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Significance of USP7 in Predicting Prognosis of Mammary Ductal Adenocarcinoma in the Turkish Population

Esra Aydemir[1], Derya Burukcu[2], Gurcan Vural[3], Taner Kivilcim[4], Fikrettin Sahin[2]

[1]Department of Biomedical Engineering, Faculty of Engineering and Natural Sciences, Biruni University, Istanbul, Turkiye
[2]Department of Genetics and Bioengineering, Faculty of Engineering, Yeditepe University, İstanbul, Turkiye
[3]Department of Pathology, Okan University Hospital, Istanbul, Turkiye
[4]Department of General Surgery, Okan University Hospital, Istanbul, Turkiye

ORCID ID: E.A. 0000-0002-6965-2838; D.B. 0000-0002-5178-0620; G.V. 0000-0002-5596-9922; T.K. 0000-0003-0088-1189; F.Ş. 0000-0003-1503-5567

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ABSTRACT

Objective: Breast cancer has the highest incidence and mortality rate among women worldwide. This study aims to analyze ubiquitin specific protease 7 (USP7) levels in mammary ductal adenocarcinoma patients in the Turkish population, to identify whether there is a relationship between the levels of USP7 protein and bad prognosis, which is indicated by enlarged tumor size, younger age, and receiving neo-adjuvant treatment.

Materials and Methods: In our study, we analyzed USP7 levels by immunohistochemistry (IHC) staining in 38 mammary adenocarcinoma cases in the Turkish population. Corelation analyses were performed to evaluate the distribution of the patients by their age, tumor size, Ki-67 levels, and the status of neo-adjuvant treatment by their USP7 levels. The IHC data concluded that the average age and tumor size are reversely proportional to the USP7 levels, insignificantly. The differences between the levels of USP7 and Ki-67 measurements were found to be statistically insignificant between the groups.

Results: Women who received the neo-adjuvant therapy prior to the operation presented much lower amount of USP7 levels when compared to their non-treated counterparts.

Conclusions: These findings highlighted that mammary ductal adenocarcinoma patients in the Turkish population present varying USP7 protein levels. Even though the levels of UPS7 are partially insignificant, the presence of USP7 protein might be a prognostic indicator in breast cancer patients in the Turkish population with a larger study set.

Keywords: Ubiquitination, USP7, mammary ductal adenocarcinoma, the Turkish population

INTRODUCTION

Breast cancer, one of the most lethal diseases among women, has the highest incidence and mortality rate that accounts for 25% of all cancer cases (1,2). Women diagnosed with breast cancer receive advantages from the latest therapeutical approaches; however, the incidents and cancer deaths continue to be a significant problem around the globe (3,4). Various parameters play a role in tumorigenesis, including family inheritance, lifestyle, age, menopause, and hormonal therapy. Mammary ductal adenocarcinomas differ widely due to the various subtypes based on the presence of Her2 receptors, treatment with a neo-adjuvant therapy, metastatic ability, and the expression of steroid hormone receptors such as progesterone and estrogen (5). Due to its complex structure at the molecular level, the treatment strategies against these carcinomas stay as troublesome. Although treatment methods vary,

Corresponding Author: Esra Aydemir **E-mail:** eaydemir@biruni.edu.tr
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finding the best option stands as challenging, and therefore alternative targets are in urge.

One of the mechanisms that steer tumorigenesis is the dysfunction of protein stability. Ubiquitin–proteasome pathway is one of the major pathways that maintain this stabilization process. Not only just degrading but also targeting other proteins that signal various cellular processes, ubiquitins are essential for the development of cancer. We know that enormous amounts of proteins do play a role in these ubiquitin pathways. Ubiquitination is a post-translational process in which enzymes are involved and an attachment of a ubiquitin-protein to another protein. This is usually done by binding of last amino acid of ubiquitin to a lysine residue on the proteins. Ubiquitin–specific protease 7 (USP7), a member of a family of proteases, is involved in removing these ubiquitins and preventing protein degradation. For a USP7 to be activated, C-terminal ubiquitin-like domains are required to be folded back into the catalytic domain, allowing the active site to be adapted to a catalytically suitable state by the C-terminal peptide. Several substrates are targeted by USP7 that play a role in a diversity of cellular events, including cell cycle, chromatin remodeling, and DNA repair. Upon its abnormal activation, USP7 can trigger or suppress tumorigenesis proposing this particular protein a double-edged sword in tumorigenesis. Up to now, many therapeutics have been used to target USP7 in the treatment of cancer, make this molecule a promising candidate for cancer therapy.

