Cell. Mol. Biol. (ISSN: 1165-158X)

Journal Homepage: www.cellmolbiol.org

Cellular and Molecular Biology

Original Article

Evaluation of TP53TG1 and PANDA lncRNAs expression in association with adjuvant chemotherapy response in the peripheral blood of invasive ductal carcinoma patients

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Article Info

Article history:

Received: February 02, 2024
Accepted: June 25, 2024
Published: October 31, 2024

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Abstract

Breast cancer is the most common malignancy in women. Breast cancer, the second leading cause of cancer deaths, affects 2.1 million women each year and is estimated to have killed 627,000 women worldwide in 2018. Unfortunately, the age of onset of this cancer in our country IRAN is about 10 years lower than the global average and is close to 45 years. Chemotherapy is one of the basic treatments for cancer. Predicting the benefits of chemotherapy is challenging. Studies are now underway to use gene expression tests to pinpoint patients who are most likely to benefit from adjuvant chemotherapy. In the present study, the expression of two long non-coding RNAs TP53TG1 and PANDA in the blood of breast cancer patients before and after receiving chemotherapy compared with this amount in the blood of normal people using Real-Time RT PCR technique to find a meaningful relationship ¬ Compared statistically. Compared to normal samples, the expression level of TP53TG1 in the blood of patients was reduced. Although it was not statistically significant. Its expression also increased after receiving chemotherapy. Compared to normal samples, the expression of PANDA in the blood of patients was increased, which was statistically significant. Also, its expression decreased after receiving chemotherapy. These findings suggest that PANDA and TP53TG1 expression levels may be possible markers associated with tumorigenesis and may also be considered as possible indicators of response to chemotherapy.

Keywords: Breast cancer, Chemotherapy, lncRNA, PANDA, TP53TG1.

1. Introduction

Breast cancer is the most common malignancy in women and is one of the three most common cancers worldwide, along with lung and colon cancer. This cancer is the most common cause of cancer death in women [1]. Chemotherapy (the use of anti-cancer drugs to treat cancer cells) is one of the most common treatments used in breast cancer patients. Specific treatment for breast cancer is based on general health, medical history, age (whether there is menstruation or not), the type and stage of cancer, and the patient's tolerance to certain medications and procedures [2].

Although a large number of RNAs are transcribed from the human genome, protein-coding sequences comprise a very small fraction of all transcripts. The rest of the transcripts are non-coding RNAs (ncRNA) and have no coding potential, except for those capable of producing small functional peptides [3]. ncRNAs were initially considered as transcriptional parasites [4]. However, it is now clear that they can play a vital role in various cellular processes, from normal development to disease processes [5].

Based on size, there are two main classes of regulatory non-coding RNAs: Short non-coding RNAs that are less than 200 nucleotides in length and long non-coding RNAs (lncRNAs) with a length of more than 200 nucleotides. Regardless of their genomic organization, it seems that almost all types of lncRNAs can regulate gene expression and therefore can be important elements in cancer biology [6]. For example, lncRNA HOTAIR and lncRNA MALAT1 have been found to have an oncogenic function, and on the other hand, tumor suppressor function has been proven in lincRNA-p21 [7].

TP53TG1 is a long non-coding RNA with a length of 751 nucleotides located in the chromosomal region 7q21 in the human genome. This molecule was originally isolated from a special cancer cell line called SW480-LOWTP53-1. This molecule plays a vital role in response to cell damage by having a function in the TP53 signaling pathway. TP53TG1 is one of the direct targets of TP53 molecules [8]. In vitro and in vivo experiments have shown that TP53TG1 has tumor suppressor activity and is active in the pathway of P53 protein response to DNA damage. In cases of DNA double-strand break damage, P53 protein activates the expression of TP53TG1 [9]. TP-53TG1 binds to DNA/RNA-binding multi-sided protein called YBX-1 and prevents its localization in the nucleus,

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Doi: http://dx.doi.org/10.14715/cmb/2024.70.10.9

thereby preventing YBX-1-dependent activation of oncogenes. Epigenetic inactivation of TP53TG1 in cancer cells releases the transcriptional inhibition of YBX-1 targeted growth-promoting genes and creates a chemotherapy-resistant tumor. Hypermethylation of TP53TG1 in primary tumors has been shown to be associated with poor outcomes. Hence, the epigenetic loss of TP53TG1 represents an altered event in a lncRNA that is not only associated with classical tumor pathways, such as p53 signaling but also with cancer cell regulatory networks [7].

