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ORIGINAL ARTICLE

Increased Expression of Gankyrin and Stemness Factor Oct-4 are Associated with Unfavorable Clinical Outcomes and Poor Benefit of Tamoxifen in Breast Carcinoma Patients

Rosa Jahangiri[1] · Fatemeh Mosaffa[2,3] · Amirnader EmamiRazavi[4] · Masoumeh Gharib[5] · Khadijeh Jamialahmadi[2]

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Abstract

Tamoxifen is the most important treatment component in estrogen receptor positive (ER+) breast carcinoma patients. Tamoxifen resistance incidence presents an important obstacle in clinical treatment. Mechanisms underlying tamoxifen refractory are not completely understood. Although elevated expression of Gankyrin (P28GANK) and stem cell markers Nanog, Oct-4 and Sox-2 have been reported in breast carcinoma, their role in tamoxifen resistance progression has not been explored. In the present study, P28GANK and stem cell markers Nanog, Oct-4 and Sox-2 expression were evaluated using quantitative RT-PCR and immunohistochemical technology in 72 breast carcinoma patients who received tamoxifen as adjuvant anti-hormone treatment. Expression data were correlated with the clinical outcome and survival of patients. Data analysis showed that P28GANK, Oct-4 and Sox-2 transcripts were significantly overexpressed in tamoxifen resistance patients. Immunohistochemical staining indicated that protein expression of P28GANK and Oct-4 were also significantly higher in tamoxifen resistance patients. We have shown a positive correlation between mRNA and protein expression of P28GANK, Oct-4 and Sox-2. Multivariate logistic regression analysis indicated that P28GANK ($P = 0.002$) and Oct-4 ($P = 0.013$) overexpression could be negative independent factors of disease outcome. Additionally, in the whole study group, multivariate Cox regression analysis revealed that high expression of P28GANK and Oct-4 remained significant and unfavorable predictive factors for patients' survival. These findings suggest that Gankyrin and Oct-4 overexpression could promote tamoxifen refractory in breast cancer patients. More studies are warranted to clarify the predictive role of these potential biomarkers for patients who don't benefit from tamoxifen treatment and their possible application as prognostic markers in ER^+ tamoxifen-treated breast carcinoma patients.

Keywords Breast Cancer · Tamoxifen Resistance · Gankyrin · Oct-4

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✉ Khadijeh Jamialahmadi
jamialahmadikh@mums.ac.ir

Rosa Jahangiri
rosajahangiri@yahoo.com

Fatemeh Mosaffa
MosaffaF@mums.ac.ir

Amirnader EmamiRazavi
aemamirazavi@irantumorbank.com

Masoumeh Gharib
GharibM@mums.ac.ir

[1] Department of Medical Biotechnology and Nanotechnology, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

[2] Biotechnology Research Center, Pharmaceutical Technology Institute, Mashhad University of Medical Sciences, Mashhad, Iran

[3] Department of Pharmaceutical Biotechnology, School of Pharmacy, Mashahd University of Medical Sciences, Mashhad, Iran

[4] Iran National Tumor Bank, Cancer Biology Research Center, Cancer Institute of Iran, Imam Khomeini Hospital Complex, Tehran University of Medical Sciences, Tehran, Iran

[5] Department of Pathology, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

Introduction

Breast cancer is the most prevalent and the second cause of cancer-related death in women worldwide. About 70% of all breast tumors are estrogen receptor positive (ER^+) [1]. Tamoxifen, the selective estrogen receptor (ER) modulator, has been used as adjuvant endocrine therapy in ER^+ patients for more than four decades. Although tamoxifen significantly reduces disease recurrence rate, unfortunately, about one-third of breast cancer patients develop resistance to tamoxifen therapy which is a major clinical obstacle in the treatment of cancer [2]. To date, multiple molecular mechanisms responsible for tamoxifen resistance have been proposed, but cancer-drug resistance progression is really a complicated and complex process. Therefore, identification of novel biomarkers that are associated with tamoxifen resistance is of crucial importance in breast cancer treatment.

Tumor heterogeneity leads to different therapeutic response patterns [3]. It has been well established that there is a considerable heterogeneity within tumors e.g. proliferation rate, differentiation state, migratory and invasive capacity and the content of Cancer Stem Cells (CSCs). Cancer stem cells are characterized by self-renewing capacity and heterogeneity. Furthermore, CSCs contribute to tumor development process, infiltration and metastasis that lead to disease recurrence and resistance to chemotherapy and radiotherapy.

Nanog, Oct-4 and Sox-2 are three stem cell markers that induce pluripotency and regulate self-renewal which leads to formation of CSCs [4]. Up-regulation of these three stem cell markers is associated with epithelial-mesenchymal transition (EMT). EMT takes a role in disease progression, invasion and metastasis induction in malignant tumors, and also progression of tamoxifen resistance in breast carcinoma patients [5]. It has been proven previously that estrogen receptor closely related to differentiation in breast tumors and provides a positive prognostic factor in patients [6]. It is considerable that overexpression of stem cell markers also leads to reduction of ER expression [7].

Gankyrin (P28GANK), 26S proteasome non-ATPase regulatory subunit 10, is a newly defined oncoprotein that has a nucleotide sequence identical to *P28* gene [8]. Overexpression of P28GANK is associated with disease progression and worse outcome in various malignancies, including hepatocarcinoma, liposarcoma, cervical and esophageal squamous cell carcinomas, and also lung, colorectal, and breast cancers [9]. This oncoprotein has an anti-apoptotic activity in cells exposed to DNA damage-inducing agents, which results in tumor progression [10]. Furthermore, it has been revealed previously that P28GANK prevents *apoptosis* by proteasomal degradation of tumor suppressor proteins including p53 and Rb [9]. Gankyrin binds to MDM2, a major E3 ubiquitin ligase for p53, facilitates p53-MDM2 conjunction and increases ubiquitylation and degradation of p53. In addition, in the absence of p53 protein, P28GANK enhances MDM2 autoubiquitylation and degradation [10]. In another pathway, P28GANK could bind to cyclin-dependent kinase 4 (CDK4) and interact with a subunit of the 26S proteasome, which leads to phosphorylation, and degradation of retinoblastoma protein and finally promote cell cycle progression [8]. Additionally, Gankyrin-CDK4 complex could enhance cell cycle progression through interaction with the inhibitory effects of INK4 proteins. INK4 involves of tumor suppressor proteins like p16INK4A and p18INK4B [11]. Interestingly, overexpression of P28GANK increases cell migration through inhibition of large focal adhesions that could leads to tumor metastasis [12]. Other studies have reported that increased expression of P28GANK drive metastasis induction in breast cancer [13]. Furthermore, Gankyrin can control stem cell behavior by regulating the expression of stemness factors [14]. However, to our knowledge, there are no reports on the association between the expression of Gankyrin and stem cell markers including Nanog, Oct-4, Sox-2 in breast cancer. Furthermore, the relation of their expression with the clinical outcome in tamoxifen treated breast carcinoma patients has not been explored previously. The present study was designed to investigate the mRNA and protein expressions of Gankyrin and stem cell markers in tamoxifen responding and non-responding breast tumors to find out if they contribute in resistance development during tamoxifen treatment or not. Besides, we evaluate the association between the expression of stem cell markers and P28GANK with patients' survival.

