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"*BIK/NBK* Gene Expression as a Possible Marker of Circulating Breast Cancer Cells in Blood"

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Abstract: The detection of circulating breast cancer cells in blood could be of special interest as an indicator of diagnosis and prognosis, and for the selection of treatment. In a previous report, our research group determined gene expression profiles in samples of breast cancer tissue, identifying over-expression of the *BIK/NBK* mRNA gene in 90% of the analyzed samples. In this paper, we analyze the *BIK/NBK* gene expression as a possible biomarker of circulating breast cancer cells in blood. We demonstrate that the *BIK/NBK* gene expression is not a significant biomarker in the detection of circulating breast cancer cells in the blood of women with breast cancer. Several studies have evaluated the regulation of apoptosis by estrogens in breast cancer cells, demonstrating the importance of BIK/NBK protein, in estrogen-regulated breast cancer cell apoptosis, which suggests that the regulation of its expression may be an important therapeutic target or strategy in the management of cancer, and, although we did not find statistically significant differences among the patient groups to demonstrate that *BIK/NBK* gene expression is a biomarker of circulating breast cancer cells in blood, we consider it necessary to continue the study of this gene in breast cancer tissue and its role in the development and progression of breast cancer, its prognostic value, and its potential use as therapeutic target.

Keywords: *BIK/NBK*, circulating breast cancer cells, biomarkers.

INTRODUCTION

Breast cancer is a serious worldwide public health problem due to its increased incidence and mortality, and Mexico is not an exception [1]. Recent developments in genomics, proteomics, and molecular pathology have advanced the understanding of some of the mechanisms involved in the development and progression of this disease; such as alterations of growth factors and their receptors, intracellular signaling molecules, adhesion molecules, cell cycle regulators and proteases, as well as the identification of potentially valuable clinical biomarkers. The detection of circulating breast cancer cells in blood could be of special interest as an indicator of diagnosis and prognosis, and for the selection of treatment [2,3].

It has been proposed that breast cancer is a systemic disease at the time of diagnosis, in which the cancer cells acquire multiple molecular alterations that allow them to migrate through the basement membrane and extracellular matrix, to introduce into and travel through the blood system or lymphatic system to other tissues [4].

With the identification of new biomarkers, the hematogenous route would facilitate the development of a less painful, more accessible, quicker and safer molecular test to detect circulating breast cancer cells with high sensitivity molecular techniques as RT-qPCR [5,6].

Generally, the selection of new biomarkers begins with expression profiles of cancer tissue compared with non-affected tissue, and then with scientific evidence of their participation in cancer pathophysiology. To date, biomarkers for the detection of circulating breast cancer cells in blood with high sensitivity have been identified, as cytokeratin 19 *(KRT19)*, or with high specificity, as mammaglobin *(SCGB2A2*) or maspin *(SERPINB5)* [7]. Nevertheless, the translation of experimental data from basic research to clinical practice has been more difficult than expected, due to the lack of, or scarcity of, available biomarkers [8]. The detection and incorporation to the available biomarkers list of new biomarkers of circulating breast cancer cells in blood will allow us to have a more sensitive, specific, cost-effective, and accessible gene panel for the identification of cancer cells that may have predictive value.

In a previous report, our research group determined gene expression profiles in samples of breast cancer tissue, identifying BCL2-interacting killer *(BIK/NBK)* mRNA over-expression in 90% of the analyzed samples [9]; furthermore, we confirmed over-expression at protein level in 44.29% of the samples (data not published). These findings attracted our attention, since several reports suggesting that the *BIK/NBK* may serve as a pro-apoptotic tumor suppressor in specific tissues [10-12]. For example, in renal cell carcinomas *BIK/NBK* gene expression was inactivated by loss of heterozygosity in chromosome 22q13.3 and by epigenetic promoter silencing [13]; in human gliomas [14], colorectal

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cancers [15] and in head and neck cancers [16], were identified 22q chromosomal deletions; and finally, BIK/NBK gene was shown to be mutated in peripheral B-cell lymphomas [17].

Due to the participation of the *BIK/NBK* gene in cancer physiopathology and for its over-expression in a high percentage of breast cancer tissue, which we had previously identified, we decided to analyze if the expression of this gene could be a possible new biomarker which could join the list of existing biomarkers, and would allow a fast, easy, and accessible detection of circulating breast cancer cells in blood.

