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Evaluation of *ER*, *HER2* and *SPATA19* Genes Expression in Clinical Samples of Breast Cancer Using NASBA-ELISA

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ABSTRACT

The most important issue in diagnosing human breast cancer is the ability to detect the early stages of cancer, which can help the healing process. Therefore, new diagnostic methods for breast cancer focus on molecular approaches. The aim of this work is to develop the NABA-ELSA method for the diagnosis of breast cancer in clinical samples, which is used for the first time to evaluate *HER2*, *ER* and *SPATA19* biomarkers simultaneously. The possibility of expressing the *SPATA19* gene, a specific biomarker for testis cancer, was also investigated in breast cancer. Specific primers and biotinylated probes were designed separately for each of the target genes. RT-PCR and NASBA reactions were done on total RNA extracted from the clinical tumor tissues. The DIG-labeled NASBA product was detected by the ELISA method using the biotinylated probe and anti-DIG antibody-enzyme conjugate. The ELISA reaction showed obvious color change and significant absorption in the positive samples. Of 15 samples tested; 11, 4 and 3 samples were positive for *ER*, *HER2* and *SPATA19* genes, respectively. Comparison of real-time RT-PCR with NASBA-ELISA showed the same results for both *ER* and *HER2* genes. There was no significant relationship in the expression of the *SPATA19* gene with *ER* and *HER2* genes in these specimens. Also, no significant relationship was observed between *SPATA19* gene expression and breast cancer in these samples. In this study, we developed a simple, fast, and reliable NASBA-ELISA method that can detect cases of breast cancer.

Keywords: Breast cancer, Molecular diagnosis, NASBA-ELISA, Tumor biomarker

INTRODUCTION

Human breast cancer is the most typical malignity among middle-aged women [1]. There are a lot of methods for the diagnosis of breast cancer in different stages of the disease. The critical point in this field is the time of detection or in other terms the ability to detect the initial stages of breast cancer to decrease the progression of cancer [2,3]. Some applicable methods in the field of diagnosis the breast cancer patient are physical examination methods such as mammography, mammaprint, and blueprint technologies [4-7]. The result of these techniques is sure, but these methods have two disadvantages first these techniques analyze the patients that have cancer symptoms and the other disadvantage of these methods is the price for each reaction. Today, improvements in molecular biology caused a new revolution in diagnosis methods in different fields such as the detection of virulence factors in infectious diseases, and the identification of biomarkers in different diseases like cancer [7,8]. Tumor biomarkers are recognized as important factors in the diagnosis of cancers. Tumor markers are protein-based or nucleic acid-based that are found in the urine or blood of the patient which may be generated by the tumor itself or by the response of the immune system to the tumor. Tumor markers such as human epidermal growth factor receptor 2 (*HER2*) and estrogen receptor (*ER*) play an important role in the rapid diagnosis of breast cancer and its origin, tracking patient treatment in response to treatment, and selecting alternative therapies [8-12]. For example, *ER* is an appropriate biomarker to identify patients who respond positively to hormone therapy. Spermatogenesis-associated protein 19 (*SPATA19*) is a specific testicular gene that belongs to the TSG family that is another biomarker in cancer [13]. *SPATA19* is defective in normal breast tissue and is

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expressed in some types of breast tumors, so recognizing this biomarker is important in the diagnosis of breast cancer [14]. These biomarkers could be detected by some molecular methods such as quantitative polymerase chain reaction (qPCR) [15], fluoresce in situ hybridization (FISH) [16], and immune histochemistry (IHC) [17]. These methods detect biomarkers on the level of RNA expression. The quantitative PCR method needs an expert operator and a real-time thermocycler device which is expensive. Therefore, other isothermal molecular methods such as Loop-mediated isothermal amplification (LAMP) or nucleic acid sequence-based amplification (NASBA) can be used due to the low cost per reaction and the lack of dependence on a specific laboratory. The LAMP method is an isothermal amplification method used as a diagnostic tool for different goals [18-22]. The NASBA technique is an isothermal, transcription-based amplification system specifically designed for the detection of RNA and also DNA targets [20,23-25]. NASBA-ELISA is a derivative approach from the NASBA method which is more sensitive than a single ELISA or NASBA reaction. This technique was used for the diagnosis of rotaviruses and carp viruses by Jean et.al and Zeng *et al*., respectively [26,27].

