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Gene Expression of OCT4 and NANOG Correlated with Advanced Stage and Worse Survival in Breast Cancer Patients

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Abstract The present study aimed to show the correlation between expression of cancer stem cell markers (OCT4 and NANOG) with both clinicopathological features and survival of breast cancer (BC) patients. **Methods**: The gene expressions of OCT4 and NANOG were quantified using real time polymerase chain reaction, clinicopathological data have been collected from patients' data records and patients were followed-up with a median duration of 110 months. **Results:** OCT4 ($p<0.001$), and NANOG ($p<0.001$) expressions were upregulated in BC tissues compared to adjacent normal tissues. OCT4 and NANOG were associated with poor histological grade ($p=0.029$, 0.025) and advanced clinical stage ($p=0.001$, 0.042 respectively). OCT4 alone showed a significant association with lymph nodes involvement ($p=0.006$), metastasis ($p=0.024$) and was significantly correlated to patients' age ($p=0.009$). NANOG also showed a significant positive correlation with ERα and PR receptors expression ($p=0.004$ and 0.005 respectively). Kaplan–Meier curves disclosed that NANOG ($p=0.028$, 0.050) positive expression was associated with worse DFS and OS, while OCT4 ($p=0.200$, 0.205) was correlated with poor DFS and OS but not significant statistically. Univariate analysis using Cox proportional hazards regression model analysis showed that OCT4 ($p = 0.002$), NANOG ($p = 0.021$), and ERα status ($p = 0.004$) had significant predictive values for poor DFS. However, the multivariate analysis did not show that any of them can be used as independent prognostic markers for DSF. **Conclusions:** From these findings, it may be concluded that the upregulated expressions of OCT4 and NANOG were associated with worse clinical outcome and could be used as predictive markers for poor DFS in BC patients.

Keywords: *breast cancer, stemness markers, Oct-4, NANOG, prognosis*

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1. Introduction

Breast cancer (BC) is one of the most common types of cancer and is the driving reason of death in women [1]. Tumors, including BC, are composed of biologically diverse cell populations. This diversity is believed to be as a result of a little subpopulation of cells that represent 1-5% of all tumor cells known as cancer stem cells [2]. Cancer stem cells (CSCs) are believed to be accountable for deriving the tumorigenesis process. There is also an expanding evidence that they may be responsible for tumor progression, metastasis, and resistance to therapy [3]. In the last few years, recognition and characterization of CSC biomarkers have been an area of growing interest.

Octamer-binding transcription factor 4 (OCT4) and Nanog homeobox protein (NANOG) are among a group of pluripotent transcription factors that work to suppress differentiation of human embryonic stem cells [4]. OCT4 gene plays an imperative part during many biological processes like proliferation, differentiation, stress response and apoptosis in stem cells [5]. NANOG functions to preserve the cell's capacity of self-renewal and suppress differentiation [6].

Mounting evidence highlights that over-expressions of OCT4 and NANOG are closely related to cell cycle control, cell reprogramming, tumorigenesis, tumor

transformation, tumor metastasis and distant recurrence after chemo-radiotherapy [7,8]. Previous studies have shown that OCT4 and NANOG are highly expressed and correlated to clinicopathological features and poor prognosis in different types of cancers including lung adenocarcinoma [9], neuroblastoma [10], and rectal cancer [11].

Several studies also have investigated the expression levels of OCT4 and NANOG in different subtypes of breast cancer [12], however, to the best of our knowledge, no previous studies have been conducted to show the correlation between quantitative expression of cancer stem cell markers (OCT4 and NANOG) with survival and prognosis of breast cancer (BC) patients. Therefore, the present study investigated the expression and the prognostic significance of CSC markers OCT4 and NANOG in BC patients. Moreover, the correlation between the expression of cancer stem makers and survival of breast cancer patients was studied.

2. Patients and Methods

2.1. Sampling

Thirty-four female BC patients aged between 40 and 62 years were enrolled in the study. All patients underwent modified radical mastectomy during which a sample from malignant breast tissue and another one from adjacent normal breast tissue were taken. Tissue samples were immediately stored at -80°C till use. The majority of patients (85%) received postoperative radiotherapy followed by 6 cycles of FAC (5-Fluorouracil, Adriamycin and Cyclophosphamide). While for the rest of patients, radiotherapy was supplied after finishing the last chemotherapy cycle. Clinicopathological data were collected from pathology reports and patients' follow-up records. This study was conducted in accordance with the principles of the Declaration of Helsinki. Ethical approvals for patients' recruitment were obtained from the local Ethics Committee of Medical Research Institute, University of Alexandria. An informed written consent was obtained from all participants included in the study.

