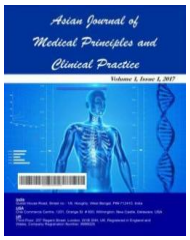

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Evaluation of DKK1 and NANOG Genes Expression as Prognostic Biomarkers in Iranian Women with Breast Cancer

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

Background: Breast cancer is the second leading cause of death in Iranian women following lung cancer with high morbidity rate among those aged 40-55 years. The aim of this study was to evaluate the expression of DKK1 and NANOG genes as prognostic biomarkers in Iranian patients with breast cancer using quantitative Real-Time PCR (RT-qPCR).
Materials and Methods: In this study, 30 clinical specimens from patients with breast cancer and 30 normal adjacent tissues from these patients were collected from Tehran Imam Khomeini Hospital in 2017 and their age ranged from 23 to 83 years (mean, 51.27 years). For evaluation of DKK1 and NANOG genes expression, following the extraction of RNA and cDNA synthesis, the expression of the genes was evaluated using Real-Time PCR.
Results: Two patients (6.6%) were in the first stage, 15 (50%) in the second stage, 11 (36.6%) in the third stage and 2 (6.6%) in the fourth stage of breast cancer. In terms of tissue grade, 4 (13.3%)

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patients were in Grade 1, 15 (50%) in Grade 2 and 10 (3.33%) were in Grade 3. The results demonstrated that the expression of DKK1gene was10.774 Fold and the expression of NANOG gene was 11.19 Fold higher in patients with breast cancer compared to those genes from normal tissues.

Conclusion: In our study, we used two biomarkers including DKK1 and NANOG genes, both of which appeared as proper biomarkers as their expression was significantly higher for the prognosis of breast cancer among patients. With the development of molecular medical techniques, the use of gene expression biomarkers in the diagnosis and prognosis of various diseases, such as cancers has become increased. By evaluation of the expression of various genes, we can prognosticate the specific types of cancers.

Keywords: Biomarkers; breast cancer; DKK1; NANOG; Real-Time PCR.

1. INTRODUCTION

Breast cancer is the second leading cause of death following lung cancer in women. One third of new cases is related to the breast cancer and is the leading cause of death among women between the ages of 40 and 55 years. One out of every eight women is diagnosed with breast cancer and the risk of death is up to 4.3%. Although the incidence of breast cancer increases with age, however, its rate decreases after menopause. This reflects the fact that breast cancer is a hormone-dependent disease, most possibly when estrogen levels downturn and progesterone is reduced in postmenopausal women [1].

Across the world, breast cancer accounts for about 10.4 percent of all cases occurred among women and accounts for the fifth cause of cancer deaths. In 2004, breast cancer killed 519,000 people worldwide (7% of deaths owing to cancer). It is approximately 100 times higher in women, although more severe in men because of postponement in diagnosis [2]. Cancer cells come from normal cells due to alterations or mutations in DNA and RNA. These alterations and mutations can occur spontaneously or may be caused by other factors, such as microwave radiation, x-rays, gamma rays and ultraviolet radiation, viruses, bacteria, fungi and parasites [3].

Approximately 20-25% of patients with breast cancer have a positive familial history, but only 5% to 10% of them are the dominant autosomal inheritance [4]. Genetic alleles have been studied with regard to clinical significance. The alleles that increase the risk of breast cancer by approximately 40 to 85% include BRCA1 and BRCA2 mutations, TP53 gene mutations due to Li-Fraumeni syndrome, PTEN due to Cowden syndrome, STK11 cause of Peutz-Jegher syndrome, neurofibromatosis (NF1) and E-cadherin (CDH-1) [5,6]. Half of the susceptible breast cancer syndromes are associated with mutations in BRCA1 and BRCA2. In women with early BRCA1 or BRCA2 mutations, the risk of breast cancer increases significantly. The risk of breast cancer for carriers is about 65-81% for BRCA1 mutations and 45-85% in BRCA2. Medium risk genes include Ataxia-telangiectasia (ATM) homozygote mutations, somatic mutations in the CHEK2 tumor suppressor gene and BRIP1 and BRIP2 genes which increase the risk of breast cancer by up to 20-40% [7-9]. A large number of low-risk alleles have also been identified through genomic studies, but their role in breast cancer has not been fully clarified [5].