Materials and Methods

Patients

Surgical pathology records of the breast cancer patients of Iran National Tumor Bank that had undergone breast surgery with lymph node dissection from 2005 to 2014 were reviewed. All patients, whose clinicopathological data could be assessed, were selected. ER negative breast tumors or patients with prior neoadjuvant therapy were excluded from the present research. Seventy-two female ER^+ patients who undergone surgery and received adjuvant radiotherapy and chemotherapy and finally tamoxifen for 6 months to 5 years or more were included. Fresh frozen tissue samples, their corresponding Formalin-Fixed Paraffin-Embedded (FFPE) breast tumor blocks and the clinicopathological data were provided by Iran National Tumor Bank, which is founded by Cancer Institute of Tehran University of Medical Sciences for Cancer Research. Each patient had signed informed consent for medical record review and tissue sample donation before surgery. The local ethical committee of Mashhad University of Medical Sciences, Mashhad, Iran, had approved this study.

Regarding to disease recurrence after 6 months of tamoxifen treatment period, recruited participants were divided into two groups: tamoxifen sensitive (TAM-S) ($n = 36$) and tamoxifen resistance (TAM-R) (n = 36). Patients included in TAM-S group, had received standard adjuvant tamoxifen treatment for 5 years or more without any symptoms of disease recurrence, while TAM-R patients had experienced cancer recurrence (local or regional recurrence, distant metastasis, or death), while receiving tamoxifen treatment for at least 6 months. Among TAM-R patients, 17/36 (47%) experience metastasis and 19/36 (53%) died during tamoxifen treatment period. Median time to recurrence for tamoxifen resistance patients was 25 months. The median follow up time for tamoxifen sensitive patients was 85 months. All of the patients were followed up until August 2016. The mean age at diagnosis time ± standard deviation of TAM-R and TAM-S patients were (48.21 ± 10.54) and (44.28 ± 8.46), respectively; with no significant difference ($P = 0.118$). More details on patients' characteristics have been described previously [15].

RNA Extraction and cDNA Synthesis

After surgery, fresh tumor samples have been stored in liquid nitrogen. The entire fresh frozen tumors underwent total RNA extraction with RiboEx Total RNA (301–001) in clean RNase-free tube, according to the manufacturer's instruction. Isolated RNA samples were eluted in DEPC-treated water. RNA integrity was verified by agarose gel electrophoresis and 28S, 18S and 5S bands have been observed. The purity of RNA samples was verified by NanoDrop2000C Spectrophotometer (Thermo Scientific). 260/280 and 260/230 ratios of each RNA sample was around 2 and 1.9–2.2, respectively. Finally, isolated RNA samples were treated with Thermo Scientific™ DNase I (RNase-free, EN0521) in order to exclude contaminated genomic DNA. Complementary DNA (cDNA) was synthesized with random hexamer primers using RevertAid First Strand cDNA Synthesis Kit (Fermentas) using 2 μg of total RNA in a final reaction volume of 20 μl.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

qRT-PCR assay was performed using reverse transcription (RT) products in order to detect P28GANK, Nanog, Oct-4 and Sox-2 expression. SYBR-Green SYBR® Premix Ex Taq ™ II (TliRNaseH Plus), Bulk, Takara (RR820L) was used as Real-Time master mix and all of the experiments performed using LightCycler ® 96 Instrument-Roche. β-actin was considered as endogenous control and reference gene to normalize qRT-PCR results [16]. Furthermore, in order to determine the PCR amplification efficiency, standard curves were generated. Serial 10-fold dilutions of pooled cDNA were prepared to produce standard curve for each primer pairs. Amplification efficiencies were approximately equivalent for all primer pairs. Comparative Ct method was used to determine relative mRNA expression level of P28GANK and stem cell markers compared to β-actin [17]. The primer sequences of Gankyrin [17] and β-actin [18] were obtained from previous studies. However, primer pairs of stem cell markers were designed. All of the splicing variants of all three stem cell markers were detected in NCBI (National Center for Biotechnology Information), as follow: Nanog (NM_001297698.1&NM_024865.3); Oct-4 (NM_001285987.1, NM_203289.5, NM_001285986.1, NM_001173531.2&NM_002701.5) and Sox-2 (NM_003106.3). The common exons were selected and Beacon Designer 8 software was used for primer design. BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi), *in silico* PCR analysis (https://genome.ucsc.edu/cgi-bin/hgPcr), and amplicon secondary structure analysis (http://unafold.rna.albany.edu/?q=mfold/DNA-Folding-Form) were performed to detect primer specificity and the feasibility of Real Time PCR experiments. Primer sequences were demonstrated in Table 1. Each qPCR reaction was performed in a total volume of 25 μl, containing 12.5 μl of SYBR-Green master mix, 10 pmol of each primer, 1 μl (100ηg) cDNA. In non-template control tube, 1 μl of water was added instead of cDNA. Real Time PCR experiments were initiated with a 5 min denaturation at 95 °C, followed by 40 cycles of denaturation at 95 °C for 30 s and annealing-extension at 60 °C for 1 min. In order to determine the specificity of the reaction and target amplification, melt curve analysis was performed at the end of each Real Time PCR reaction by increasing the temperature from 60 to 95 °C with a temperature transition rate of 0.5 °C/s. Single sharp peaks were obtained for each target gene with no amplification of non-specific products or primer-dimer. No melting peaks were observed in no-template controls.