The aim of this study was to evaluate the expression of breast cancer biomarkers in clinical specimens in order to develop the NASBA-ELISA method for breast cancer diagnosis. Therefore, the expression of tumor biomarkers *SPATA19*, *HER2* and *ER* in breast cancer samples compared to healthy samples has been investigated using the NASBA-ELISA method. A specific hybridization reaction with oligonucleotide probes was used to increase the sensitivity of the approach at the detection stage by using the ELISAn method for the detection. For this, the digoxigenin molecule is used to label the NASBA products. After that, these products were evaluated by hybridization between a biotin-labeled oligonucleotide probe and streptavidin-coated ELISA plates.

MATERIAL AND METHODS

Sample Preparation

In this study, 15 breast tumor tissues and 15 breast healthy tissue samples were obtained from the tumor bank of Imam Khomeini Hospital. Also, 3 testis tumor tissue was taken to confirm the function of the *SPATA19* primers.

RNA Extraction and RT-PCR Amplification

Total RNA was extracted by using the GeneJET™ RNA Purification Kit (Fermentas) according to the manufacturer's protocol. Extracted RNAs were treated with DNaseI (Fermentas) at 37 °C for 30 min, to remove contaminating DNA. For the inactivation of DNaseI, the preparation was cleaned up according to the kit protocol. Then, 1 µg from each total RNA was reverse-transcribed with RevertAid™ First strand cDNA synthesis Kit (Fermentas) using random hexamer primers (Fermentas). Reverse transcription was performed in a thermal cycler (Techne) with a total reaction volume of 20 µl at 37 °C for 90 min and followed by 90 °C for 5 min. For confirmation of the target genes primers, RT products were amplified using their specific primers (Table 1) in a thermal cycler with a total reaction volume of 10 µl at 94 °C for 5 min, followed by 30 cycles containing 45 s at 95 °C and 45 s at 65 °C and 45 s at 72 °C, and 5 min at 72 °C for the final extension. All PCR products were analyzed by 2% agarose gel electrophoresis.

Primers and Probes Designining for NASBA Reaction

In this study, 3 sets of primers and 3 DNA probes were designed to perform NASBA reactions (Table 1). For the NASBA reaction, it is necessary to design two specific primers from every target sequence. The first primer, known as P1, is a downstream primer that reacts in the first step and binds to the 3′ end of the RNA molecule to initiate the cDNA synthesis by the RT enzyme. P1 primer contains a 28-nucleotide locus on the 5′ end as the T7 RNA Polymerase promoter region. The second primer, named P2, is an upstream primer that reacts in the third step of the reaction and binds to the 3′ end of cDNA to initiate the synthesis of the second strand cDNA by the RT enzyme. For designing specific primers, firstly the mRNA of the target sequences of *SPATA19* (XR_428970.1), *HER2* (XR_426961.1) and *ER* (XM_006721768.1) tumor markers were retrieved from Gene Bank. After multiple alignments of the target sequences using mega 6 Blast (Molecular Evolutionary Genetics Analysis Version 6.0) offline software, the specific primers, and probes were designed according to the target sequences within the regions that had no homology with other known genes in Gene Bank, and analyzed using the Primer Express software v.3.0 (Applied Biosystems) and Gene Runner

Table 1. The Primers and Probes Used in this Study

Target name	Primer sequence (5′→3′)	Primer length (bp)
P1-SP19	AATTCTAATACGACTCACTATAGGGAGAAGGAGG ACTTAGAGAGGTGGTGCTTCA	55
P2-SP19	GGAAAGTGAGGCTGTGTCTGTA	22
Probe-SP19	CAGGGTGTAAGGGAGAAGATGTC	23
P1-ER	AATTCTAATACGACTCACTATAGGGAGAAGGA AGAGCATTGTGGCCATTGTCA	53
P2-ER	GCCTGAAGGATGCTGTACCA	20
Probe-ER	CTCCTCGTGGGGGTCAAGTC	20
P1-V-ERB-B2	AATTCTAATACGACTCACTATAGGGAGAAGGA AGGGCTTGCTGCACTTCTCA	52
P2-V-ERB-B2	AGGGCTTGCTGCACTTCTCA	20
Probe-V-ERB-B2	CCCTACAACTACCTTTCTACGGAC	24

software v.3.0 (Hastings Software, Inc). The primers were synthesized by Bioneer Company (South Korea).