The gene inhibiting the dickkopf WNT (DKK1) signalling pathway or the SK gene, encodes a member of the dickkopf family of proteins. The members of this family are secreted proteins characterised by two cysteine-rich domains. The encoded protein is coupled to the LRP6 receptor and inhibits the beta-ketamine-dependent Wnt signalling pathway. This gene plays an important role in the development of the fetus and may be pivotal in bone formation in adults. High expression levels of this gene have been observed in numerous cancer types, and the protein can increase proliferation, invasion and growth of cancer cells. The DKK1 expression has increased in patients with breast cancer and is considered as a useful indicator/biomarker of breast cancer [10].

The NANOG or Nanog homeobox gene is another tumour prognostic biomarker, a protein encoded by this gene is aDNA-binding protein and a transcription factor involved in the proliferation, regeneration, and evolution of embryonic cells. The coded protein can inhibit the differentiation of the fetal cells and also able to process/modulate its expression in

differentiated cells. The aim of this study was to evaluate the expression of DKK1 and NANOG genes as prognostic biomarkers in Iranian patients with breast cancer using Real-Time PCR [11,12].

2. MATERIALS AND METHODS

2.1 Collection of Clinical Samples

In this study, 30 clinical specimens from patients with breast cancer and 30 adjacent normal tissue sections were collected from these patients in Tehran Imam Khomeini Hospital in 2017. Sample confirmation was fulfilled by a pathologist. The samples also belonged to women aged 23 to 83 years (mean, 27.21 years). The samples were preserved in Cryotubes at -80C for further investigation.

2.2 Extraction of RNA from Tumor Tissues

For RNA extraction, the Cinagen Co. (Iran) protocol was followed. Firstly, 100 mg of tissue was removed by the scalpel device and placed it in a plate. We then milled and homogenise the tissue. In the next step, we transferred the tissue to a Cryotubes and added 1 ml trizole into the tube, and the cells were entirely lysed and a homogeneous solution was obtained. Then 200 ml of chloroform was added to the trizol-containing microtube to separate the phases and then centrifuged several times in invert for 10 minutes at 13000 rpm at 4°C. Following centrifugation, three phases were formed including the pink trizol the below, a white sedimentary layer containing blood proteins and supernatant or the upper aqueous phase containing RNA. The supernatant containing the RNA was transferred into a microtube accurately not contacting the lower layer, and the same amount of isopropanol was added into the microtube and incubated for 10 minutes at room temperature.

In the next step, centrifugation was performed for 5 minutes at a 4°C temperature for 5 minutes. The supernatant was then left to completely be evaporated and finally only the RNA sedimentation remained in the microtube. RNA was then added to one milliliter of ethanol 75% for washing, and centrifugation was carried out for 5 minutes at about 10,000 rpm at 4°C. In the next step, the microtube was placed on ice for 10 minutes to completely dry the RNA sediment. At the end, 30-50 µl of DEPC water was added and pipetting was performed to completely dissolve the RNA sediment. Finally, the optical density (OD) value and the extracted RNA concentrations were checked out to evaluate its quality using a Nanodrop device, and the RNA was transferred immediately to the -70°C.

2.3 The cDNA Synthesis

According to the manufacturer's guidelines (Cinagen Co. Iran), in the first step toward the Real-Time PCR performance, the total RNA molecules are used as a template for the synthesis of the cDNA molecule. For this purpose, the reverse transcriptase enzyme was used, along with a suitable method for initiating the polymerisation reaction. In the next step, with PCR, the cDNA molecule increases logarithmically. Following extraction of total RNA, the quantity and quality of RNA were evaluated using spectrophotometry and agarose gel. For this purpose, the enzyme Primescipt (Takara Co. Japan) and specific primers and oligomer dioxymidine (Oligo-dt) were used to synthesise of cDNA.