Initially, we determined the *BIK/NBK* gene expression by RT-PCR in the blood of women with and without breast cancer, to demonstrate if the proposed gene was or was not differentially expressed in both groups.

Later, we performed a relative quantification of the *BIK/NBK* gene expression in blood by RT-qPCR (a highly sensitive method) to identify differences in expression in a second group of women with sporadic breast cancer classified by clinical stages.

Additionally, we performed a relative quantification of the *BIK/NBK* gene expression in blood and breast cancer tissue by RT-qPCR with LUX Fluorogenic Primers in a third group of women with breast cancer, to correlate the *BIK/NBK* gene expression in different tissues. Finally, we compared the *BIK/NBK* gene expression in blood of these women with another group of women without breast cancer, using the same system.

MATERIALS AND METHODOLOGY

Patients

A total of 85 samples were analyzed. All participants were Mexican by at least three previous generations, and patients were obtained from the Breast Tumor Service, Oncology Hospital, Centro Médico Nacional Siglo XXI, IMSS.

In the first group, 28 women without breast cancer and without personal or family history of cancer [aged 23-52 years (mean age, 33.96 years; standard deviation, $\pm$ 8.30)], were compared to 11 women with sporadic breast cancer, no family history of cancer or with a different location of the primary cancer than the breast, and without previous treatment [aged 31-82 years (mean age, 58.36; standard deviation, $\pm$ 17.59)].

In the second group, we included 29 women with sporadic breast cancer classified by clinical stages, without family history of cancer or primary cancer in a different location than the breast, and without previous treatment [aged 31-80 years (mean age, 56.69; standard deviation, $\pm$ 13.56)].

In the third group, we included 11 women with sporadic breast cancer, without family history of cancer or primary cancer in a different location than the breast, and without previous treatment [aged 34-83 years (mean age, 41.50; standard deviation, $\pm$ 10.79)]. They were compared with 6 women without breast cancer, and without personal or family history of cancer [aged 28-60 years (mean age, 39.33 years; standard deviation, $\pm$ 11.86)].

Ethics

Protocols and procedures were approved by the Local Research and Ethical Committee of the Pediatrics Hospital, Centro Médico Nacional Siglo XXI, IMSS (Protocol number 2005-3603-0042); and by the National Scientific Research and Ethical Committee, Centro Médico Nacional Siglo XXI, IMSS (Protocol numbers C01-74 and 2007-785-063). Everything was performed in accordance with the Helsinki Declaration of 1975 as revised in 1983. All participants signed a letter of informed consent before any procedure.

Sample Collection

Blood Samples

A total of 8 ml of venous blood was collected from each participant in BD Vacutainer K3 EDTA tubes. To avoid inclusion of venipuncture-cored skin epithelial cells in the blood sample, the first tube was discarded. White cells were separated using Ficoll-Paque PLUS (Amersham Biosciences) according to the manufacturers instructions.

Breast Cancer Tissues

Breast cancer samples from 11 women were collected with the Vacora Breast Biopsy System (C.R. Bard, Inc) through biopsies performed for histopathologic diagnosis. Each sample was snap-frozen and transported in liquid nitrogen, and stored at -70°C until use. Samples were selected for analysis by the presence of sufficient material and histopathologic confirmation of breast cancer by two pathologists so that each sample consisted of at least 70% tumor cells.

Total RNA Extraction

Blood Samples

Total RNA extraction was performed using the RNeasy Mini Kit (QIAGEN) according to the manufacturers instructions, and eluted in *RNase-free* water for a final concentration of 100 ng/µl. RNA was stored at -70°C until use. The integrity of each sample was assessed by agarose gel electrophoresis. The purity and concentration of the samples were determined by spectrophotometry (NanoDrop 1000, Thermo Fisher Scientific).

Breast Cancer Samples

A minimum of 30 mg of breast cancer tissue was placed in a mortar with liquid nitrogen for its disruption and thoroughly ground with a pestle. The powdered tissue and liquid nitrogen were decanted into a 1.5 ml liquid nitrogen-cooled Eppendorf tube (*RNase-free*). Total RNA extraction was performed using the RNeasy Mini Kit (QIAGEN) according to the manufacturers instructions and eluted in *RNase-free* water for a final concentration of 100 ng/µl. The extracted RNA was stored at -70°C until use. The integrity of each sample was assessed by agarose gel electrophoresis. Purity and concentration were determined by spectrophotometry (NanoDrop 1000, Thermo Fisher Scientific).

cDNA Synthesis

The synthesis of cDNA was achieved from 100 ng total RNA, using the Superscript First-Strand Synthesis System (Invitrogen Life Technologies) and oligo-dT, according to the manufacturers instructions. The cDNA was stored at -70°C until use.