NASBA Amplification

First, the NASBA reaction mixture was prepared in a total volume of 25 µl containing 5 µl of total RNA, 300 mM $MgCl_2$, 1.25 M KCl, 1 M Tris-HCl (pH = 8.5), 23 mM DTT, 34.5% (v/v) DMSO, 2.3 mM each dNTP, 4.6 mM each NTP, 0.2 mM Digoxigenin-11-UTP, and 5 pmol each primer. The reaction mixture was incubated at 65 ºC for 10 min and immediately transferred onto the ice. Then, an enzyme mixture containing 2.6 µg of BSA in 50% glycerol, 0.2 U RNaseH, 40 U T7 RNA polymerase, and 8 U AMV-RT, was added into the first mixture, and the resulting reaction was incubated at 41 ºC for 150 min. NASBA products were analyzed by 2% agarose gel electrophoresis.

Detection of NASBA Products Using the ELISA Method

In this study, three biotinylated probes were designed according to the three target genes. To perform the ELISA method, a dilution of 10 pM in 100 µl of the probe was prepared and incubated at 4 °C for 16 h. The blocking process was done with BSA at 37 °C for 1 h after the washing step. After washing, 2.5 µl from each NASBA DIG-labeled product of the tumor and the healthy samples were mixed with 97.5 µl of the hybridization solution and each one was added to the wells, separately. Then, the ELISA plate was incubated at 49 °C for three hours. After washing, 100 µl of the conjugated anti-Digoxigenin-HRP antibody was diluted 1:1000 in PBS solution and added to each well, and placed on a shaker at room temperature for 40 min. Then, the washing step was repeated and 100 µl of H_2O_2 substrate containing TMB chromogen was added to each well and placed at room temperature for 20 min in a dark room. Finally, 100 µl of stop buffer was added to each well and the absorbance was determined for each sample at 450-630 nm.

Statistical Analysis

The statistical analysis was performed using SPSS software, v.19 (SPSS Inc, USA) and Microsoft Excel 2010 (Microsoft corp.). The *P*-value of <0.01 was considered statistically significant for the student's *t-test* analysis.

RESULTS

RT-PCR and NASBA Amplification

Total RNA was extracted from tumor and healthy tissue samples and used to determine the appropriate reaction conditions and evaluate the RT-PCR and NASBA assays. The size of RT-PCR and NASBA products were 223, 233, and 238 nucleotides for *ER*, *HER2,* and *SPATA19* biomarker

genes, respectively. Agarose gel electrophoresis stained by ethidium bromide was used for the detection of the RT-PCR (Fig. 1a) and NASBA products (Fig. 1b). As shown in Fig. 1a, the expected fragments have been amplified in the RT-PCR reaction using P1 and P2 primers for *SPATA19* biomarker from testis tumor tissue (lane 1), and *ER* and *HER2* biomarkers (lanes 2 and 3, respectively) from breast tumor tissue. Also, as shown in Fig. 1b, the expected fragments have been amplified in the NASBA reaction using P1 and P2 primers for *ER*, *HER2,* and *SPATA19* biomarkers (lanes 4, 5, and 6, respectively) from tumor tissue, and any fragment from breast healthy tissues (lanes 1, 2 and 3).