2.4 Real-Time PCR Technique

Real-Time PCR is a quantitative PCR method for the detection of genes expression. The reaction conditions of the PCR were performed using the Applied Biosystems StepOnePlus Real-Time PCR Systems. The SYBR GREENdye was also used as the fluorescent reporter. The fluorescence colour is attached to the double-stranded DNA sequences, which, if multiplied, produces light that increases the intensity of the light recorded in the device with a proportional amount of the product obtained. The cDNAs synthesised from specimens with specific primers and the target aimed for RT-PCR was amplified using standard PCR. The GAPDH enzyme was used as the internal control or housekeeping gene. Since this gene is one of the genes having a permanent expression in the cell, it is therefore an appropriate target for internal control. Primers and materials and temperature conditions used in set up of the Real-Time PCR are depicted in Tables 1, 2 and 3. The gene expression was calculated with $2^{-\Delta\Delta CT}$ method.

2.5 Statistical Analysis

This study was statistically analysed using SPSS version 20 where p value <0.05 was considered significant. The student t-test and ANOVA were employed for comparison of genes expression differences.

Table 1. Primers used to study the expression of DKK1 and NANOG genes

Primer	Nucleotide sequence	Reference
NANOG-F	5′- TGTGATTTGTGGGCCTGAAG -3′	[13]
NANOG-R	5′- TTTGGGACTGGTGGAAGAATC -3′	[13]
DDK1-F	5′-GATCCTTACAGCTTCTTTTGCATTACAATGTCCATGGTGGA-3′	[13]
DDK1-R	5′-GATCTCCACCATGGACATTGTAATGCAAAAGAAGCTGTAAG -3′	[13]
GAPDH	F: TGA AGG TCG GAG TCA ACG GAT TTG GT R: CAT GTG GGC CAT GAG GTC CAC CAC	[13]

Table 2. Materials used in the RTR time reaction for the genes used in this study

Reaction ingredients	Volume (µl)
RealQ Plus 2x Master Mix Green - Amplicon	7.5
Primer Mix Forward and Reverse (3µM each)	1
cDNA (10 ng/µL)	1
Distilled Deionised Water	5.5
Total reaction volume	15

Table 3. Temperature and time used for RTK timing for DKK1 and disgrace genes, min: minute, s: second

Stage	Temperature (°C)	time	Repeat/cycles
Hot Start Activation	95	15 min	-
Denaturation	95	15 s	40
Annealing and Extension	62	1 min	

3. RESULTS

3.1 Clinical Information of Patients

Of the patients studied, 2 (6.6%) were in the first stage, 15 (50%) in the second stage, 11 (36.6%) in the third stage and 2 (6.6%) in the fourth stage history of breast cancer. In terms of tissue grade, 4 (13.3%) were in Grade 1, 15 (50%) in Grade 2 and 10 (33.3%) in Grade 3 of cancer. The type of breast cancer was primary. The onset age of them was between 12 to 16 years old and menopausal age ranging from 38 to 55 years. Two of the patients were single and the rest were married. Three (10%) had a history of abortion and had the first gestational age between 17-34 years old. Ten percent of the patients had a history of smoking.

3.2 Real-time qPCR

Linear and logarithmic graphs of quantitative real-time PCR (RT-qPCR) amplification and melt curves for the negative control and the DKK1 gene are shown in Figs. 1 and 2. The results demonstrated that the expression of DKK1 was increased 10. 774 Fold and expression of NANOG increased 11.19 Fold in patients with breast cancer compared to the normal group (Table 4).

