL EDTA-anticoagulated tubes. Subjects who underwent surgical intervention had blood collection performed before anesthesia.

Then, blood was centrifuged to obtain the plasma, which was separated in 1 mL eppendorf tubes and stored at -80°C until analysis. For DNA extraction, High Pure PCR Template Preparation Kit (diagnostics Roche®, Germany) (according to the manufacturer's instructions) was used to analyze 200 µL of plasma. Gene sequences that codify the GAPDH enzyme (forward 5'-CCACCCATGGCAAATTCC-3' and reverse 5'-GATGGGATTTCCATTGATGACA-3', Sigma® Life Science) and ß-actin (forward 5'-ATGTTTGAGACCTTCAACA-3' and reverse 5'-CACGTCAGACTTCATGATGG-3', Bionner®) were utilized to quantify the plasmatic FDNAF. All samples were analyzed in duplicate, using the equipment Step one PlusTM Real Time PCR System Thermal Cycling Block (Applied Bio-systems®).

Real time PCR reaction using the GAPDH was performed using 25 µl of total reaction (12.5 µl MaximaTM SYBR Green/ROX qP CR Master Mix (2X), 1 µl DNA, 7.5 µl water, 10 pmol of primers). For the ß-actin reaction, 25 µl of total reaction were also used (12.5 µl MaximaTM SYBR Green/ROX qPCR Master Mix (2X), 1 µl DNA, 1.5 µl water and 10 pmol of primers). The amplification reaction was conducted with the initial denaturing of 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C.

To calculate the quantity of FLDNA, it was used the equation C=Q x.Vdna/Vpcr x 1/Vext (C concentration of DNA in plasma in copies by milliliter, Q copies of genome, Vdna total volume of the extraction (10 µl); Vext, volume of extracted plasma. The FLDNA equivalent was calculated by using dilution curves, calculated with known concentrations of human DNA(16).

C. Statistical Analysis

The data were input in a Microsoft® Excel database and analyzed using the Statistical Package for the Social Science (SPSS) software, version 17.0.

The normality of variables was verified by the Kolmogorov-Smirnov test. The association between the multiple independent variables and the presence of breast cancer was evaluated using the Pearson's chi-square test. In addition, statistical significances of the differences between the two groups were determined by Student's t test, for variables with normal distribution, or Mann-Whitney U test, in case of asymmetric variables. Odds ratio was calculated with 95% confidence interval. Results were presented as mean ± SD and median (min-max). Significance level of 5% was adopted.

III. Results

A. Sample Characterization

Table 1 shows anthropometric, sociodemographic and reproductive characteristics of the patients. Case and control groups were comparable for age. In addition, no statistical significance was observed with regard to anthropometric variables. The mean BMI of the subjects was 26.98±4.75 in the first group and 26.26±4.80 in the control group (p=0.60). Case and control group patients were mainly obese and overweight, respectively.

Table 1: Anthropometric, sociodemographic and reproductive characteristics of the evaluated volunteers.

	Case group (n = 30)	Control group (n = 20)	*P*
	Mean±SD	Mean±ED	
Weight (kg)	65,93±13,50	64.17±14.40	0,66[a]
BMI (kg/m²)	26,98±4,75	26.26±4.80	0,60[a]
Age (years)	53,40±12,06	47.75±8.39	0,075[b]
Age of menopause (years)	45,62±6,87	47.14±5.04	0,59[a]
Age of menarche (years)	13,00±1,70	12.6±1.60	0,40[a]
Breast feeding (months)	6,38±7,76	7.52±9.58	0.81[b]

SD: standard deviation; Min: minimum; Max: maximum; BMI: Body mass index. [a]Student's *t* test, [b]Mann Whitney *U* test.

Women in the GCO presented RCQ higher than 0.8 in relation to the GCA, 95% and 73%, respectively, with a significant difference between the groups (p=0.044) (Table 2).

In relation to the factors of reproductive risks, a larger number of the women of the breast cancer group used oral contraceptives (GCA=63.3% and GCO 45%, p=0.14), experienced menopause (GCA=6.6 and GCO=35%, p=0.0001), and used hormone reposition therapy (TRH) (GCA=20% and GCO=10%, p=0.21) (Table 2).

The women of the control group breast fed (GCO=80% and GCA=77%, p=0.43), but with an average breastfeeding time period per infant child (GCO=8.78 and GCA=10.73 months, p=0.8). In relation to their offspring, the quantity of nulliparous women was similar in both groups (20%, p=0.49), as well as for the menarche average age (GCO 12.6 and GCA 13 years, p=0.58) (Tables 1 and 2).

The control group consisting of younger women (47.7 x 53.4 years old, p=0.075), did not present a previous history of benign breast disease (GCO=0 and GCA=20%, p=0.01 or breast cancer (GCO=0 and GCA=46%, p=0.00008), presented a larger number of women who did physical activity in relation to the GCA (GCO=20% and GCA=10%, p=0.23), considered protective factors (17, 18) (Table 1 and 2).