Detection of NASBA Products Using the ELISA Method

The NASBA products were analyzed by using the ELISA method based on the interaction of streptavidin-coated in microplate and specific biotinylated-probes, and the detection of DIG-labeled NASBA products hybridized with specific probes. The results were visualized by a color-shifting of the reaction mixtures in positive samples. In the next step, samples were used to determine the absorbance at 450-630 nm (Table 2). The mean absorbance was obtained from duplicate reactions in two independent assays. As shown in Fig. 2, the expected results have been obtained in

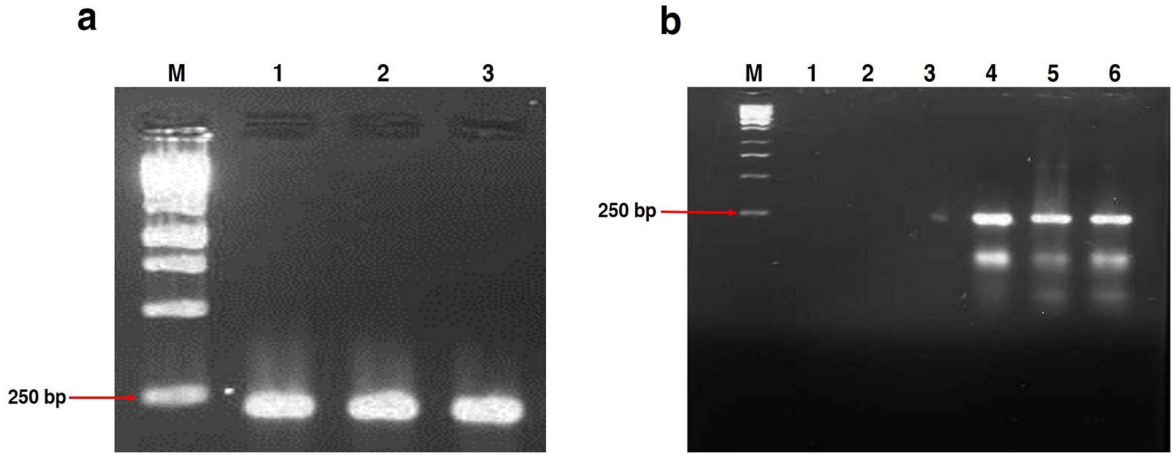

Fig. 1. Analysis of the RT-PCR (a) and NASBA (b) products in 2% agarose gel electrophoresis. M: 1kb DNA ladder.

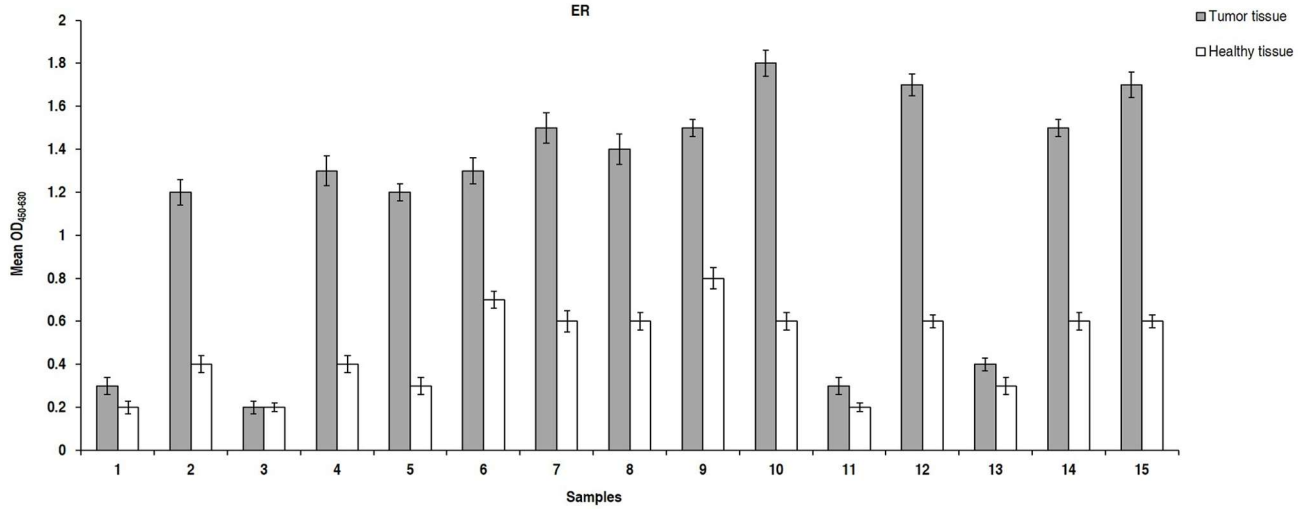

Fig. 2. Analysis of the mean absorbance at 450-630 nm for NASBA-ELISA product of *ER*. The mean absorbance was obtained from duplicate reactions of two independent assays.