4. DISCUSSION

Breast cancer is the most common cancer among women and is the second dominant ailment in all cancers. It is currently the most common cancer in developed and developing countries and the incidence of breast cancer varies across the globe, with a range of 27 individuals per 100,000 population in Central and Eastern Asia, up to 96 individuals per 100,000 population in Western Europe [14]. Those genes involved in the control of the cell cycle are pivotal among patients affected by genetic alterations in cancer. In addition, the mechanism of cancer and carcinogenicity development, clearly increasing the number of cells and alterations in mechanisms regulating cell proliferation, is only one aspect of the mechanism of cancer [15]. Reducing cell death or apoptosis also contributes to certain types of cancer. Cancer is distinct from other processes of tumor formation because of its ability to attack surrounding tissues. Several causes including genetic and epigenetic alterations under the influence of environmental, physicochemical factors and viruses can lead to cancer conditions. The improper regulation of

multiple genes within various cell pathways leads to the transformation of normal cells into cancerous cells. The emergence of a tumor is a multi-stage process of genetic mutations that controls the activity of genes controlling cell growth and regulates cellular death susceptibility and maintains genetic stability [16,17]. Currently, many new molecular profile tools have been developed to accurately predict breast cancer outcomes and assess the benefits of using them. With the advent of genomic diagnostic technologies and targeted selective molecular therapies, biomarkers play an important role in the clinical management of cancer patients [18]. In this study, we also used tumor markers such as DKK1 and NANOG genes to examine breast cancer prognosis in order to evaluate the expression of these genes in patients with Real-Time PCR.

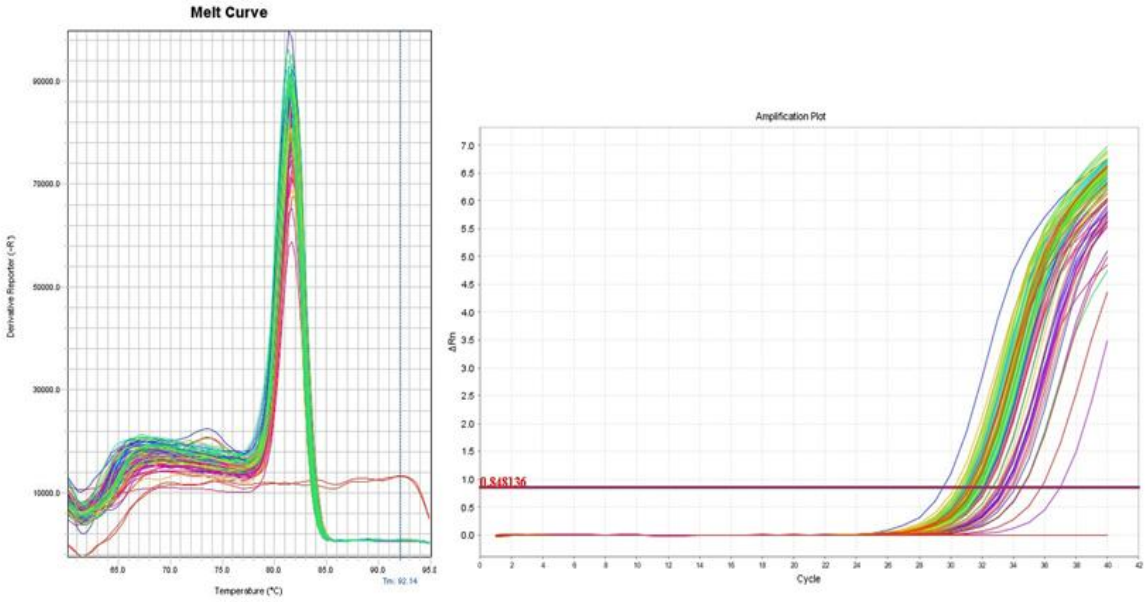

Fig. 1. Linear and logarithmic diagrams for RT-qPCR amplification and melt curves for negative control gene