p21-associated ncRNA DNA damage-activated (PANDA) lncRNA has been identified as a lncRNA that is induced in response to DNA damage using high-resolution tiling array analysis [10]. PANDA is located approximately 5 kb upstream of the transcription start site (TSS) of p21, which is a cyclin-dependent kinase (CDK) inhibitor. PANDA genomic DNA consists of one exon and is transcribed in the opposite direction of p21, the product of which is a 1.5 kilobase transcript. The genomic region containing p21 and PANDA is regulated by tumor suppressor p53 [11]. By binding to the upstream of the p21 transcription start site, p53 activates both p21 and PANDA genes in response to DNA damage. PANDA interacts with nuclear transcription factor Y (NF-Y) subunit A. NF-Y, which is a three-subunit complex consisting of subunits A, B, and C, induces the transcription of the apoptotic gene FAS (cell surface death receptor fas) by binding to its promoter [12]. PANDA suppresses the transcription of apoptosis activators such as APAF1, BIK, FAS, and LDD by inhibiting the binding of NF-YA to their promoters, thereby suppressing apoptosis [13]. When apoptosis is suppressed, cancer cells can get more mutations without getting forced to encounter removal from the system and as a result, the tumor becomes more aggressive and can use these cells during progression and invasion phase [14].

In recent years studies have shown that PANDAR and TP53TG1 are dysregulated in many cancer types and this dysregulation has been correlated with progression of cancers [7, 15-17]. In 2016 a study performed by Sang and colleagues showed that PANDAR is upregulated in Breast cancer tissues [18]. In 2020, Shao et al showed that in breast cancer, TP53TG1 is activated by wild-type TP53, and further, TP53TG1 affects the PI3Ks family by binding to YBX2. They proposed TP53TG1 as an anti-oncogenic target in breast cancer [16].

As the upregulation of PANDA in breast cancer tissues has been shown before, and the possible anti-oncogenic role was proposed for TP53TG1 by studies mentioned above we tried to evaluate the possible changes of lncRNA PANDA and TP53TG1 expressions in peripheral blood of BC patients in order to check whether these changes will be seen in the blood samples or not. In the next step, we evaluated the PANDA and TP53TG1 expression changes in blood samples in response to chemotherapy in order to get a better understanding of the certain chemotherapy drugs have been the right choice for patients or not.

2. Material and methods

The total number of participants in this research was 25 people, which consisted of 5 healthy women (mean age of 49±1.7) and 20 women with invasive ductal carcinoma (IDC) breast cancer (mean age of 52±1.7). None of the participants in this study had a history of breast cancer or other related cancers in the family. All patients underwent surgery after diagnosis and did not receive any other treatment such as neoadjuvant chemotherapy before surgery. The chemotherapy regimen of these patients consisted of 4 to 7 courses of CMF (cyclophosphamide, methotrexate, and fluorouracil) whose dose was calculated according to the weight of the patients by the attending physician. To carry out this study, blood was taken from the patients in two stages: the first time before the start of the first chemotherapy regimen and the second time before the start of the second chemotherapy regimen. The time interval between two blood draws was about 20 days. All participants signed the consent form to approve the use of their blood and information before entering this project. This project was approved to be in accordance with the ethical principles and the national norms and standards in Iran on 2019-10-23 under the approval ID IR.MODARES.REC.1398.148.

2.1. Quantitative Real-time PCR

Total RNA was extracted from peripheral blood samples using the RNxplus blood RNA extraction kit (Tehran Cavosh Clon, Iran). In order to eliminate the possible DNA contamination the DNase I treatment was applied. We used a NanoDrop assessment (IMPLEN) to check the quality and quantity of RNA extractions. In order to evaluate the quality, we checked the ratio of absorption at 260 to 280 nm. All the extractions had ratios between 1.8 and 2. Since the study of gene expression by Real-Time PCR is done on DNA (and not RNA), we must convert the extracted RNA into cDNA. To synthesize cDNA from RNA, the Easy cDNA Synthesis kit (ParsTous) was used. We performed RT-PCR to check the synthesized cDNA using the control gene (GAPDH). The primers were designed to bind only to cDNA and not to DNA. Finally, after RT-PCR, the product was loaded with 100 bp markers in the wells and electrophoresis was performed. After staining with ethidium bromide, the desired fragment was observed under UV, which indicated the correctness of RT-PCR. To assess relative PANDA gene expression, we used 2X Real-Time PCR master mix (cat. No: DQ385-40h) (BIOFACT), which contains Cyber Green dye and is High ROX. GAPDH gene was selected and used as internal control gene. PCR program consisted of a denaturation step at 95 °C for 300 seconds, followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 45 seconds and one final step at 72 °C for 30 seconds. The specific sequences of primers used in this study are as follows: GAPDH-F 5-ATGAGAAGTATGACAACAGCCTC-3, GAPDH-R 5-CATGAGTCCTTCCACGATACC-3, PANDA-F 5-GTTTTCCTGTTCGTCGATTCTGG-3, PANDA-R 5-GGAAAGCTGAGAGAGACTTTGAAC-3, TP53TG1-F 5-TGGGCTCTTTCCTTTAATCTTCG-3 and TP53TG1-R 5-GTGAAGAGAATTGTTACCAGG-3. Real time equipment used in this survey was Applied Biosystems StepOnePlus (USA). All the primers were provided by Pishgam Biotech Company (IRI).