Tissue Microarray Construction and Immunostaining

Seventy two formalin-fixed paraffin-embedded (FFPE) breast tumor tissues were recruited for TMA construction which was defined previously [19]. In brief, representative tumor areas of each FFPE block were carefully selected regarding H&E-stained sections by an expert pathologist. Three 1.5 mm tissue cores of representative tumor area were retrieved from each specimen and then inserted into ready-made holes in three recipients TMA blocks randomly. Finally, three TMA blocks were prepared for each group of patients. Each TMA blocks contained 36 tumors. In the second step, the pathologist reviewed hematoxylin and eosin- stained slides of constructed TMA blocks in order to confirm the presence of tumor representative areas of the original blocks. In order to perform immunohistochemical analysis, 4-μm-thick sections of TMA

Table 1 Real Time PCR primer sequences

Oligonuleotide	Sequence	Length (bp)	PCR product size (bp)
P28GANK	Forward: 5'-AGCAGCCAAGGGTAACTTGA-3'	20	155
	Reverse:5'-TACTTGCTCCTTGGGACACC-3'	20	
Nanog	Forward:5'-GCAGAGAAGAGTGTCGCAAA −3'	20	83
	Reverse:5'-AGCTGGGTGGAAGAGAACAC -3'	20	
Oct-4	Forward:5'-TATTCAGCCAAACGACCATC-3'	20	109
	Reverse:5'-TTGTTGTCAGCTTCCTCCAC-3'	20	
Sox-2	Forward:5'-GCAAAAGAGGAGAGTAAGAAACAGC -3'	25	132
	Reverse:5'-CGTGAGTGTGGATGGGATTGG −3'	21	
β-actin	Forward:5'-TCATGAAGTGTGACGTGGACATC-3'	22	156
	Reverse:5'-CAGGAGGAGCAATGATCTTGATCT-3'	24	

blocks were prepared, mounted on slides, heated in a dry oven at 60 °C for 30 min, deparaffinized with xylene (100%) twice and rehydrated through graded concentrations of ethanol. For blocking endogenous peroxidase activity, TMA slides were treated with 3% hydrogen peroxide for 15 min and then heat-induced antigen retrieval was performed using EDTA Tris buffer (pH 7.4) in a 94–98 °C water bath for 30 min for Gankyrin. In immonohistochemical analysis for Nanog, Oct-4 and Sox-2, antigen retrieval was performed through cooking the sections in 10 mm sodium citrate buffer, pH 6.0, for 10 min in an autoclave. The sections were then incubated with the primary antibody against Gankyrin (Gankyrin, sc-8991, 1:200 dilution; Nanog, ab80892, 1:400 dilution; Oct-4, sc-5279, 1:250 dilution; Sox-2, sc-365,823, 1:150 dilution) followed by incubation with a secondary antibody (Biogenex) at room temperature. The immunohistochemical reactions were visualized using by 3, 3′ -diaminobenzidine DAB reagent (Gene Tech) at room temperature and finally counterstained with hematoxylin for 30 s. The slides were dehydrated and mounted for microscopic examination under a light microscope (Nikon, Elipse E200). The light microscope was coupled with a digital camera. Negative control for each antibody was prepared by replacement of primary antibody with PBS. Human urinary bladder tissue, testis, adrenal gland tissue and human squamous epithelial cells of esophagus tissue blocks were stained immunohistochemically as appropriate positive control tissues for Gankyrin, Nanog, Oct-4 and Sox-2, respectively.

Evaluation of Immunohistochemical Staining

In order to analyze protein expression of Gankyrin and three stem cell markers, immunostained tissue microarrays were reviewed and scored by two pathologists, independently, who were blinded to the clinical features and outcomes of the study. A good concordance >85% of cases have been observed between the two pathologists. Both pathologists reassessed all of the immunostained sections with contradictory explanation under a multi-head microscope and finally they reached a full agreement. Assessment of the expression of Gankyrin and all three stem cell markerswere performed by semi-quantitative scoring system. The percentage of positive tumor cells and the staining intensity were included in the scoring of each case. Immunopositivity of Gankyrin and stem cell markers including, Nanog, Oct-4 and Sox-2 were identified as follow: the percentage of tissue with positive staining (0, negative; 1, positive in <25%; 2, positive in 25–50%; 3, positive in 50–75% and 4, positive in>75% of tumor cells) and the staining intensity (0, negative;1, weakly positive;2, moderate staining and 3, high staining). IHC scores were determined by multiplying the staining intensity by the percent of positive-staining tissue [20, 21]. Finally, expression of all studied genes including Gankyrin and stem cell markers was graded as: score 0, negative; score 1–4, weak expression; score 5–8, moderate expression; and score 9–12, strong expression.

Survival Analysis

Survival analyses were performed using Kaplan–Meier and Cox regression analysis to study the association between the expression of desired genes and the risk of disease recurrence or death. For survival analysis, patients were divided into two main groups: low expression versus high expression regarding median of mRNA expression. Cox regression analysis were applied to determine whether expression of desired genes had predictive value when added to the base model of other factors or not. Disease free survival (DFS) was defined as the time between the date of surgery to the date of first confirmed disease recurrence. In DFS analysis, local or regional disease recurrence or distant metastasis were considered as event. Overall survival (OS) was defined by the duration between the dates of surgery to death date. In OS analysis, death was defined as event. The patients had no disease other than breast cancer influencing survival.

Statistical Analysis

Statistical analyses were performed using SPSS software version 20. Clinical parameters as well as mRNA expression of Gankyrin and stem cell markers were collected. Mann-Whitney U Test was used to assess the differential expression of nonparametric data of mRNA and protein expressions between TAM-S and TAM-R patients. Spearman rank correlation test was performed to analyze the association between the expressions of all four studied transcripts. The relationship between protein and mRNA expression levels of each studied gene was examined using the spearman correlation coefficient analysis. The crude and adjusted odds ratios (ORs) and 95% confidence intervals (CIs) were calculated by performance of univariate and multivariate logistic regression analyses in order to define relation between expression levels of desired genes and tamoxifen response. The effects of elevated mRNA expression levels of P28GANK and stem cell markers on patient survival were estimated by the Kaplan–Meier method and the differences between the two groups were compared using the log-rank test. To compute the hazard ratio (HR) in the analysis of disease free survival and overall survival, the clinicopathological variables as well as mRNA expression of target genes were included in Cox univariate regression analysis. Those clinical indicators that had statistical significance in the Cox univariate regression analyses were subsequently examined in multivariate Cox regression models to determine independent predictors for DFS and OS. HR was represented with 95% confidence intervals (95% CI). $P < 0.05$ was considered as statistically significant.