2.2. RNA Extraction

Total RNA was extracted from 100 mg of the collected tissues (tumor as well as normal tissues) using miRNeasy mini kit (QIAGEN Co, Hilden, Germany), according to the manufacturer's instructions. The purity and concentration of extracted RNA were evaluated by NanoDrop$^{(R)}$ ND-1000 UV-Visible Spectrophotometer (Thermo Fischer Scientific, USA).

2.3. cDNA Synthesis from Total RNA

Total RNA was reversely transcribed using Quantitect RT kit (QIAGEN Co, Hilden, Germany) for OCT4 and NANOG, according to the manufacturer's instructions. The obtained cDNA was stored at -20°C immediately till real-time PCR was performed.

2.4. Real Time Quantitative PCR Analysis for Oct4 and Nanog

Real-time PCR was then performed with the cDNA, using QuantiTect SYBR Green RT-PCR Kit and specific pre-designed QuantiTect primers for OCT4 and NANOG (QIAGEN Co, Hilden, Germany). The reactions were carried out in 25 µl final volume by adding 12.5 µl Master Mix, 2.5 µl primers, 8 µl RNAse free water and 2 µl cDNA. The reaction tubes were incubated at 95°C for 10 min, followed by 45 cycles of 95°C for 30 sec, 60°C for 40 sec and 72°C for 30 sec. After the reactions were completed, the CT values were determined by setting a fixed threshold. The ΔCT of both malignant and control groups were calculated using the level of GADPH expression in the same sample as a housekeeping gene. ΔΔCT for the gene expression in each patient was calculated by subtracting the ΔCTs of malignant and adjacent normal tissue. OCT4 and NANOG levels were expressed as $2^{-\Delta\Delta CT}$.

2.5. Statistical Analyses

Data were fed to the computer and analyzed using SPSS software package version 20.0 (IBM Corporation, Chicago, Illinois, USA). Quantitative data were described using mean ± standard deviation. The distributions of quantitative variables were tested for normality using Kolmogorov-Smirnov test. Mann-Whitney test was used to compare between two studied groups, for Kruskal Wallis test was used to compare between more than two groups. Wilcoxon signed ranks test was used to compare expression levels between cancer and adjacent normal tissues, Spearman correlation test was used to study the correlation between OCT4 and NANOG expression and the clinicopathological parameters. Kaplan-Meier survival curves were done to investigate the association of studied parameters with disease-free and overall survival and Cox proportional hazards regression model analysis was done to investigate the prognostic value of studied parameters. At all statistical analyses, p value was considered significant at ≤0.05.

3. Results

3.1. Patients' Clinical Characteristics

The clinicopathological characteristics of enrolled patients are presented in Table 1. All thirty-four BC patients that were included in the present study were diagnosed with invasive ductal carcinoma. The age of enrolled patients ranged from 40 to 62 years with a mean value 54.3±6.6 years. The majority of patients were post-menopausal representing 88.2% and the rest were pre-menopausal. Tumor size was categorized into 3 groups: T1 ≤ 2 cm, T2 ≤ 2 -5 cm, and T3 > 5 cm. Most patients had a T3 tumor size 64.7%, while 32.4% were T2 and 2.9% were T1. Regarding the clinical stage, 32.4% of specimens were stage II, 52.9% stage III, 14.7% and stage IV. Moreover, 5.9% of specimens were of histological grade I, 82.4% grade II, and 11.7% grade III. All cases

represented with positive vascular invasion while lymph node involvement was negative in 14.7% of cases, however the rest of cases were positive ranging from N1 to N3 (29.4, 20.6 and 32.3% respectively). Regarding receptor expression, 94.1% of tumors were ERα +/PR+, however, all tumors were Her2/neu negative.

3.2 Upregulation of OCT4 and NANOG Expression in Breast Cancer Tissues Compared to Normal Tissues

Quantitative determination of OCT4 and NANOG expression in BC tissues revealed that OCT4 was upregulated in 79% of cases with a mean value of 28.28±63.00 and down-regulated in 21% of cases with a mean value of 0.59±0.30, hence the overall fold change was significantly higher than control tissue ($p<0.001$*). NANOG showed a similar pattern where it was upregulated in 82% of cases with a mean value of 33.7±55.7 and down-regulated in the rest of cases with a mean value of 0.37±0.26 and the overall fold change was also significantly higher than normal tissues ($p<0.001$*) as presented in Figure 1a and Figure 1b.