2.2. Statistical analysis of Real-Time PCR

Graph Pad Prism 7.03 software was used to analyze Real-Time PCR results and design graphs in this project, which is based on the use of ΔΔCT. Here, a p-value threshold of less than 0.05 is considered as a significance level. Kolmogorov-Smirnov test was also used to check the normality of the samples.

3. Results

3.1. Evaluation of TP53TG1 expression levels in IDC Breast Cancer Patients and Normal subjects.

TP53TG1 expression was decreased in IDC breast cancer patients compared to healthy individuals (fold change: 0.3928) (although this change was not significant (p-value: 0.1317). Also, this rate increased in people with breast cancer after receiving a course of chemotherapy (fold change: 0.8786), which was significant (p-value: 0.0398). As shown in the picture, the expression level of TP53TG1 is the lowest in cancer patients before chemotherapy and the highest in normal people. Also, the standard deviation (SD) in the two affected groups before and after receiving chemotherapy was equal to 1.90 and 1.11, respectively (Fig. 1). The ROC diagram shows that TP53TG1 expression can be used to distinguish between BC patients and normal people (Fig. 2).

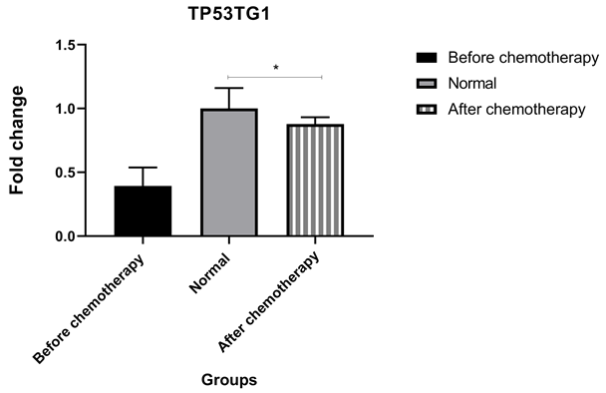


Fig. 1. Graph of TP53TG1 expression changes in normal subjects and patients with invasive ductal breast cancer before and after receiving CMF treatment.

Area under the ROC curve	
Area	0.9800
Std. Error	0.02478
95% confidence interval	0.9314 to 1.000
P value	0.0011
Data	
Controls	5
Patients	20
Missing Controls	0
Missing Patients	0

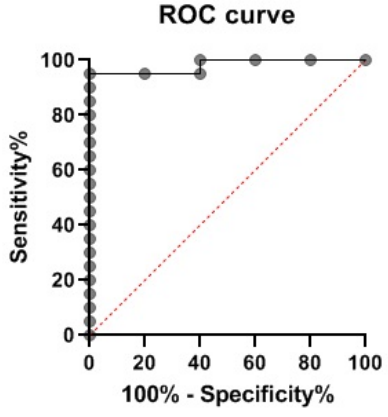


Fig. 2. . ROC diagram of TP53TG1 lncRNA in groups of normal subjects and breast cancer patients.

3.2. Evaluation of TP53TG1 expression changes in triple-negative breast cancer and other subtypes

Comparing the changes in the expression of TP53TG1 in patients whose breast cancer was of the triple-negative type (TNBC) (6 people) with patients with breast cancer of other subtypes (Non-TNBC) (14 people) showed a decrease in the expression in both groups compared to normal subjects. However, this reduction was more severe in the TNBC group (fold change in TNBC group is equal to 0.126, while its value for Non-TNBC group was equal to 0.507 (p-value>0.05)). Also, the standard deviation (SD) in the two triple negative groups and the non-triple negative group is equal to 0.32 and 0.41, respectively (Fig. 3).