RT-PCR Assay

The use of hypoxanthine phosphoribosyltransferase 1 *(HPRT1)* and *BIK/NBK* primers has been previously reported [9]. A Gene Amp PCR System 9700 thermocycler was used to amplify the control and target genes in separated tubes, the *HPRT1* control gene with a 387 bp fragment length, and the *BIK/NBK* target gene with a 290 bp fragment length. The cDNA aliquots (1 µl) were used as templates for PCR in a total volume of 20 µl including with 1X Buffer (20 mM Tris-HCl (pH 8.0), 40 mM NaCl, 2 mM Sodium Phosphate, 0.1 mM EDTA, 1 mM DTT, stabilizers, 50% (v/v) glycerol), 2.5 mM $MgCl_2$ for *HPRT1* and 1.25 mM $MgCl_2$ for *BIK/NBK*, 0.2 mM of each one of the dNTPs, 20 pmol forward primer, 20 pmol reverse primer, and 1.0 UI Taq polymerase. The mixtures were pre-incubated at 94ºC for 5 min. and then 45 cycles of three-step incubation: denaturing at 94ºC for 20 sec., alignment at 58ºC for 1 min. (*HPRT1*) and 55ºC for 1 min. (*BIK/NBK*) and elongation at 72ºC for 1 min.; followed by a final elongation phase at 72ºC for 7 minutes. As negative control, the cDNA of MCF-7 cellular line, which does not express the *BIK/NBK* gene was used, and as positive control the cDNA of breast cancer tissue, which expresses *BIK/NBK* gene was used. Amplification products were analyzed by agarose gel electrophoresis.

RT-qPCR Assay

A Real Time Amplification Rotor-Gene 3000 (Corbett Research) was used to amplify the control gene and the target gene using the previously described primers, in separate reactions. The cDNA aliquots (1 µl) were used as templates for qPCR in a total volume of 25 µl including 2X Platinum SYBR Super Green qPCR Mix-UDG (Invitrogen Life Technologies), 4 mM $MgCl_2$ for *HPRT1* and 6 mM $MgCl_2$ for *BIK/NBK*, 200 nM forward primer, and 200 nM reverse primer. The mixtures were pre-incubated at 94ºC for 5 min. and then 45 cycles of three-step incubation were performed; denaturing at 94ºC for 20 sec., alignment at 58ºC for 59 sec. (*HPRT1*) and 56ºC for 59 sec. (*BIK/NBK*), elongation at 72ºC for 59 sec. and signal acquisition, followed by melting curves analysis (67-95ºC with a ramp time of 1ºC/step, wait 45 sec. on first step and 5 sec./step after). All reactions were performed in triplicate. Relative quantification was performed with the Rotor-Gene 6.0.19 Software (Corbett Research) for the Δ_{CT} method [18].

RT-qPCR Assay with LUX Fluorogenic Primers

LUX fluorogenic primers for PCR amplification of *HPRT1*, *BIK/NBK*, *KRT19* and *SCGB2A2* genes (to validate the results), were designed by the D-LUX Designer program (Invitrogen life Technologies). Sequences of the primers are as follows:

HPRT1: 5´GCTGAGGATTTGGAAAGGGTGT3´, JOE-labeled 5´cgagatCCATCTCCTTCATCACATCTcG3´;

BIK/NBK: 5´CCTGAGGCTCACGTCCATCTC3´, FAM-labeled 5´cgaagtGGACCCTATGGAGGACTTcG3´;

KRT19: 5´CCCGCGACTACAGCCACTACT3´, FAM-labeled 5´cgaacTCCGTCTCAAACTTGGTTcG3´;

SCGB2A2: 5´CACTTGTGGATTGATTGTCTTGGA3´, FAM-labeled 5´gtagcaCCCTCTCCCAGCACTGCcAC3´.