In relation to schooling, the number of women with complete primary education (grades 1 to 8) was fewer in the

GCO (GCO=60% and GCA=63.4%, p=0.40), as well as their origin from urban areas (GCO=50% and GCA=60%, p=0.63), two risking factors which cannot be independently analyzed, but which reflect changes in reproductive patterns, such as parity, number of children, breast feeding, mammograms done, among others; with increased risk for women from urban areas and with a higher socioeconomic status (19) (Table 2).

The alcohol and the tobacco variables had a representative number of women whose data had not been informed (alcohol, GCO=40% and GCA=3.3% and smoking GCA=13%), and these data should be carefully analyzed (Table 2).

Table 2: Risk factors of the Evaluated Volunteers

Risk factors	Cancer group (n = 30) n (%)	Control group (n = 20) n (%)	*P*
Urban Zone	18 (60%)	10 (50%)	0.42 #
Education (elementary school)	19 (63.4%)	12 (60%)	0.81 #
Past history of breast benign disease	6 (20%)	0.0 (0.0%)	0.03 *#
Family history of breast cancer	14 (46.6%)	0.0 (0.0%)	0.000 *#
Children	24 (80%)	16 (80%)	1.00 #
Absence of breast-feeding	7 (23%)	5 (20%)	0.87 #
Hormonal Contraception	19 (63.3%)	9 (45%)	0.27 #
Menopause	26 (86.6%)	7 (35%)	0.000 *#
Menarche ≤13 years	17 (56.6%)	14 (70%)	0.34 #
Hormone replacement therapy	6 (20%)	2 (10%)	0.38 #
Physical activity	4 (13.3%)	4 (20%)	0.48 #
Excess weight (overweight / obesity)	17 (56.6%)	11 (55%)	0.86 #
WHR> 80	22 (73.3%)	19 (95%)	0.08 #
Alcoholism	8 (26.6%)	6 (30%)	0.38 #
Smoking	8 (26.6)	6 (30%)	0.59 #

* p<0,05, # Qui quadrado.

IV. DNA Quantification

The average plasma DNA concentration obtained by the real-time PCR technique using the GAPDH was 0.198 copies/ul for the control group (maximum 23.904, minimum 0.0866), and 0.661 copies/ul (maximum 17.974, minimum 0.001) in the group of breast cancer cases with significant difference between the values found for each group (P = 0.0020). Using the ß-actin, these values were respectively 0.060 copies/uL for the control group (maximum 17.005, minimum 0.007) and 0.009 copies/uL for the cancer group (maximum 0.996, minimum 0.0009), with significant difference between the groups (=0.0001). The found data using the GAPDH gene showed higher levels for the group with breast cancer cases in relation to the values found for the control group, while the values found using the ß-actin were inferior in the cases with controls.

V. Discussion

In this study, the control and case group patients presented similar characteristics in several aspects, such as origin, schooling, children, breast feeding, hormonal contraceptive use, hormone replacement therapy, physical activity practice, overweight, and alcohol and tobacco use habits. There was a difference between the groups on the variables, with a greater number of menopausal women (p=0.000), with breast cancer family history (p=0.000) and previous history of malignant breast disease (p=0.03) in the GCA. As for the HFCA and HPBD variables, the difference found was significant, with a larger number of women with both characteristics in the GCA, but it was not possible to do the evaluation of the associated risks because there were no women in the GCO exposed to this factor. In relation to the RCQ, this variable had been previously described in association to breast cancer, but the results found in this study did not show any association with pathology. The difference between the groups was not significant; being the GCO more exposed, this did not confer increased or reduced risks (20).

The association between the previous personal history of benign breast disease and the development of mammary neoplasia is described in the literature (21). The only variable associated with increased risks in this study was the presence of menopause (p=0.000, OR 11.14, IC 2.73 – 45.46). It has already been described in the literature the increase in the incidence of breast cancer with the woman's natural aging, mainly after 50 (22). On the studied population, 86.6% of the women from the GCA were menopausal and their average age was 53.4, while the ones from the GCO, 35% were already at menopause, the group was younger and the average age was 47.5.

The diagnosis of solid tumors, such as of the breast, is done in an invasive manner, through the surgical removal of the tumor or by the biopsy of the primary injury or metastatic sites. The possibility of using peripheral blood samples for the cancer diagnosis or for monitoring and quantification of tumor would bring comfort to the patient, convenience, expedient evaluation, because of the easy access to the material to be analyzed and the less invasive procedure for drawing of peripheral blood (23).

In this research, the fractions of free DNA in the plasma of patients with breast cancer and under healthy controls were quantified using real-time PCR.

The studies which have been previously presented used a sole marker to quantify the FLDNA. It had not been described before the use and the comparison between different genes used for this quantification in the same sample. The values found in breast cancer patients were higher in relation to the values found in patients with benign disease or control group (24, 25), even using different markers in an isolated way, as the ß-globin (24, 25) and GAPDH (26, 27), but the variety of

results found in the studies could be explained by the difference in the clinical stages among the patients or by the different methodologies used by the authors.