Results

Comparison of mRNA Expression of P28GANK and Stemness Factors Between TAM-S and TAM-R Breast Carcinoma Patients

The expression of P28GANK, Nanog, Oct-4 and Sox-2 in TAM-S and TAM-R breast tumor tissues was evaluated using qRT-PCR experiments. The expressions of P28GANK and stemness factors were observed in all breast tumor tissues regardless of whether they were TAM-R or TAM-S.

Mean fold increase in the mRNA expression of P28GANK in tamoxifen resistant compared to tamoxifen sensitive patients were about 4.89 and the observed difference was statistically significant ($P = 0.003$). Mean fold increase in the mRNA expression of stem cell markers in tamoxifen resistant breast tumors in comparison to tamoxifen sensitive ones were 1.69, 2.13, and 2.62 for Nanog, Oct-4, and Sox-2, respectively (Table 2). Expression of Oct-4 ($P < 0.001$) and Sox-2 ($P = 0.035$) were significantly higher in TAM-R in comparison to TAM-S patients. The observed difference was not statistically significant about Nanog expression ($P = 0.096$).

Correlations Between mRNA Expression of P28GANK, Nanog, Oct-4 and Sox-2 in Breast Carcinoma Patients

Spearman correlation coefficient analyses were performed to assess the correlation between the expressions of all studied genes. Our results showed that there was no significant association between expression of P28GANK and two stemness markers including Nanog ($r_s = 0.192$, $P = 0.118$) and Sox-2 ($r_s = 0.137$, $P = 0.256$), although it was near the significance criterion about Oct-4 ($r_s = 0.224$, $P = 0.067$). Nanog expression was positively correlated with the expression of Sox-2 ($r_s = 0.385$, $P = 0.001$) and Oct-4 ($r_s = 0.53$, $P < 0.001$). Furthermore, significant positive correlation between the expression of Sox-2 and Oct-4 was found ($r_s = 0.477$, $P < 0.001$).

Gankyrin and Stemcell Markers Immunostaining in Breast Carcinoma Tumors

Protein expressions of all studied genes were assessed through immunohistochemical staining on TMA blocks (Fig. 1). Protein expression of P28GANK was observed in 33/36(92%) of TAM-R and 19/36(52%) of TAM-S breast tumor tissues. Higher expression of P28GANK in TAM-R samples was statistically significant ($P < 0.001$). Nanog expression was detected in 26/36(72%) of tamoxifen sensitive tumors compared to 29/36(80%) of tamoxifen resistant ones ($P = 0.173$). Oct-4 expression was assessable in 23/36(64%) of TAM-S and 29/36(80%) of TAM-R tissue specimens ($P = 0.011$). The positivity of Sox-2 was seen in 29 out of 36 TAM-S tumors (80%) compared to 30/36(83%) of TAM-R cases ($P = 0.064$). Differential expressions of Gankyrin and three studied stem cell markers in TAM-R and TAM-S breast carcinoma patients have been summarized in Supplementary Table S1.

Correlation Between mRNA and Protein Expression Levels of Gankyrin and Stemcell Markers

The relationship between mRNA and protein expression level of each studied gene was analyzed. Spearman rank correlation revealed a statistically significant correlation between mRNA and protein expression of Gankyrin (r_s: 0.371, $P = 0.002$), Oct-4 (r_s: 0.277, $P = 0.018$) and Sox-2 (r_s: 0.428, $P < 0.001$) while close to but not quite statistically significant correlation was found between Nanog mRNA and protein expression level in recruited patients (r_s: 0.220, $P = 0.063$).

Table 2 Mean fold increase of expression levels of P28GANK and stem cell markers in tamoxifen resistant tumor tissues ($N = 36$) compared to tamoxifen sensitive ones (N = 36)

Gene		TAM-R[a] tumors	TAM-S[b] tumors	Fold increase($2^{-\Delta\Delta CT}$)[c]
P28GANK	Mean of ΔCT ± SD[d]	3.18 ± 2.57	5.47 ± 2.85	4.89
Nanog	Mean of ΔCT ± SD	9.92 ± 1.85	10.68 ± 2.15	1.69
Oct-4	Mean of ΔCT ± SD	7.19 ± 2.37	8.28 ± 2.07	2.13
Sox-2	Mean of ΔCT ± SD	9.86 ± 3.99	11.25 ± 3.29	2.62

a.Tamoxifen Resistance, b. Tamoxifen Sensitive, c. TAM-R in comparison to tamoxifen sensitive tumors, d. Standard Deviation

Correlation Between Clinicopathological Characteristics and Tamoxifen Treatment Outcome

Considering that the different subtypes of breast cancer show different prognostic value, logistic regression analysis was performed to evaluate the effects of P28GANK and stemness markers expression on treatment outcome after adjustment of other confounding factors on ER^+ tamoxifen-treated breast carcinoma patients ($N = 72$). In each categorical variable, the first-ordered category was considered as the reference level. In the first step, univariate logistic regression analysis indicated that N stage N2, N3 ~ N0, N1 (OR = 2.906, 95% CI: 1.091–7.741; $P = 0.033$), Extracapsular nodal extension (ECE) (OR = 3.52, 95% CI: 1–12.38; $P = 0.049$), Perineural invasion (PNI) (OR = 3.250, 95% CI: 1.217–8.676; $P = 0.019$), P28GANK mRNA expression high expression~ low expression (OR = 9.286, 95% CI: 3.057–28.205; $P < 0.001$), Nanog mRNA expression high expression~ low expression (OR = 4, 95% CI: 1.501–10.658; $P = 0.006$), Oct-4 mRNA expression high expression~ low expression (OR = 6, 95% CI: 2.154–16.712; $P = 0.001$) and Sox-2 mRNA expression high expression~ low expression (OR = 3.385, 95% CI: 1.277–8.972; $P = 0.014$) were the important considering features that could affect on disease recurrence in patients.