Table 2. Comparison of the Results of Real Time RT-PCR and NASBA-ELISA

Sample	Biomarker	Mean $OD_{450-630}$[a]		Assay	
		Tumor tissue	Healthy tissue	RT-PCR[b]	NASBA-ELISA
1	ER	0.3	0.2	-	-
	HER2	1.6	0.4	+	+
	SPATA19	0.2	0.1	NA	-
2	ER	1.2	0.4	+	+
	HER2	0.3	0.3	-	-
	SPATA19	1.1	0.2	NA	+
3	ER	0.2	0.2	-	-
	HER2	0.2	0.2	-	-
	SPATA19	0.2	0.1	NA	-
4	ER	1.3	0.4	+	+
	HER2	1.8	0.4	+	+
	SPATA19	0.4	0.2	NA	-
5	ER	1.2	0.3	+	+
	HER2	0.2	0.2	-	-
	SPATA19	0.3	0.2	NA	-
6	ER	1.3	0.7	+	+
	HER2	0.3	0.4	-	-
	SPATA19	0.4	0.2	NA	-
7	ER	1.5	0.6	+	+
	HER2	0.4	0.4	-	-
	SPATA19	0.2	0.1	NA	-
8	ER	1.4	0.6	+	+
	HER2	0.3	0.3	-	-
	SPATA19	0.2	0.2	NA	-
9	ER	1.5	0.8	+	+
	HER2	0.3	0.4	-	-
	SPATA19	0.3	0.2	NA	-
10	ER	1.8	0.6	+	+
	HER2	0.4	0.3	-	-
	SPATA19	1.2	0.2	NA	+
11	ER	0.3	0.2	-	-
	HER2	2	0.4	+	+
	SPATA19	0.2	0.1	NA	-
12	ER	1.7	0.6	+	+
	HER2	0.3	0.3	-	-
	SPATA19	0.4	0.2	NA	-
13	ER	0.4	0.3	-	-
	HER2	2.2	0.4	+	+
	SPATA19	1.2	0.3	NA	+
14	ER	1.5	0.6	+	+
	HER2	0.3	0.3	-	-
	SPATA19	0.2	0.1	NA	-
15	ER	1.7	0.6	+	+
	HER2	0.4	0.3	-	-
	SPATA19	0.3	0.2	NA	-

[a]The mean absorbance was obtained from duplicate reactions of two independent assay, *t* test: ($p < 0.01$). [b]Results were obtained from the tumor bank of Imam Khomeini Hospital. NA: Not available.

NASBA-ELISA reactions for *ER* biomarker from positive breast tumor tissues (samples 2, 4-10, 12, 14, and 15), and negative breast tumor tissues (samples 1, 3, 11 and 13) and breast healthy tissues (samples 1-15). Also, as shown in Fig. 3, the expected results have been obtained in NASBA-ELISA reactions for *HER2* biomarker from positive breast tumor tissues (samples 1, 4, 11, and 13), and negative breast tumor tissues (samples 2, 3, 5-10, 12, 14 and 15) and breast healthy tissues (samples 1-15). These results were completely consistent with the results obtained from the real-time RT-PCR in the tumor bank of Imam Khomeini Hospital (Table 2). NASBA-ELISA results were unexpected for the *SPATA19* biomarker. As shown in Fig. 4, positive results have been obtained in NASBA-ELISA reactions for *SPATA19* biomarker from breast tumor tissues (samples 2, 3 and 10), and negative results from breast tumor tissues (samples 1, 4-9 and 11-15) and breast healthy tissues (samples 1-15), respectively. As can be seen, in the positive results of all three biomarkers, there is a significant difference between tumor tissue and healthy tissue samples ($P < 0.01$).