A Real Time Amplification Rotor-Gene 3000 (Corbett Research) was used to amplify the *HPRT1* gene in a diplex reaction with the *BIK/NBK* or *KRT19* or *SCGB2A2* genes. cDNA aliquots (1 µl) were used as templates for qPCR in a total volume of 25 µl with 2X Super Platinum qPCR Mix-UDG (Invitrogen Life Technologies), 3 mM $MgCl_2$, 50 nM LUX labeled primer of *HPRT1*, 50 nM LUX unlabeled primer of *HPRT1*, 100 nM LUX labeled primer, and 100 nM LUX unlabeled primer of *BIK/NBK* or *KRT19* or *SCGB2A2*. The mixtures were pre-incubated with the following conditions: UDG Reaction at 50°C for 2 min., UDG inactivation/denaturation at 95°C for 2 min., and then 40 cycles of three-step incubation: denaturing at 95°C for 5 sec., alignment 55°C for 15 sec. with signal acquisition and elongation at 72°C for 10 sec.; followed by melting curves analysis (60-95°C with a ramp time of 1°C/step, wait 60 sec. on first step and 5 sec./step after). All the reactions were performed in triplicate. Relative quantification was performed with the Rotor-Gene 6.0.19 Software (Corbett Research) for the Δ_{CT} method.

Statistical Analysis

In the first group, the *BIK/NBK* gene expression was measured using a nominal scale by the presence or absence of the expected amplification fragment. To determine expression differences between women with and without breast cancer, a Fisher Exact Test was performed.

In the second group, the *BIK/NBK* gene expression was measured using a quantitative scale. To determine expression differences among women with breast cancer at different clinical stages, a Krhuskall-Wallis Test was performed.

In the third group, the *BIK/NBK* gene expression was measured using a quantitative scale. To determine expression correlation between blood and breast cancer tissue in women with breast cancer, a Spearman Correlation Test was performed.

To determine expression differences between women with and without breast cancer, a Mann-Whitney U Test was performed.

A P-value of < 0.05 was considered statistically significant. All statistical analyses were performed using SPSS for Windows v12.0 (SPSS Inc., Chicago, IL).

RESULTS

Determination of the BIK/NBK Gene Expression by RT-PCR in Blood Samples from Women with and without Breast Cancer

RT-PCR assays were performed on 39 blood samples from women with breast cancer (11 samples) and without breast cancer (28 samples). The transcript of the *HPRT1* gene was found in all samples. Only one blood sample from a woman with breast cancer amplified the *BIK/NBK* transcript (data not shown). For statistical analysis of expression differences among the groups, a Fisher Exact Test was performed, obtaining a p=0.282.

Relative Quantification of BIK/NBK Gene Expression by RT-qPCR in Blood Samples from Women with Breast Cancer in Different Clinical Stages

RT-qPCR assays were performed on 29 blood samples from women with breast cancer in different clinical stages.

Table 1. Absolute and Relative Frequency of Women with Breast Cancer at Different Clinical Stages

Clinical Stage	Absolute Frequency	Relative Frequency
I	6	20.7
IIA	8	27.6
IIB	7	24.1
IIIA	3	10.3
IIIB	2	6.9
IV	3	10.3
Total	29	100

The absolute and relative frequencies are shown in Table **1**, with the *HPRT1* and *BIK/NBK* genes expressed in all samples. In Figs. (**1** and **2**) the amplification curves and melting curves analysis of the two genes are shown. Finally, a relative quantification of the *BIK/NBK* gene expression by the Δ_{CT} method was performed. For statistical analysis of expression differences among women at different breast cancer clinical stages, a Kruskall-Wallis Test was performed, obtaining a p=0.469.

Relative Quantification of BIK/NBK Gene Expression by RT-qPCR in Breast Cancer Tissue and its Correlation with BIK/NBK Gene Expression in Blood Samples from Women with Breast Cancer

RT-qPCR in 11 breast cancer tissue and respective blood samples using LUX Fluorogenic Primers was performed. The *HPRT1* and *BIK/NBK* genes were expressed in all breast cancer tissue and blood samples. For statistical analysis of correlation among the breast cancer tissue and blood *BIK/NBK* gene expression (Fig. **3**), a Spearman Correlation Test was performed, obtaining a p=0.278.

Relative Quantification of BIK/NBK Gene Expression by RT-qPCR in Blood of Women with and without Breast Cancer

We performed RT-qPCR assays in 17 blood samples from women with breast cancer (11 samples) and without breast cancer (6 samples), using LUX Fluorogenic Primers. *HPRT1* and *BIK/NBK* genes were expressed in all samples. For statistical analysis of expression differences between these groups, a Mann-Whitney U Test was performed, obtaining a p=0.615.