3.3. Evaluation of PANDA expression levels in IDC Breast Cancer Patients and Normal subjects

PANDA expression level is increased in people with IDC breast cancer compared to normal subjects (fold change: 3.298) and this change was meaningful (p-value: 0.0001). Also, the expression level of this long non-coding RNA has decreased after receiving a course of chemotherapy (CMF regiment) (fold change: 1.879) this change was meaningful (p-value: 0.0098) (Fig. 4). The ROC diagram shows that PANDA expression can be used to distinguish between BC patients and normal people (Fig. 5).

3.4. Evaluation of PANDA expression changes in triple-negative breast cancer and other subtypes

Comparison of PANDA expression changes in patients whose breast cancer was of triple-negative type (TNBC)

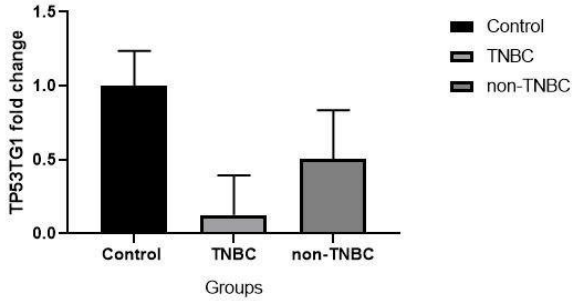


Fig. 3. Graph of TP53TG1 expression changes in TNBC and non-TNBC invasive ductal breast cancer before and after receiving CMF treatment.

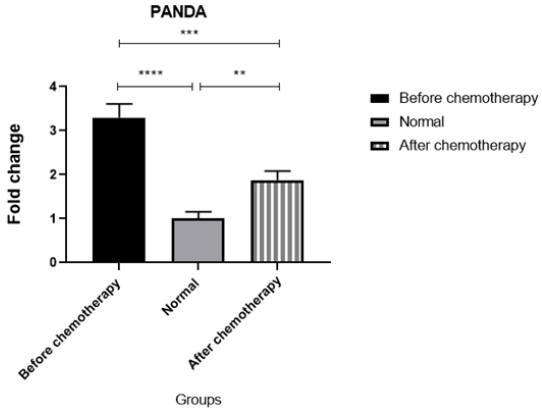
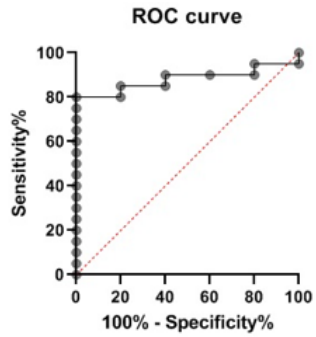


Fig. 4. Graph of PANDA expression changes in normal subjects and patients with invasive ductal breast cancer before and after receiving CMF treatment.



Area under the ROC curve	
Area	0.8800
Std. Error	0.06822
95% confidence interval	0.7463 to 1.000
P value	0.0098
Data	
Controls (normal)	5
Patients (P-G 0)	20
Missing Controls	0
Missing Patients	0

Fig. 5. . ROC diagram of PANDA lncRNA in groups of normal subjects and breast cancer patients.

(6 people) with patients with breast cancer of other subgroups (Non-TNBC) (14 people) showed the level of expression in both groups (compared to normal subjects) has increased, although this increase was more intense in the TNBC group (fold change in the TNBC group is equal to 4.227, while its value for the Non-TNBC group was equal to 2.9). These changes were significant in both groups compared to the normal group (p-value for changes in TNBC group was equal to 0.001 and for changes in non-TNBC group was less than 0.001). Also, the standard deviation (SD) in the triple-negative group and the non-triple negative group is equal to 0.93 and 0.47, respectively (Fig. 6).

4. Discussion

Precision medicine (personalized medicine) is an approach that allows physicians to choose a treatment that is more likely to benefit patients, based on a genetic understanding of their disease. Precision medicine has a number of targets, including genes and transcripts, proteins and their metabolites. In precision medicine, not only the relatively static genetic codes of people but also the dynamic and heterogeneous genetic codes of cancers should be considered. Therefore, precision medicine relies not only on the discovery of identifiable targets to modify treatment and monitoring but also on reliable and non-invasive methods to detect changes in these targets over time.[19]

The term biomarker refers to a broad subset of medical symptoms that can be accurately and reproducibly measured. Currently, a number of molecular biomarkers are used in standard clinical practice, including hormone receptors for breast cancer subtyping and several genes involved in genome maintenance to predict breast cancer susceptibility. In addition, a number of biomarkers of multigene or monogenic signatures have been approved for clinical use in breast cancer prognosis. An increasing number of molecular biomarkers are being studied and tested to facilitate