MATERIALS AND METHODS

Patients and Tissue Samples

Thirty-eight mammary adenocarcinoma patients, who had been regularly followed up between 2018 and 2020 at the Department of General Surgery in Okan University Hospital, and whose slides and paraffin blocks were extracted from the archives of the Department of Pathology, were evaluated in the study. We collected all the samples by the approved ethical standards of the responsible committee of Yeditepe University Hospital. During the follow-up, we obtained the patient information about their age, tumor size, and receiving any neo-adjuvant therapy.

Immunohistochemistry

Immunohistochemical (IHC) staining was achieved to investigate the expression of USP7 in breast cancer tissues. Collectively tissues went under a series of processes, including fixation in 10% buffered formalin, embedding in paraffin, and further slicing in 4-µm thickness. Deparaffinization by incubating at 70°C for 15 minutes was followed by the dehydration step with xylene three times. Later, sections were rehydrated gradually with diluted alcohol solution ranging from 100% to 70%. Samples were then washed with phosphate-buffered saline (PBS) two times for 5 minutes and permeabilized in a solution containing tri-sodium citrate dehydrate and triton X-100 for 8 minutes at +4°C. Upon washing with PBS, samples were treated with antigen unmasking solution (pH: 6) in the microwave for 1 minute. Samples were washed sequentially with a pre-cooled PBS and room temperature (RT), respectively. Endogenous peroxidase activity was inhibited by incubating the sections in 0.3% hydrogen peroxide (H_2O_2) for 30 minutes at RT. Upon washing, the samples were treated with goat sera and incubated in a humidified chamber for 30 minutes. Primary antibody USP7 (ab108931, Abcam, USA) was applied at 4°C overnight. Subsequently, diaminobenzidine (DAB) reaction and peroxidase (HRP) detection were conducted with SignalStain® DAB Substrate Kit (8059, Cell Signaling Technologies, USA) according to the manufacturer's instructions. Lastly, all sections were counterstained with Gill's hematoxylin solution (1051740500, Sigma Aldrich, USA), dehydrated, air-dried, and mounted.

Statistical Analysis

In the beginning, the conditions for ensuring the normality assumptions of the age variable, tumor size, and Ki-67 levels were examined by looking following parameters; Skewness-Kurtosis values, Shapiro-Wilks significance value due to the sample size being 50 or below, and Q-Q plot chart. After the normality examination, the age variable was analyzed with a one-way ANOVA test, which is one of the parametric tests. Tumor size and Ki-67 level variables were examined with a non-parametric Kruskal-Wallis test. Lastly, the Chi-square test was applied to data that belonged to the status of neo-adjuvant treatment because of comparing two categorical groups. The p-value was taken as < 0.05 in all analyses.

RESULTS

Evaluation of Immunohistochemical Staining

Figure 1 (Figure1, A-C) demonstrates that based on the staining levels of the nuclei. The power of staining of each sample was imaged and depicted as follows. The level of staining for sample 1 showed the lowest staining, while the second and third samples expressed the medium, the most levels of staining, respectively.

Immunohistochemical Findings Related to Age, Tumor Size, Ki-67 levels, and Neo-adjuvant Treatment

Based on the intensity of USP7 staining, the distribution of people and their corresponding parameters of interest were depicted in Table 1 accordingly.

Skewness-Kurtosis values of the age variable met the criteria of normal distribution (6). Considering the significance value of the Shapiro Wilk result, it was seen that the age variable was normally distributed (p=0.497). The distribution of the age variable within the group was controlled by looking at the Q-Q Plot chart. It is seen that the series has a linear distribution, provides a normal distribution due to the absence of outliers. After normality examination, one-way ANOVA showed that the mean age decreases as the USP7 level increases although not significantly.

For the tumor size and Ki-67 levels, Skewness-Kurtosis, Shapiro-Wilk statistical value was invalid for normal distribution. Q-Q

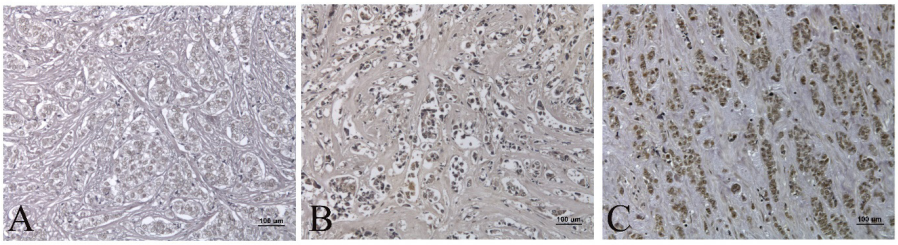


Figure 1. Images were taken with a light microscope with various staining levels of the nuclei (20x). A: Weak nuclei staining by USP7, score 1, B: Medium value nuclei staining by USP7, score 2, and C: Strong nuclei staining by USP7, score 3.

plot of these variables had non-linearity. Kruskal-Wallis results showed that the patients who possess medium, high levels of UPS7 levels had tumor sizes bigger than 20 mm. On the other hand, low levels of USP7 were observed among the ones with tumor sizes less than 20 mm.