Additionally, in these last samples, the *KRT19* and *SCGB2A2* gene expressions were measured with LUX Fluorogenic Primers. In women with breast cancer, we found both genes expressed in all breast tissue samples and almost all blood samples. On the other hand, we did not find the expression of either gene in any of the blood samples from women without cancer.

DISCUSSION

Breast cancer constitutes a worldwide public health problem [1]. In Mexico, this neoplasia occupies first place as the cause of death in women 40 years old and older [19]. One of the factors that has a considerable influence on the prognosis and life expectancy of these patients is the clinical stage at which the disease is diagnosed [20].

In recent years, several studies have been performed to identify new molecular methods and biomarkers with potential clinical use for the detection of circulating breast cancer cells in blood in the early stages of the disease [5]. Due to considerable limitations that have been observed with immunohistochemistry and flow cytometry, the use of high

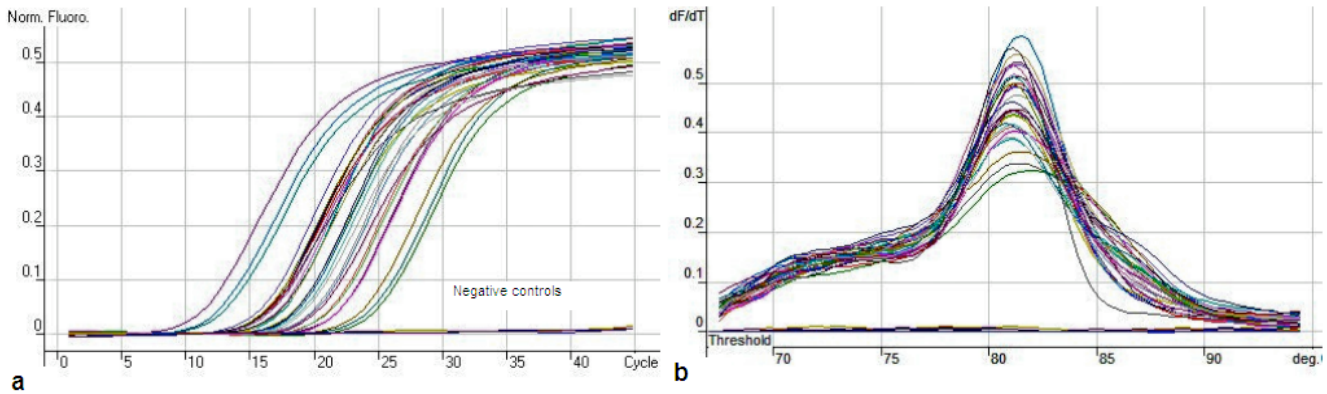

Fig. (1). qPCR of *HPRT1* gene expression in 29 blood samples of women with breast cancer. a) Amplification curves of *HPRT1* control gene and three negative controls (without cDNA). The abscissa shows fluorescence relative units and ordinate cycle number. **b)** Melting curves analysis of *HPRT1* control gene and three negative controls. The fluorescence signal (F) was monitored continuously during the temperature ramp and then plotted against the temperature (*T*). These curves were transformed to derivative melting curves [(2dF/d*T*) vs *T*]. Graphic generated by Rotor Gene 3000 real time PCR thermocycler (Corbett Research).