DISCUSSION

Today, breast cancer is the most incident malignity in

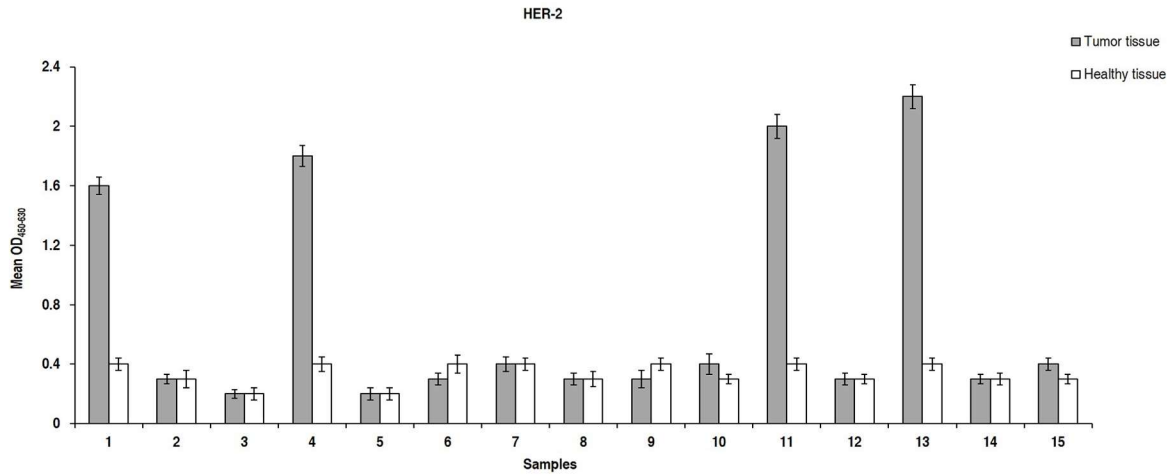

Fig. 3. Analysis of the mean absorbance at 450-630 nm for NASBA-ELISA product of *HER2*. The mean absorbance was obtained from duplicate reactions of two independent assays.

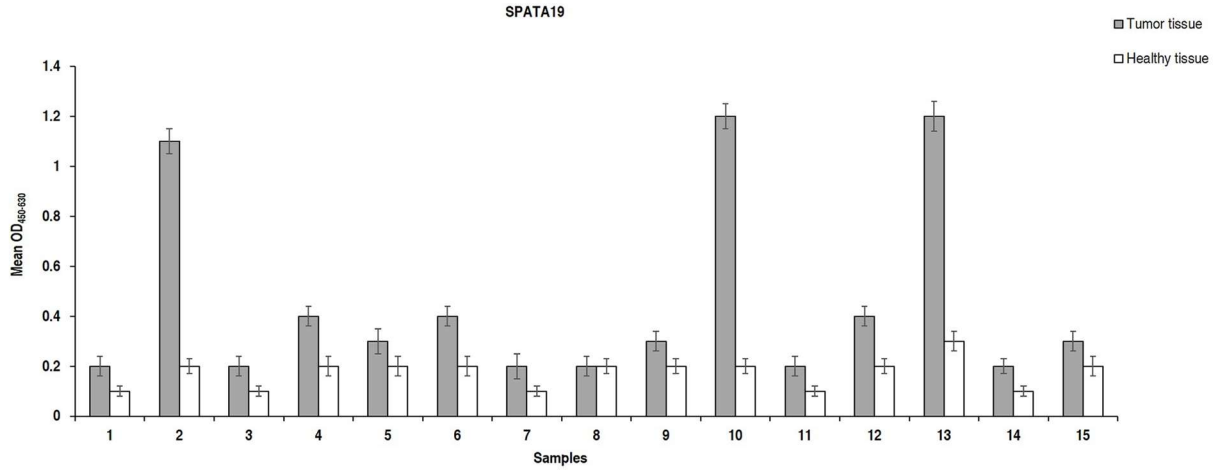

Fig. 4. Analysis of the mean absorbance at 450-630 nm for NASBA-ELISA product of *SPATA19*. The mean absorbance was obtained from duplicate reactions of two independent assays.