As an important proliferation marker, only one out of 38 patients who had the Ki-67 measurement less than 10% was found to express low levels of USP7 whereas 7 of them had medium and high levels of USP7. Eight patients who had the Ki-67 measurement more than 10% had the lowest level of USP7, however, 22 of them had the medium and high levels of USP7.

Women who received the neo-adjuvant therapy before their operations were also subjected to the quantifications of USP7 levels. Accordingly, it seems that neo-adjuvant therapy overall has a downregulating effect on the USP7 levels. Out of 38 patients who had received the therapy, 2 patients expressed low USP7 levels while 4 of the total numbers of patients expressed USP7 levels at medium/high levels. Patients who did not receive any treatment expressed higher amounts of USP7 levels.

DISCUSSION

Discovered by Everett et al., the herpes virus-associated ubiquitin-specific protease (HAUSP), also known as USP7, was characterized in the late 1990s (7). This enzyme is involved in many cellular disorders, including cancers, neurological and metabolic syndromes, and immune dysfunctions. P53 is one of the substrates bound by this enzyme, and the reason for it to have a tumor suppressor role causes a decrease in the growth of tumor cells (8). However, further studies showed that mouse double minute 2 homolog (MDM2) was regulated by HAUSP-mediated deubiquitination, caused degradation of p53, reactivation of survival of the tumor cells (9). A recent study indicated the effect of USP7 on promoting the chemoresistance of triple-negative breast cancers (10), hence USP7 inhibitors could be potent agents in inhibiting several cancers (11). Therefore, the double-sword effect of USP7 stands as an open field to study in deep.

In our study, we have examined a total of 38 patients in the Turkish population based on their USP7 protein levels in histological specimens. An extensive analysis of the tumor size and its relationship with the levels of USP7 was performed. Although the size of the tumor might correlate inversely with

Table 1. Distribution and USP7 staining of all patients in accordance with the parameters including age, tumor size, Ki-67 levels, and receiving neo-adjuvant therapy.

Parameters	Limits	USP7 Levels			Total number of cases (n)
		Low (1)	Medium (2)	High (3)	
Age	< 40	0	3	2	38
	≥ 40	11	12	10	
Tumor size	< 20 mm	5	4	2	38
	≥ 20 mm	7	12	8	
KI-67%	≤ 10%	1	4	3	38
	> 10%	8	11	11	
NEO-ADJ treatment	-	8	13	11	38
	+	2	1	3	

the levels of USP7, no significant relationship was detected. The overexpression of USP7 levels in patients with epithelial ovarian cancer holds a bad prognosis and therefore, a high prognostic value in prediction (12). In addition, enhancer of zeste homolog 2 (EZH2) upregulation along with the high expression of USP7 was found to be related to bad prognosis in laryngeal squamous cell carcinoma (13).

One of the first parameters that we have taken into consideration is the age of the patients. According to our data, the average age is found to decrease while the USP7 protein level increases. These findings correlate with others as they explain how women at younger ages tend to present with breast cancer at an advanced stage than older ones, ultimately elucidating the worse outcome (14).

Ki-67 is one of the most valuable biomarkers in predicting tumor progression. A previous study indicates that Ki-67 might be a prognostic marker in breast cancer patients concerning the process and recurrence. Although we had the highest mean value of the Ki-67 measurement at a medium level, the lowest mean value at a low level, it was seen that the difference between the groups was not statistically significant (p=0.324).

In our study, we wanted to inspect whether USP7 levels could have a prognostic relevance of USP7 in breast cancer patients treated with neo-adjuvant therapy. Our results indicate that upon receiving neo-adjuvant treatment, the USP7 levels of the patients were found to be much lesser than the ones who had not received the therapy prior to the surgery. A similar study done by Giovinazzi et al. confirmed that USP7 inhibition improved the outcome of chemotherapy response in larynx carcinoma patients who were resistant to taxane treatment (15). However, an opposing study done by Cartel et al. shows that an increased expression of USP7 might be related to the elevated chemoresistance in acute myeloid leukemia (AML) patient-derived xenograft (PDX) models treated with cytarabine (16). These two conflicting data, along with our findings, assure that USP7 might be a changing prognostic criterion depending on the tumor type. Even though the levels of UPS7 are partially insignificant, the presence of USP7 protein might be a prognostic indicator in breast cancer patients in the Turkish population with a larger study set.

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Ethical approval: This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Yeditepe University of Medical Sciences under code KAEK:984.

Consent to Participate: Informed consent was obtained from all individual participants included in the study.

Consent to Publish: The authors affirm that human research participants provided informed consent for the publication of the images in Figure 1(A-C) and Table 1.

Availability of Data and Material: The authors affirm the data they share and its availability.

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