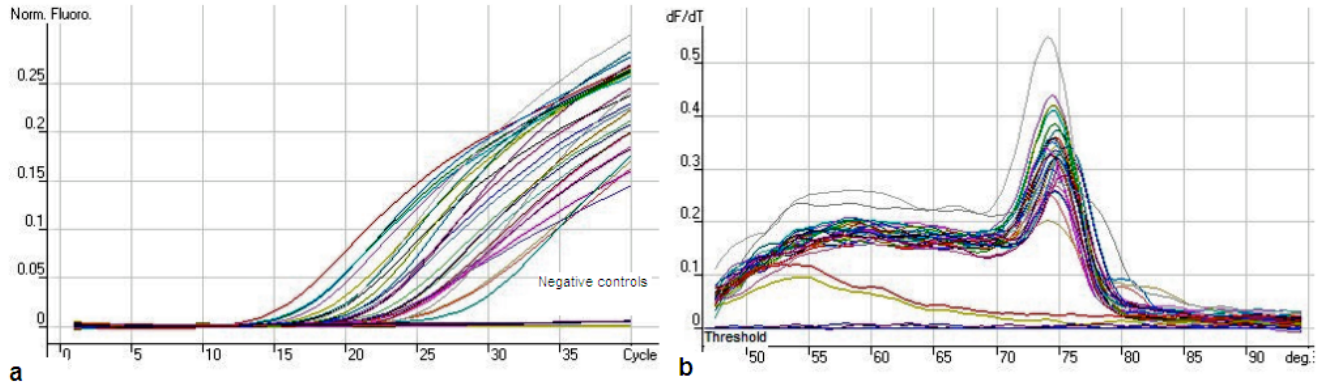

Fig. (2). qPCR of *BIK/NBK* gene expression in 29 blood samples of women with breast cancer. a) Amplification curves of *BIK/NBK* target gene and three negative controls (without cDNA). The abscissa shows fluorescence relative units and ordinate cycle number. **b)** Melting curves analysis of *BIK/NBK* target gene and three negative controls. The fluorescence signal (F) was monitored continuously during the temperature ramp and then plotted against the temperature (*T*). These curves were transformed to derivative melting curves [(2dF/d*T*) vs *T*]. Graphic generated by Rotor Gene 3000 real time PCR thermocycler (Corbett Research).

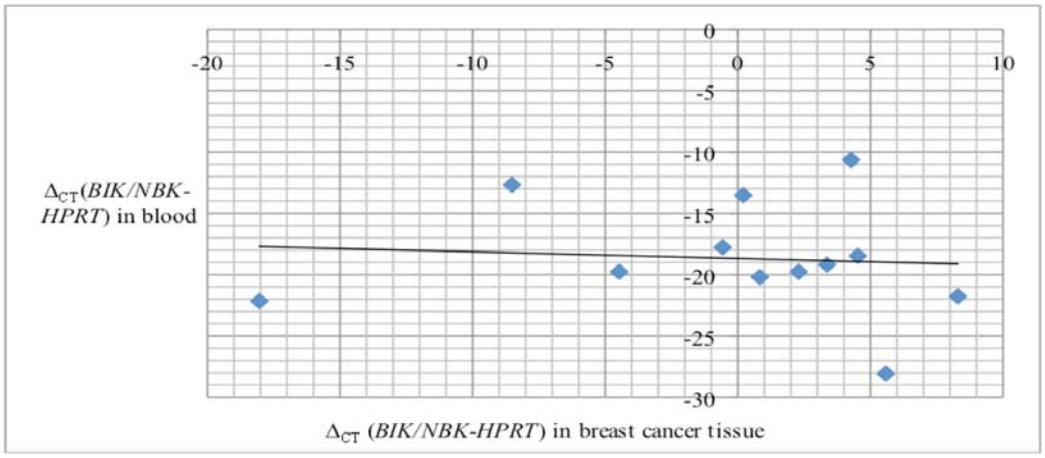

Fig. (3). Spearman Correlation Test among the Δ_{CT} (*BIK/NBK-HPRT*) in blood and the Δ_{CT} (*BIK/NBK-HPRT*) in breast cancer tissue of 11 women with breast cancer. Dispersion graph of Δ_{CT} (*BIK/NBK-HPRT*) in blood and Δ_{CT} (*BIK/NBK-HPRT*) in breast cancer tissue. We did not find significant correlation (p=0.278).

sensibility molecular techniques, as RT-qPCR, has been preferred for the detection of specific biomarkers of circulating breast cancer cells in blood. However, although multiple gene expressions have been proved, only some of them, such as *KRT19*, *SCGB2A2* and *SERPINB5*, have been identified with greater sensibility and specificity for the detection of circulating breast cancer cells in blood [7,21-25]. The need for new biomarkers to improve the detection of circulating breast cancer cells in blood led us to search for new molecules with potential clinical use. We decided to determine if the *BIK/NBK* gene expression (which we observed as over-expressed in the mRNA and protein levels) could be used as a biomarker for the detection of circulating breast cancer cells in blood [20].