In the second step, independent predictive factors which were statistically significant in univariate analysis were involved in multivariate logistic regression model in order to identify independent predictors of treatment after adjustment of other clinicapathological features. The results showed that P28GANK mRNA expression high expression~ low expression (OR = 15.861, 95% CI: 2.837–88.675; $P = 0.002$) was remained as independent predictors of treatment after adjustment of other clinicopathological features. The same results were observed about Oct-4 (OR = 9.344, 95% CI: 1.61–54.22; $P = 0.013$) (Table 3).

Correlation of P28GANK and Stemness Factors Expressions with Survival of Breast Carcinoma Patients

Kaplan-Meier survival curves based upon mRNA expression of P28GANK, Nanog, Oct-4 and sox-2 were generated for analysis of disease free survival and overall survival in all recruited patients ($N = 72$). Survival comparison of the patients with high or low level expression of studied genes indicated that patients with higher expression of P28GANK in their tumors did not benefit from tamoxifen treatment. Overexpression of P28GANK transcript predicted poorer DFS ($P < 0.001$, Fig. 2a) and OS ($P = 0.002$, Fig. 2b) compared to low expression. Comparison survival of the patients with high or low expression of stem cell markers indicated that overexpression of Nanog significantly associated with poor disease free survival ($P = 0.006$, Fig. 2c). In contrast, no significant correlation was found between overexpression of Nanog and overall survival ($P = 0.071$, Fig. 2d). Higher expression of Oct-4 negatively associated with disease free survival ($P < 0.001$, Fig. 2e) and overall survival ($P = 0.004$, Fig. 2f). Survival analysis of Sox-2 demonstrated that patients with low expression of Sox-2 have a trend to better disease free survival ($P = 0.018$, Fig. 2g); however, no significant association was found between Sox-2 expression and overall survival ($P = 0.228$, Fig. 2h).

Univariate and Multivariate Cox Regression Analysis

Multivariate Cox regression models were used to ascertain relevant predictors for disease free survival and overall survival in tamoxifen-treated breast carcinoma patients ($N = 72$) after adjustment of other effective factors. In the first step, univariate Cox proportional hazard analysis was performed. The first-ordered category was considered as reference group in each categorical variable. Data analysis indicated that patents with low mRNA expression of P28GANK (HR = 3.572, 95% CI: 1.718–7.428; $P = 0.001$), Nanog (HR = 2.546, 95%CI: 1.268–5.110; $P = 0.009$), Oct-4 (HR = 3.904, 95%CI: 1.917–7.948; $P < 0.001$) and Sox-2 (HR = 2.184, 95%CI: 1.090–4.375; $P = 0.028$) showed significant trend of better DFS. Besides, patients with low mRNA expression of P28GANK (HR = 4.778, 95% CI: 1.583–14.420; $P = 0.006$) and Oct-4 (HR = 4.033, 95%CI: 1.447–11.243; $P = 0.008$) have significantly longer OS. Other clinicopathological factors, which were statistically significant in univariate Cox regression analysis were presented in Table 4.