women and its curing process is closely related to the diagnosis time. The methods of diagnosis the breast cancer such as mammography need special and expensive devices with expert personnel for testing patients and checking the results. Therefore, novel breast cancer therapies focus on the diagnosis using molecular methods and breast cancer biomarkers. Molecular diagnosis of breast cancer has been reviewed in several reports [28,29,30,31]. In these studies, a number of molecular diagnosis methods are based on the nucleic acids amplification using breast cancer biomarkers such as *ER* and *HER2*. PCR and its derivatives techniques such as real time PCR are considered the "gold standard" in molecular analysis of nucleic acids. Considering that the mentioned methods are expensive and require equipment, it is necessary to use simple molecular detection methods such as NASBA technique. Today, few reports have been presented for the molecular detection of biomarkers in breast cancer using NASBA technique. Verjat *et al.* have developed multiparametric duplex real-time NASBA assays to detect mRNA coding for the estrogen receptor α (*ESR1*) and the progesterone receptor (*PGR*) in breast tumors. They stated that the NASBA technique can be a suitable tool for analyzing the expression of genes in small clinical samples and preparing an expression profile for each gene [32]. Lami and coworkers have evaluated the mRNA expression of three clinically relevant markers estrogen receptor a (*ERa*), progesterone receptor (*PR*) and human epidermal growth factor receptor (*ERBB2*) in breast cancer clinical samples using the NASBA technique. They were able to amplify and identify three markers with as little as 3 × 50 ng of total RNA [33]. Their results showed that NASBA is suitable for evaluating the status of ER, PR and ERBB2 in breast tumor samples and is a fast, sensitive and standard approach complementary to existing techniques in small tumors. In other study, Lami and colleagues have investigated the expression of urokinase plasminogen activator (*uPA*) and and its main inhibitor, plasminogen activator inhibitor type-1 (*PAI-1*) in 77 clinical samples of breast cancer using duplex real-time NASBA method. They were able to quantify gene expression for these markers using as little as 50 ng of total RNA per reaction [34]. Kijanka *et al.* have demonstrated a novel biological microprocessor for highly efficient cell capture, culture, and analysis that provides a versatile platform for a wide range of molecular and cell biology methods. They assessed the expression of the estrogen receptor alpha (*ESR1*) marker in the MCF7 breast cancer cell line based on real-time NASBA and used fluorescent staining to analyze protein expression [35]. Their device was able to run an integrated real time NASBA protocol from as few as five cells. Riehle and his colleagues have used the NASBA technique to amplify the mRNA genes of ribosomal protein S18 (*RPS18*), epidermal growth factor receptor 2 (*HER2*), estrogen receptor alpha (*ERa*), Y box binding protein (*YBX-1*), matrix metallopeptidase 11 (*MMP11*), caspase (*CASP8*), and superoxide dismutase 2 (*SOD2*), isolated from formalin-fixed and paraffin-embedded (FFPE) breast cancer tissues. They showed that in the NASBA reaction, an input of 100 ng of fragmented RNA was sufficient to generate a strong hybridization signal for all genes tested in the respective tumor samples. Their results indicated that NASBA is suitable to amplify with high specificity and sensitivity, even strongly degraded RNA isolated from FFPE tissues, and therefore can complement RT-PCR for analysis of such tissues [36].

In this study, we have developed a NASBA-ELISA method for the detection of breast cancer in clinical samples, which has been used for the first time to evaluate *HER2*, *ER* and *SPATA19* biomarkers simultaneously. This method has high sensitivity and specificity but its main property is the optical visual which can be detected by the color change in positive samples. We developed a simple diagnostic molecular method that is able to detect human breast cancer patients. The data were showed, of 15 samples tested, 11 and 4 samples were positive for *ER* and *HER2* genes, respectively. Our results were completely consistent with the results obtained from the real-time RT-PCR in the tumor bank of Imam Khomeini Hospital and also, our results were showed that NASBA-ELISA using *ER* and *HER2* breast-specific biomarkers offers a simple, fast, and reliable molecular method that can detect cases of breast cancer. On the other hand, there is no significant relationship in the expression of the *SPATA19* gene with *ER* and *HER2* genes in breast tumor tissues. The *SPATA19* gene, a specific biomarker for testis cancer, may be expressed in cases of breast cancer in which specific markers such as *ER* and *HER2* are negative. However, because of the few tumor tissues analyzed, our results should be interpreted with caution until verified from an ongoing subsequent analysis including a

larger group of patients' tissues.

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