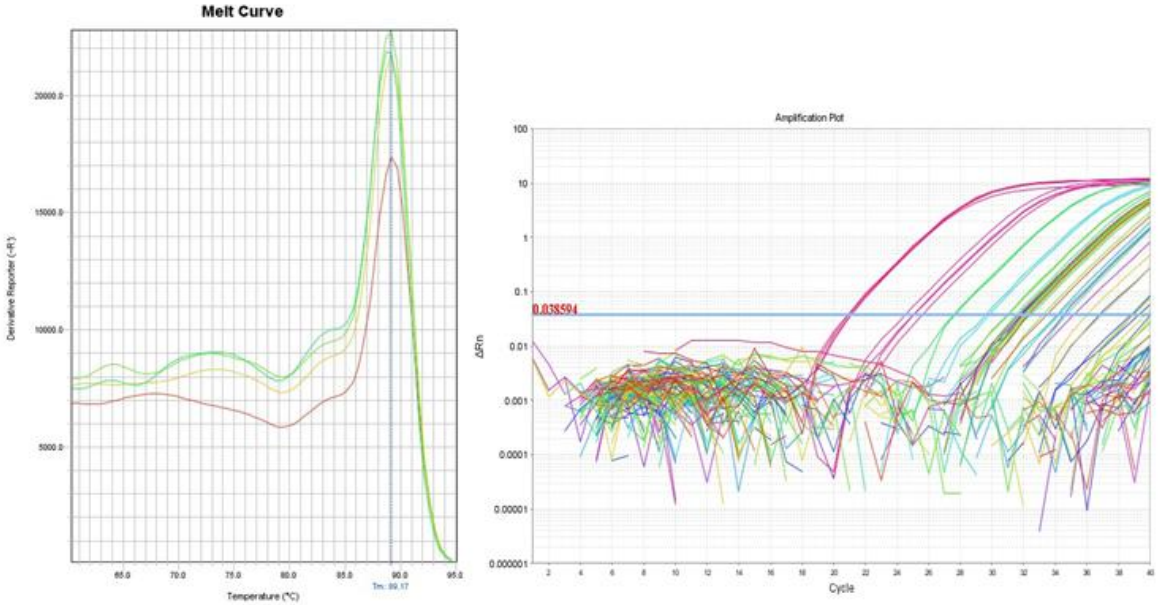

Fig. 2. Linear and logarithmic graphs of RT- qPCR amplification and melt curve for DKK1 gene