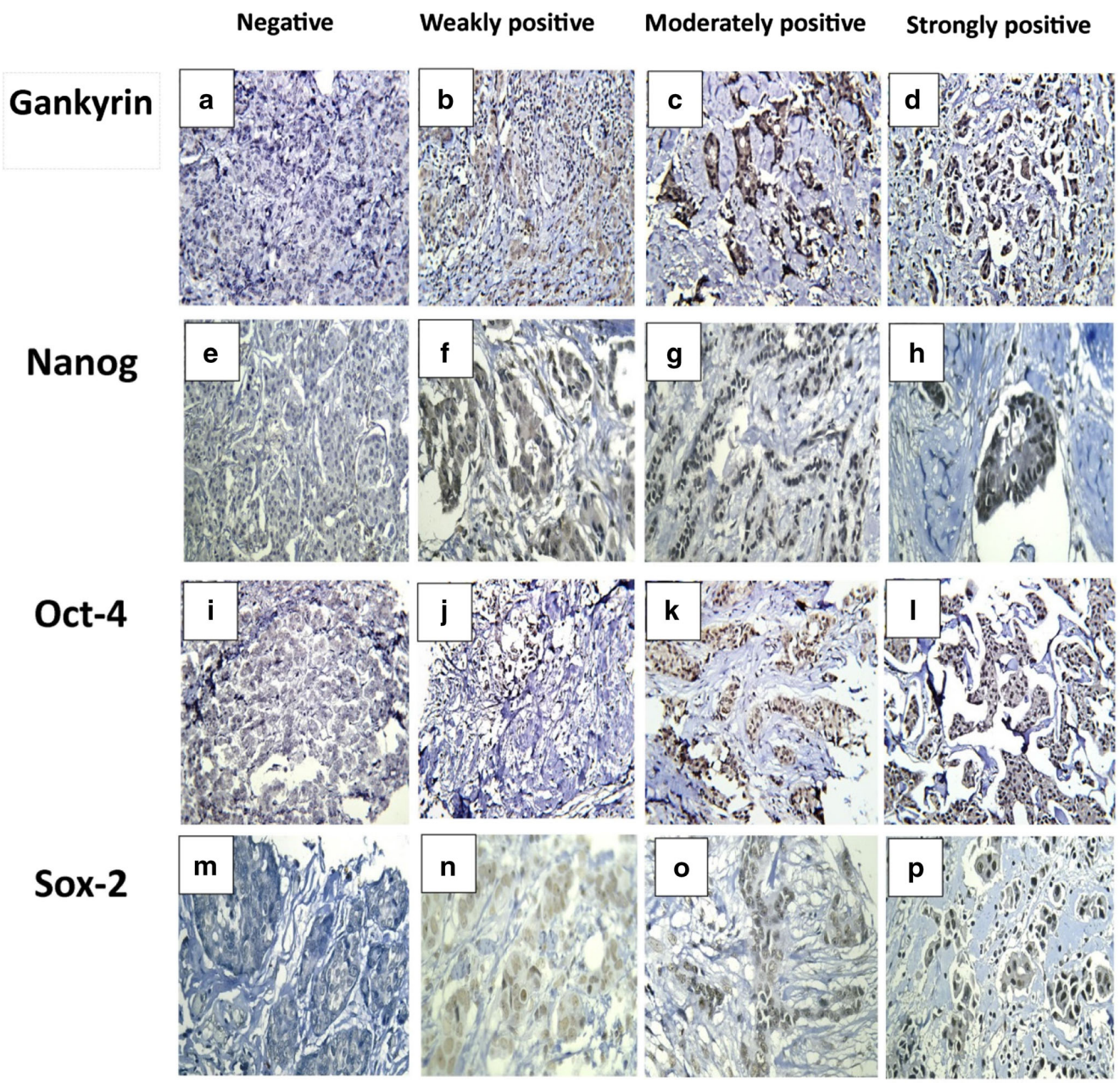

Fig. 1 Immunohistochemical staining of P28GANK (a, b, c, d), Nanog (e, f, g, h), Oct-4 (i, j, k, l) and Sox-2 (m, n, o, P) proteins in estrogen receptor positive breast tumors. Positive staining was observed as brown color. Negative (a, e, i, m), weakly positive (1+) (b, f, j, n), moderately positive (2+) (c, g, k, o), and strongly positive (3+) (d, h, l, p) staining of all studied genes (400 × magnification)

In the second step, multivariate Cox regression analysis was performed using categorical indicators, which were statistically significant in univariate analysis. N stages, Extracapsular nodal extension (ECE), DCIS histology, Perineural invasion (PNI) and mRNA expression of P28GANK, Nanog, Oct-4 and Sox-2 were involved in multivariate Cox regression model for DFS. As shown in Table 4, N stage (HR = 2.128, 95% CI: 1.048–4.323; $P = 0.037$), Perineural invasion (PNI) (HR = 2.411, 95% CI: 1.132–5.135; $P = 0.022$), Oct-4 mRNA expression (HR = 2.779, 95% CI: 1.330–5.809; $P = 0.007$) remained as independent prognostic factors for DFS after adjustment of other criteria. In the presented model P28GANK mRNA expression was near to the significant level (HR = 2.184, 95% CI: 0.958–4.980; $P = 0.063$). In multivariate Cox regression model for OS histological grade and mRNA expression of P28GANK and Oct-4 were considered. Data analysis indicated that overexpression of P28GANK transcript (HR = 4.004, 95% CI:

Table 3 Univariate and multivariate logistic regression models for tamoxifen response in estrogen receptor positive tamoxifen-treated breast carcinoma patients ($N = 72$)

Factor of base model	Univariate analysis			Multivariate analysis		
	Odds Ratio	95% CI	*P* value	Odds Ratio	95% CI	*P* value
Histological grade (MBR[a])						
Grade I						
Grade II	2.252	0.780–6.505	0.134			
Grade III	2.844	0.713–11.351	0.139			
T stage						
T1						
T2	0.608	0.144–2.576	0.499			
T3	1.467	0.282–7.627	0.649			
T4	0.800	0.037–17.196	0.887			
N stage						
N0, N1						
N2, N3	2.906	1.091–7.741	0.033*	2.826	0.570–14.021	0.204
Extracapsular nodal extension (ECE)						
No						
Yes	3.52	1–12.38	0.049*	2.657	0.334–21.120	0.356
DCIS histology						
Non-Comedo type						
Comedo type	1.290	0.317–5.256	0.722			
Nipple involvement						
No						
Yes	1	0.290–3.45	>0.99			
Lymphatic invasion						
No						
Yes	2.2	0.712–6.793	0.17			
Perineural invasion (PNI)						
No						
Yes	3.250	1.217–8.676	0.019*	2.484	0.56–11.013	0.231
PR status						
Positive						
Negative	1.130	0.428–2.985	0.805			
HER-2 status						
Positive						
Negative	1.540	0.534–4.438	0.424			
p53 status						
Positive						
Negative	1.909	0.696–5.236	0.209			
P28GANK mRNA expression						
Low expression						
High expression	9.286	3.057–28.205	$P < 0.001$*	15.861	2.837–88.675	0.002**
Nanog mRNA expression						
Low expression						
High expression	4	1.501–10.658	0.006*	1.664	0.391–7.080	0.490
Oct-4 mRNA expression						
Low expression						
High expression	6	2.154–16.712	0.001*	9.344	1.610–54.22	0.013**
Sox-2 mRNA expression						
Low expression						
High expression	3.385	1.277–8.972	0.014*	2.616	0.518–13.205	0.244

[a] MBR: Modified-Bloom-Richardson

$P < 0.05$ was considered statistically significant

* Clinicopathological features which were statistically significant in univariate analysis and were included in multivariate logistic regression model

** Clinicopathological features which were statistically significant in multivariate logistic regression model

1.293–12.397; $P = 0.016$) and Oct-4 transcript (HR =3.437, 95% CI: 1.194–9.896; $P = 0.022$) were effective predictive factors of overall survival after adjustment of other significant variables in ER^+ tamoxifen-treated breast carcinoma patients (Table 5).

Discussion

Progression of drug resistance is one of the most important obstacles in cancer treatment. Although tamoxifen has improved survival of countless ER^+ breast carcinoma patients,