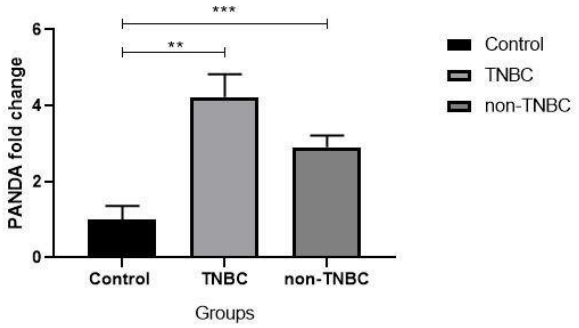


Fig. 6. Graph of PANDA expression changes in TNBC and non-TNBC invasive ductal breast cancer before and after receiving CMF treatment

disease diagnosis and disease management, especially for early detection of breast cancer, accurate prediction of metastatic behaviors and selection of treatment methods. However, many of them are still in preclinical stages. Biomarkers with non-invasive protocols, such as serological molecules, have advantages in ease of detection over other types of biomarkers and therefore are of particular importance in terms of clinical development to improve diagnosis, prognosis and treatment.[20]

lncRNAs perform diverse biological functions by regulating gene expression and functions at the levels of transcription, translation and post-translation. In the past decade, the misregulated lncRNA profile has been shown to be widely involved in the pathogenesis of many diseases, including cancer, metabolic disorders, and cardiovascular diseases. In particular, lncRNAs have been shown to play an important role in tumor growth and metastasis. It has been shown that many lncRNAs are potential biomarkers and important targets for the diagnosis and treatment of cancers.[21]

In the present study, the expression of long non-coding RNAs PANDA and TP53TG1 was investigated for the first time in the peripheral blood samples of breast cancer patients before and after receiving chemotherapy. The reason for choosing the blood sample to check the expression of this gene was the non-invasiveness of this method, which made the sampling and, if necessary, subsequent screening of the patients simple and needless invasive methods.

In this study, a decrease in the expression of TP53TG1 was observed in the blood samples of patients with IDC breast cancer compared to normal individuals. However, this change was not significant. Also, comparing the expression levels of this lncRNA between cancer patients before and after receiving chemotherapy shows an increase in its expression after receiving chemotherapy, which suggests its tumor-inhibitory role in this cancer. As shown in the studies conducted by other researchers, TP53TG1 is deactivated in human cancers in different ways, the final result of which is the reduction of the activity of this lncRNA in cancer. Our study showed that this decrease in expression is also evident in the blood of these people, and people with lower TP53TG1 are likely to have a poorer prognosis for example, TNBC type compared to Non-TNBC).

In evaluating the expression of PANDA in the blood samples of people with IDC breast cancer, we observed an increase in its expression compared to normal people, which suggests the oncogenic role of this long non-coding

RNA in breast cancer. Also, comparing the expression levels of this lncRNA between cancer patients before and after receiving chemotherapy shows a decrease in its expression after receiving chemotherapy, which suggests the oncogenic role of this lncRNA in breast cancer. As shown in previous studies, PANDA has increased activity in human cancers. In this study, we confirmed the hypothesis of PANDA being oncogenic in breast cancer by showing the increase in the expression of this lncRNA in the blood of IDC breast cancer patients. Our study showed that a higher level of PANDA in the blood of people with breast cancer can suggest a poorer prognosis in these people (for example, the probability of getting TNBC compared to the probability of getting Non-TNBC). On the other hand, since the expression level of this oncogenic lncRNA increases in people with breast cancer and decreases after receiving CMF chemotherapy regimen, it can be concluded that patients who experience a smaller decrease in PANDA levels after receiving this treatment are probably patients who benefit less from this regimen and maybe changing the chemotherapy regimen is a logical option for them.

Acknowledgments
This article has been extracted from the MSc thesis of the first author and was supported by the Research Department of the Faculty of Medical Sciences, Tarbiat Modares University. The authors are grateful to the patients and normal individuals for participating in this study.

Funding information
This study was funded by the Tarbiat Modares University Tehran, Iran, by grant number IG-39711.

Author contributions
M.N. performed the experiments, analyzed the data, and wrote the manuscript. H.M. supervised, designed and edited the manuscript. R.R.P. performed experiments and statistical analysis. M.L. selected the patients and confirmed the clinical diagnosis. All authors contributed to the study design and approved the final manuscript.

Ethics approval
The questionnaire and methodology for this study were approved by the Human Research Ethics Committee of the Faculty of Medical Sciences Tarbiat Modares. (Ethics approval number: IR.MODARES.REC.REC.1398.148).

Conflicts of interest
The authors declare no conflict of interest.

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