This study obtained values for the FLDNA dosage for the group with cancer inferior to those published. While the mean values found in this study on the GCA and the GCO were of 9.39 and 8.47 ng/ml, respectively, data from the literature showed results of 221 ng/ml and 63 ng/ml on GCA and GCO (24); and 13, 19 and 65 ng/ml on GCO, benign disease group and GCA (26). Possibly, the difference in the applied methodology could explain this result. In this study, the fluorescent composite used was the SYBR® Green, while other studies used the TaqMan®, and the primers used were the GAPDH and ß-actin; the latter was not used in patients with breast cancer.

There is no more detailed report about a marker or any other in relation to breast pathology, or to any other specific pathology. In this work, the comparison of the same samples with different markers/primers demonstrated different results in relation to the same pathology. Concerning the GAPDH, it can be inferred a better correlation between the higher values obtained in the GCA and the presence of malignant breast pathology, similar data to those present in the literature (26-28). The results found with the ß-actin do not allow this correlation because they differ from the ones demonstrated in the literature with other markers (26-28). While higher levels of patients with breast cancer were described in the literature, in this study, the values found with the use of ß-actin in the control groups were higher in relation to the women with breast neoplasia (0.060 copies/ul for the control group and 0.009 copies/ul for the cancer group, p=0.0001).

While compared with the values obtained with ß-actin and GAPDH, the use of the GAPDH for the quantification of the FLDNA demonstrated more capacity to mark the FLDNA in the patients with breast cancer. The result with the use of ß-actin was unexpected, showing the behavior of this marker opposite to those previously found with other markers.

In the applied methodology, the choice of the SYBR® Green was justified by previous efficient experiences in quantifying the free DNA fractions in other tumors, as well as the use of the ß-actin (29-31), whose results presented increased levels of these fractions in patients with urological neoplasia.

In order that works with similar results could be used to justify the application of this methodology as a diagnostic or even as a prognostic method, requiring the use of similar methodologies, among the diverse options, this is the most adequate and reproducible for this objective, since this is a method for easy population scale applications, minimally invasive and capable of aiding the physician to plan the most adequate therapy for each patient.

Further studies are necessary to prove this hypothesis, as well as for the standardization of the methodology, since positive results with the correlation of increased FLDNA with diverse neoplasia were found by several authors (4, 7, 24-26, 28, 30, 32).

VI. Conclusion

The ß-actin and the GAPDH were efficient in enlarging free fragments of plasma DNA of women with breast cancer and healthy controls. The GAPDH was the most adequate marker to identify the patients with breast cancer, because they presented higher levels of fragments of free DNA, when compared to healthy controls. The ß-actin was not considered a good biomarker, once it presented higher levels in the control group, in this research. According to the data presented, the dosage of FLDNA could not, in the light of current knowledge, be considered an irrefutable cancer diagnostic method, since one of the markers used presented a result contrary to the data previously published.

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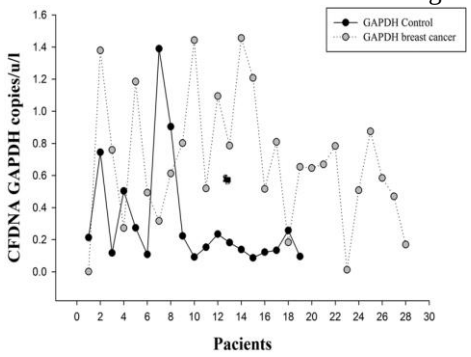
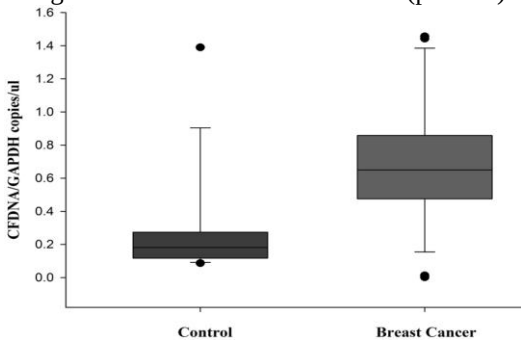
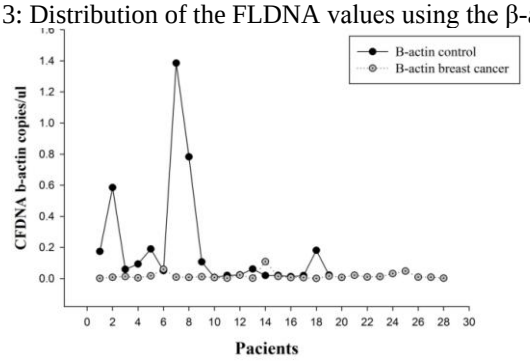
Graph 1: Distribution of the FLDNA values using GAPDH



Graph 2: Mean and standard deviation of the FLDNA values found using GAPDH for cases and controls (p <0.05).



Graph 3: Distribution of the FLDNA values using the β-actin



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