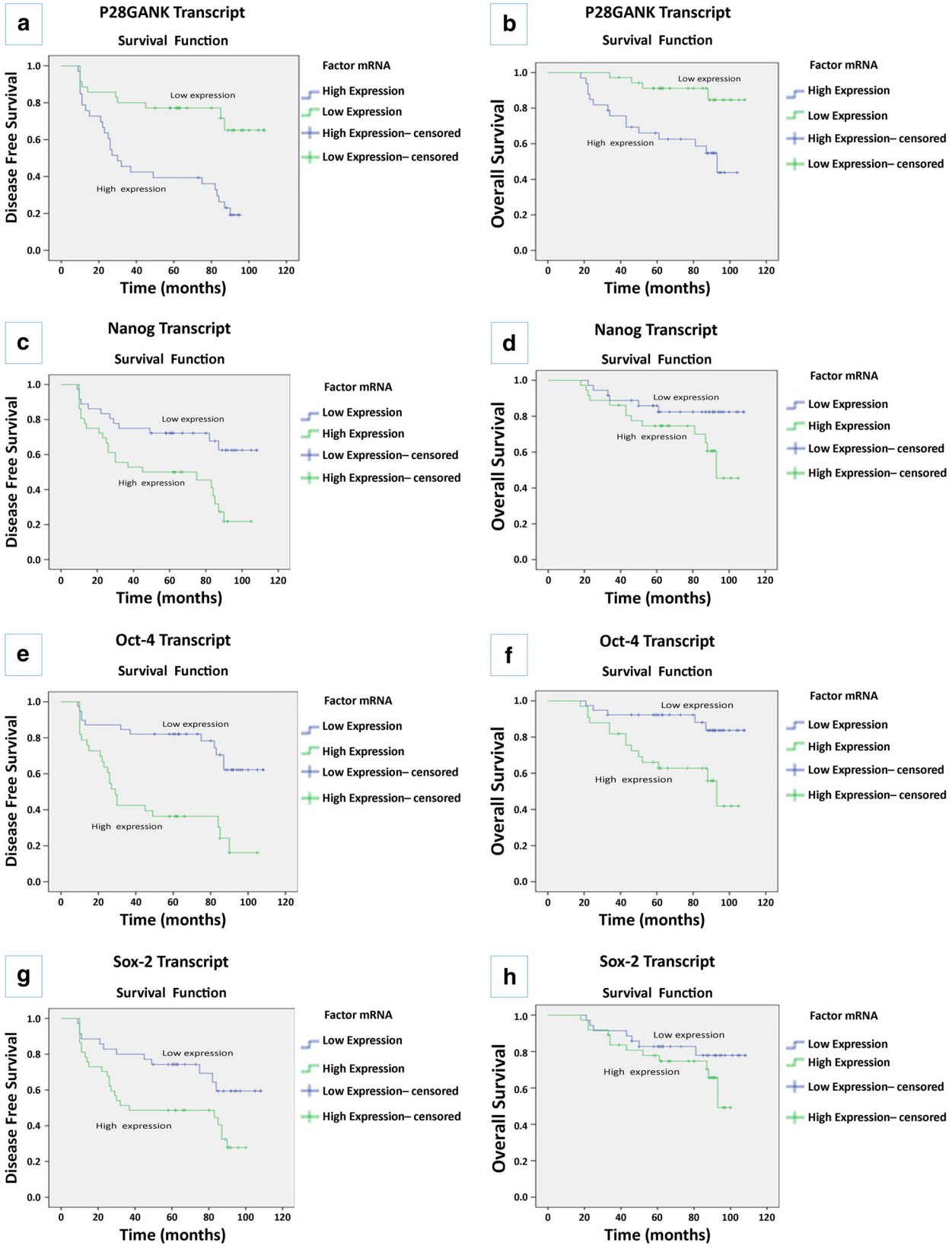

Fig. 2 Kaplan–Meier (KM) survival curves of breast carcinoma patients stratified by the median values of mRNA expression of P28GANK (a, b), Nanog (c, d), Oct-4 (e, f) and Sox-2 (g, h) for disease free survival (a, c, e, e), and overall survival (b, d, f, h) in 72 estrogen receptor positive tamoxifen-treated breast carcinoma patients

Table 4 Univariate Cox regression models for disease free survival and overall survival in estrogen receptor positive tamoxifen –treated breast carcinoma patients($N = 72$)

Factor of base model	Univariate Cox regression model for DFS			Univariate Cox regression model for OS		
	HR	95% CI	*P* value	HR	95% CI	*P* value
Histological grade (MBR[a])						
Grade I						
Grade II	2.034	0.915–4.520	0.081	3.969	1.104–14.261	0.035**
Grade III	2.016	0.777–5.229	0.149	3.207	0.715–14.328	0.128
T stage						
T1						
T2	0.691	0.257–1.857	0.464	0.526	0.142–1.954	0.338
T3	1.190	0.413–3.432	0.747	1.001	0.248–4.032	0.999
T4	1.438	0.167–12.39	0.741	2.481	0.256–24.071	0.433
N stage						
N0, N1						
N2, N3	2.325	1.2–4.506	0.012*	1.852	0.750–4.574	0.182
Extracapsular nodal extension (ECE)						
Absent						
Present	2.174	1.057–4.469	0.035*	2.002	0.76–5.274	0.160
DCIS histology						
Non-Comedo type						
Comedo type	1.017	0.395–2.620	0.972	1.840	0.609–5.557	0.280
Nipple involvement						
Absent						
Present	1.417	0.585–3.434	0.441	1.099	0.317–3.810	0.881
Lymphatic invasion						
Absent						
Present	1.785	0.742–4.296	0.196	1.825	0.531–6.268	0.339
Perineural invasion (PNI)						
Absent						
Present	2.322	1.199–4.499	0.013*	0.787	0.310–1.999	0.614
PR status						
Positive						
Negative	1.156	0.585–2.283	0.677	2.466	0.997–6.102	0.051
HER-2 status						
Positive						
Negative	1.144	0.519–2.521	0.739	1.223	0.403–3.706	0.723
p53 status						
Positive						
Negative	0.570	0.268–1.214	0.145	1.004	0.381–2.645	0.994
P28GANK mRNA expression						
Low expression						
High expression	3.572	1.718–7.428	0.001*	4.778	1.583–14.420	0.006**
Nanog mRNA expression						
Low expression						
High expression	2.546	1.268–5.110	0.009*	2.374	0.899–6.270	0.081
Oct-4 mRNA expression						
Low expression						
High expression	3.904	1.917–7.948	$P < 0.001$*	4.033	1.447–11.243	0.008**
Sox-2 mRNA expression						
Low expression						
High expression	2.184	1.090–4.375	0.028*	1.720	0.674–4.390	0.257

[a] MBR: Modified-Bloom-Richardson

$P < 0.05$ was considered statistically significant

* Statistically significant variables affecting disease free survival in univariate Cox regression model

** Statistically significant variables affecting overall survival in univariate Cox regression model

a large proportion of tamoxifen-treated patients experience disease recurrence [22]. Therefore, looking for novel biomarkers that are associated with tamoxifen-refractory is an important research area for scientists who work on breast malignancies [23]. It has been well established that overexpression of some oncogenes and low expression of some tumor suppressor proteins are involved in resistance development [3]. P28GANK is a newly defined oncoprotein with anti-apoptotic activity that mediates selective destruction of different cell cycle regulators and tumor suppressor proteins

[24]. It also plays important roles in proliferation, invasion and metastasis of different cancers [9].