3.3. OCT4 and NANOG Expression Levels are Associated with Clinicopathological Parameters in Breast Cancer Patients

Stratification analysis revealed that OCT4 and NANOG expressions are not associated with menopausal status or tumor size. However, they were associated with histological grade ($p=0.029$ and 0.025 respectively) and clinical stage ($p=0.001$ and 0.042 respectively). OCT4 alone showed a significant association with lymph nodes involvement ($p=0.006$) and distant metastasis ($p=0.024$) while NANOG was associated with ERα and PR receptors expression ($p=0.007$ and 0.019, respectively) as presented in Table 2. Furthermore, NANOG showed a significant positive correlation with ERα and PR receptors expression ($p=0.004$ and 0.005, respectively) and OCT4 was significantly correlated to patients' age ($p=0.009$) as showed in Table 3.

Table 1. Clinicopathological Parameters of BC Patients

	BC Patients	
Age	40-62 (54.3±6.6)	
Type of Surgery		
Modified Radical Mastectomy	34	100%
Menopausal Status		
Pre	4	11.8%
Post	30	88.2%
Estrogen Receptor (ERα) Expression		
-	2	5.9%
++	21	61.8%
+++	11	32.3%
Progesterone Receptor (PR) Expression		
-	2	5.9%
+	7	20.6%
++	16	47.1%
+++	9	26.4%
Her2/neu Expression		
-	34	100%
Tumor size		
T1 (≤ 2)	1 *	2.9%
T2(2 - ≤5)	11	32.4%
T3(>5)	22	64.7%
Lymph Nodes Involvement		
N0	5	14.7%
N1(1-3)	10	29.4%
N2(3-6)	7	20.6%
N3(>6)	11	32.3%
Vascular Invasion		
+	34	100%
Histological Grade		
I	2	5.9%
II	28	82.4%
III	4	11.7%
Clinical Stage		
II	11	32.4%
III	18	52.9%
IV	5	14.7%
Metastasis		
No metastasis	26	76.5%
Metastasis	6	17.6%
Lost follow up	2	5.9%
Mortality		
Alive	27	79.4%
Died	5	17.7%
Lost follow up	2	5.9%

ERα: Estrogen receptor alpha, PR: Progesterone receptor.

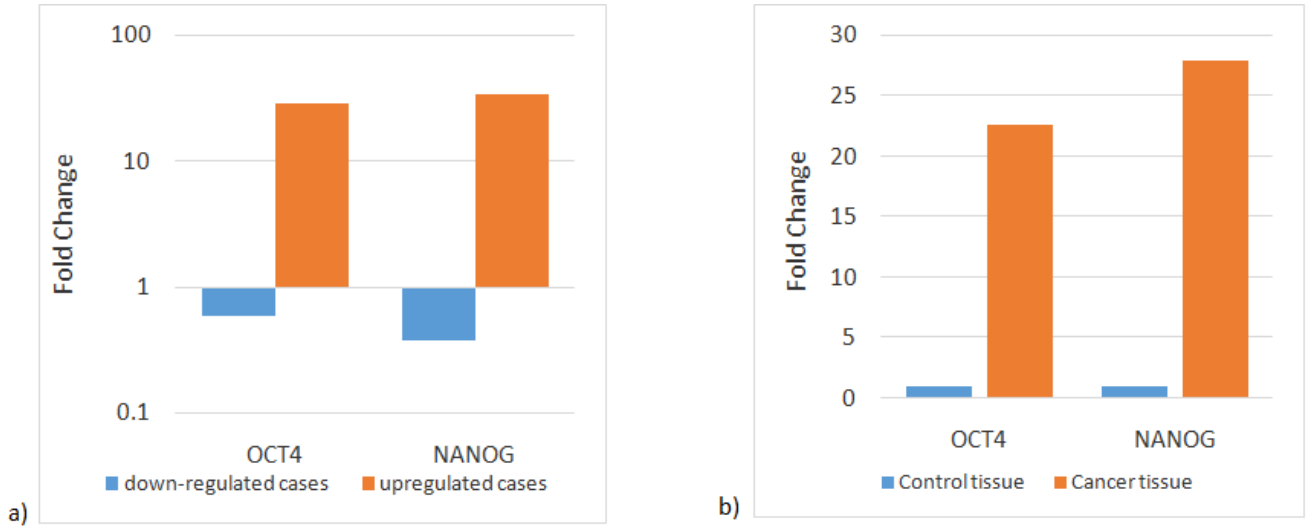

Figure 1. Upregulation of OCT4 and NANOG expression in BC tissues where (a) represents the mean fold change in upregulated cases compared to down-regulated cases and (b) represents the mean overall fold change in cancer tissues compared to control tissues