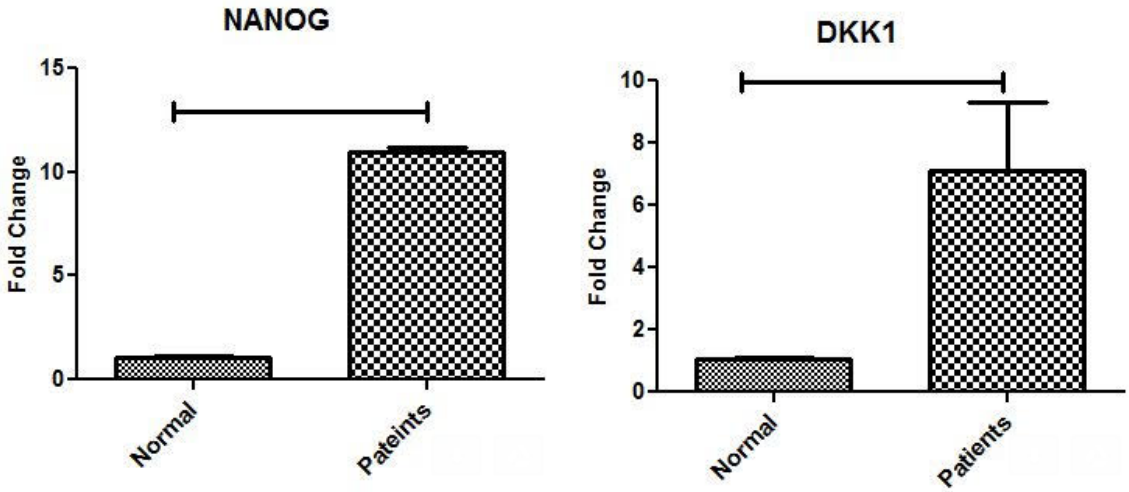

Fig. 3. The fold change expression of NANOG and DKK1 genes

Table 4. The fold change expression of NANOG and DKK1 genes

	Adjacent normal breast tissue N=30	Breast cancer N=30
Fold expression of DKK1	1.06±0.19	10. 774±0.33
Fold expression of NANOG	0.994 ±0.17	11.19±0.21

The DKK-1 was initially recognised as a secreted inhibitor of the WNT signaling pathway. The DKK-1 interacts with the LRP5 /6 and the Kremen receptors by inhibiting Wnt activity. Inhibition of the of Wnt function enhances the activity of glycosin synthase kinase-3-beta (GSK-3β), which results in increased phosphorylation of the beta-catenin-stable state and is important for the destruction of protease-dependent proteins. Therefore, increasing in DKK-1 expression leads to the inhibition of beta-catenin-dependent alterations in gene transcription. A large number of studies have revealed the association of family members of the DKK gene in a variety of cancers, including breast cancer [19,20].

A study by Shao-ji Zhou et al in 2014 found that the DKK-1 serum level could be used as a non-invasive biomarker for breast cancer prognosis. Data from this study showed that DKK-1 Serum may be an efficient molecular marker for breast cancer diagnosis [21]. The expression and role of DKK-1 is different in various cancer types. Various studies have shown that excessive expression of DKK-1 in many malignant tumors, including lung cancer, esophageal carcinoma, cervical cancer as well as breast cancer, gastric cancer, and Melanoma infers the potential for oncogenic function of DKK-1, and the different function of DKK-1 in a variety of cancers depends on the type of tissue of the cancer cells and the microscopic environment of the tissue [22,23]. In a study that we conducted on breast cancer patients in Tehran with the aim of examining the expression of the DKK1 gene, it was found that the expression of this gene in the studied patients increased and could be useful as a biomarker in the prognosis of these patients in the country. Another biomarker that was studied in cancer patients in this study was an investigation of the expression of NANOG gene. Studies that examined the role of this biomarker in cancer patients, including breast cancer, were related to the Lu X study in 2014 that brought NANOG to as a major factor in the prognosis of tumorigenicity and metastatic breast cancer. Recent studies have also shown that NANOG has a high expression in many types of human cancers, including breast cancer. In addition, NANOG also increases the risk of migration and invasion of breast cancer cells [24]. In a study by Takuya Nagata and colleagues in 100 patients with breast cancer, they examined the expression of NANOG and KLF-4 using immunohistochemistry and microarray analysis. According to the results, they showed a strong expression of NANOG and thus being a poor prognosis for patients with breast cancer, on the contrary to NANOG, KLF4 was a better prognostic factor. The results indicated that NANOG stimulated the growth and metastasis of

breast cancer cells, while KLF4 inhibited these processes [25]. In our studies, we were able to predict NANOG as a biomarker in the prognosis of these patients in the RT-qPCR.

5. CONCLUSION

With the advancement of science and the development of molecular medical techniques, the application of biomarkers in the diagnosis and prognosis of various diseases, including cancers, is in advancement increasingly. By examining the expression of various genes, we can prognosticate the types of cancers. In our study, we used two biomarkers DKK1 and NANOG, both of which were advantageous biomarkers for the prognosis of breast cancer patients.

CONSENT

The patients had the consent for this study by filling related form.

ETHICAL APPROVAL

As per international standard or university standard ethical approval has been collected and preserved by the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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