Taheri et al. reported that P28GANK expression was upregulated in multidrug resistance (MDR) breast cancer cell line MCF-7/MX in comparison to their non-MDR counterparts (MCF-7) [25]. In another study, Chen et al. showed that P28GANK overexpression contributes to arsenic trioxide resistance in liver and gastric cancer cells [26]. Furthermore, P28GANK contributes to induction of drug resistance in ovarian cancer and gastric cancer cells [9]. These results are consistent with and support the findings of this study that expression of P28GANK was significantly increased in TAM-R tumor tissues in comparison to TAM-S ones and breast cancer patients with higher expression of P28GANK don't benefit from tamoxifen treatment.

Furthemore, multivariate logistic regression analysis showed that overexpression of P28GANK and Oct-4 transcripts could have independent predictive value in tamoxifen response in ER^+ breast carcinoma patients after adjustment of other clinicopathological features. In the present study, correlation between mRNA expression of P28GANK and Oct-4 was near significant level, although a significant positive association was observed between protein expression levels of P28GANK and Oct-4 (r: 0.271, $P = 0.021$) (data not shown). Qian et al. reported that P28GANK prevents degradation of Oct-4 in hepatocarcinogenesis and promotes expansion of cancer stem cells. P28GANK prevents the direct binding between WWP, an E3 ubiquitin ligase, and Oct-4. WWP2 mediates Oct-4 ubiquitination and degradation [27]. In addition, P28GANK knockdown leads to reduction of Nanog expression, suggesting that P28GANK could

Table 5 Multivariable Cox regression models for disease free survival and overall survival in estrogen receptor positive tamoxifen–treated breast carcinoma patients ($N = 72$)

Factor of base model	Multivariate analysis for DFS			Multivariate analysis for OS		
	Hazard Ratio	95% CI	P value	Hazard Ratio	95% CI	P value
Histological grade (MBR[a])						
Grade I						
Grade II				2.794	0.77–10.137	0.118
Grade III				2.540	0.543–11.870	0.236
N stage						
N0, N1						
N2, N3	2.128	1.048–4.323	0.037*			
Extracapsular nodal extension (ECE)						
Absent						
Present	1.336	0.599–2.981	0.479			
Perineural invasion (PNI)						
Absent						
Present	2.411	1.132–5.135	0.022*			
P28GANK mRNA expression						
Low expression						
High expression	2.184	0.958–4.980	0.063	4.004	1.293–12.397	0.016**
Nanog mRNA expression						
Low expression						
High expression	1.390	0.630–3.069	0.415			
Oct-4 mRNA expression						
Low expression						
High expression	2.779	1.330–5.809	0.007*	3.437	1.194–9.896	0.022**
Sox-2 mRNA expression						
Low expression						
High expression	1.918	0.879–4.187	0.102			

[a] MBR: Modified-Bloom-Richardson

$P < 0.05$ was considered statistically significant

* Statistically significant variables affecting disease free survival in multivariate Cox regression model

** Statistically significant variables affecting overall survival in multivariate Cox regression model

control stem cell behavior and expansion of tumor-initiating cells [14]. Our results showed high-level of P28GANK and Oct-4 proteins expression in TAM-R tumors compared to TAM-S samples. Furthermore, a significant correlation was observed between mRNA and protein expression of P28GANK, Oct-4 and Sox-2.

It is becoming increasingly evident that induction the expression of stem cell markers, e.g. Oct-4 and Sox-2 take a role in acquired drug resistance and disease recurrence in different cancers. Additionally, inhibiting of stemness in cancer cells could reverse acquired drug resistance [28]. It has been indicated previousely that overexpression of stem cell markers promotes tamoxifen resistance in breast cancer cells [29]. The current study demonstrated a significant higher expression of Oct-4 and Sox-2 stemness factors in TAM-R tumor tissues in comparison to TAM-S ones. Our findings were confirmed by Arif et al. who observed overexpression of Nanog, Oct-4 and Sox-2 in tamoxifen resistance breast cancer cells (MDA-MB-231 and MCF7). Furthermore, their expriments indicated that ransfection of tamoxifen resistance breast cancer cells with Nanog siRNA could lead to apoptosis induction and inhibition of cell proliferation [30]. In the same manner, Leung et al. showed an increased potential for mammosphere formation, the stem-like character, and expression of stemness factors including Nanog, Oct-4 and Sox-2 in breast cancer cells (MCF-7) after hormone therapy [31], therefore, they suggested that as an early response to endocrine therapy, breast cancer cells acquire stem cell-like properties.

Kaplan-meire survival analysis indicated that increased expression of P28GANK and Oct-4 significantly associated with poorer survival function. Similarly, multivariate Cox regression models demonstrated that overexpression of P28GANK and Oct-4 independently predict worse disease free survival and overall survival in surgical breast carcinoma patients receiving adjuvant tamoxifen. Our results were consistent by other studies that show Oct-4 is a negative prognostic factor in patients with esophageal squamous cell carcinoma, gastric, breast and bladder cancer [32]. Similar negative associations have been identified between P28GANK expression and survival function in various malignancies including, hepatocellular carcinoma, colorectal cancer, esophageal squamous cell carcinoma (ESCC), and glioma [33].

Furthermore, Kaplan-meire survival analysis revealed that patients with higher expression of Nanog and Sox-2 have a trend to poorer disease free survival. In line with our results, Yang et al. indicated that expression of Nanog, Oct-4 and Sox-2 associated with worse overall survival in HER-2 positive breast cancer patients [34]. In contrast, overexpression of Sox-2 was associated with better survival in squamous cell lung cancer [35]. It has also been exhibited that patients with low Nanog and Oct-4 expressions in renal cell carcinoma tissues had significantly higher survival rates [36].

In summary, the present study confirmed that the expression of the stemness marker Oct-4 and P28GANK oncoprotein are significantly associated with tamoxifen response. Moreover, survival analyses suggest that patients with overexpression of P28GANK and Oct-4 were more likely to experience poorer survival during tamoxifen treatment period. It is considerable that more studies are still needed to elucidate the exact functions of these molecules in progression of tamoxifen resistance and their possible application as prognostic markers.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval All experiments performed in this study were in accordance with the ethical standards of the local ethical committee at Mashhad University of Medical Sciences, Mashhad, Iran.

Informed Consent Each recruited patient has signed written informed consent for retention and analysis of her tissue and long-term follow-up.

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