We initially determined what was the *BIK/NBK* gene expression by RT-PCR in blood samples from women with and without breast cancer. All the samples expressed *HPRT1* gene control, demonstrating an appropriate cDNA synthesis and amplification system. Regarding the *BIK/NBK* gene, we found its expression in only one of the women with breast cancer. Since none of the samples of non-cancerous women had the *BIK/NBK* gene expression, and the statistical test did not show significant differences between the groups, this result led us to think that the detection system used had not been sufficiently sensitive or that patients could be in an incipient or early clinical stage. Therefore, we decided to determine the *BIK/NBK* gene expression with a more sensitive molecular technique in blood samples from women with sporadic breast cancer, taking into consideration the clinical stage of each one [21].

A relative quantification of the *BIK/NBK* gene expression by RT-qPCR in blood samples from women with breast cancer classified by clinical stages, using *HPRT1* as gene control was performed. Surprisingly, all the samples amplified both genes, and when we determined the differences in gene expression among women with breast cancer in different clinical stages, we did not find significant differences. One of the possible reasons why differences were not found could be that not all the analyzed blood samples had *BIK/NBK* gene over-expression because not all tumors had this gene

over-expressed. This result motivated us to try the LUX Fluorogenic Primers system, a molecular methodology that has been reported with high specificity detection of amplification products [26], in another group of breast cancer patients to determine the correlation of the *BIK/NBK* gene expression between breast cancer tissue and blood. We expected that the women with a higher expression in breast cancer tissue also would present a higher expression in their blood. However, we did not find a statistically significant correlation in the *BIK/NBK* gene expression in either type of analyzed tissues from women with breast cancer, which indicated that the expression of this gene in blood is apparently independent from the expression of the gene in breast cancer tissue.

Finally, to confirm the possible function of *BIK/NBK* gene expression as a biomarker of circulating breast cancer cells in blood by means of a high sensibility molecular technique (RT-qPCR) and a system with high specificity detection of amplification products (LUX Fluorogenic Primers system), we compared the *BIK/NBK* gene expression in 11 blood samples from women with breast cancer, with the expression in 6 blood samples from women without breast cancer. The unexpected result was contrary to what has been previously reported [12] and to what we observed by RT-PCR. We found that all blood samples from women with and without cancer expressed both genes, the *HPRT1* control gene and the *BIK/NBK* target gene. The statistical test did not find significant differences between both groups, which indicated that *BIK/NBK* gene has a basal level expression in blood.

Additionally, to validate that our results originated from epithelial breast cells, we determined the *KRT19* and *SCGB2A2* gene expressions, finding that both genes were expressed in all breast cancer tissues, and in some blood samples. On the other hand, we did not find both genes expressed in any blood samples from women without breast cancer [7,23,25].

CONCLUSION

We did not find statistically significant differences among the patient groups to demonstrate that *BIK/NBK* gene expression is a biomarker of circulating breast cancer cells in blood. A statistical significance might be found with a larger number of samples, nevertheless the need of biomarkers with high sensibility and specificity demands significant differences, even among a small number of patients. However, we consider it necessary to continue the study of this gene in breast cancer tissue and its role in the development and progression of breast cancer, its prognostic value, and its potential use as therapeutic target.

Although several reports suggest that *BIK/NBK* may serve as a pro-apoptotic tumor suppressor gene in specific tissues [12-17], correlation of *BIK/NBK* high expression with poor prognosis of non-small cell lung cancer (NSCLC) [27] has also been reported. Over-expression of another BH3-only member, *BNIP3* has been reported to be associated with poor prognosis in breast cancer [28] and in NSCLC [29]. This might suggest that there is an adaptive phenomenon of tumor cells despite the over-expression of proapoptotic genes and subsequent progression towards aggressive tumor behavior [30].

Several studies have evaluated the regulation of apoptosis by estrogens in breast cancer cells, demonstrating the importance of the mitochondrial dependent apoptotic or intrinsic pathway which involves the BCL-2 family of proteins, and among these BIK/NBK, in estrogen-regulated breast cancer cell apoptosis, which suggests that the regulation of its expression may be an important therapeutic target or strategy in the management of cancer [31, 32].

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS CONTRIBUTIONS

ELM participated in the design of the study, carried out qPCR assays, performed statistical analysis and drafted the manuscript. NGH participated in the analysis and interpretation of data, carried out qPCR assays and revising critically the manuscript for important intellectual content. RIPE participated in the collection of non breast cancer samples and acquisition of data. MEGT and GZE participated in the collection of breast cancer samples and acquisition of data. FSG and DJAA participated in the conception and design of the study and revising critically the manuscript for important intellectual content. All authors read and approved the final manuscript.

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