3.4. OCT4 and NANOG are Associated with Poor Breast Cancer Patient Survival

The association between disease-free survival (DFS) and overall survival (OS) with OCT4 and NANOG expression in BC patients were evaluated as shown in (Figures 2a-2d). The median follow-up time of the surviving patients in DFS was 89.3 months; while the median follow-up time for OS was 111.6 months. During the follow-up, 14.7% of patients had history of metastasis while disease-related death occurred in 11.8% of patients. Of the patients. 85.3% had no history of recurrence, metastasis, or disease-related death. Both OS and DFS were significantly poorer in BC patients with high NANOG expression (p=0.028, 0.050 for DFS and OS respectively). Compared to patients with low NANOG expression. Regarding OCT4, the association with patients' poor survival was insignificant (p=0.200, 0.205 for DFS and OS respectively).

Table 2. Stratification Analysis of OCT4 and NANOG in Patients with Different Clinicopathological Status

	No.	OCT4	NANOG
Menopausal			
Pre	**4**	2.6(1.8–6.6)	1.4(0.1–3.1)
Post	**30**	3.2(0.2–284.0)	4.3(0.0–203.7)
U(p)		57.0(0.897)	29.50(0.105)
ER			
-	**2**	7.4(1.6–13.2)	4.2(2.0–6.5)
++	**21**	2.5(0.6–43.7)	2.0(0.0–18.4)
+++	**11**	3.9(0.2–284.0)	101.1(0.5–203.7)
H(p)		2.336(0.311)	9.790*(0.007*)
PR			
-	**2**	7.4(1.6–13.2)	4.2(2.0–6.5)
+	**7**	3.1(1.0–28.4)	1.0(0.0–3.1)
++	**16**	2.7(0.6–43.7)	3.1(0.5–138.1)
+++	**9**	3.9(0.2–284.0)	26.7(0.5–203.7)
H(p)		2.137(0.545)	10.002*(0.019*)
Tumor size			
T1 (≤2)	**1***	42.8	1.5
T2(2-≤5)	**11**	2.0(0.2–40.8)	2.0(0.1–138.1)
T3(>5)	**22**	3.1(0.2–284)	5.6(0.0–203.7)
U(p)		96.50(0.355)	96.50(0.355)
Lymph nodes involvement			
N0	**5**	27.9(1.0–40.8)	1.1(0.0–138.1)
N1(1-3)	**10**	2.6(0.2–13.2)	2.3(0.1–101.1)
N2(3-6)	**7**	0.8(0.2–3.9)	2.0(0.5–25.3)
N3(>6)	**11**	3.4(2.5–284)	18.1(1.5–203.7)
H(p)		12.413*(0.006*)	6.170(0.104)
Histological Grade			
I	**2**	21.8(2.8–40.8)	138.1(138.1–138.1)
II	**28**	2.7(0.2–43.7)	2.3(0.0–101.1)
III	**4**	114.8(3.4–284)	102.2(26.7–203.7)
H(p)		7.108*(0.029*)	14.113*(0.001*)
Clinical Stage			
II	**11**	3.9(0.2–40.8)	1.7(0.0–138.1)
III	**18**	2.7(0.2–42.8)	2.8(0.5–102.5)
IV	**5**	43.7(1.6–284.0)	26.7(6.5–203.7)
H(p)		7.392*(0.025*)	6.349*(0.042*)
Metastasis			
No metastasis	**26**	2.8(0.2–42.8)	2.5(0.0–138.1)
Metastasis	**6**	43.0(1.6–284)	22.4(2.0–203.7)
Lost follow up	**2**	2.9(1.9–3.9)	51.5(2.0–101.1)
H(p)		7.426*(0.024*)	5.254(0.072)
Mortality			
Lost follow up	**2**	2.9(1.9–3.9)	51.5(2.0–101.1)
Died	**5**	43.7(1.6–284)	18.1(2.0–203.7)
Alive	**27**	2.9(0.2–42.8)	2.5(0.0–138.1)
H(p)		5.378(0.068)	3.788(0.150)

U: Mann Whitney test, H: H for Kruskal Wallis test
*: Statistically significant at $p \leq 0.05$
#: Excluded from the relation due to small number of case (n = 1).

Table 3. Correlation between OCT4 and NANOG with Clinicopathological parameters

		OCT4	NANOG	CA15-3
Age	r_s	-0.443*	0.067	0.124
	p	0.009	0.708	0.483
Menopausal	r_s	0.028	0.284	0.121
	p	0.875	0.104	0.496
ER	r_s	0.228	0.476*	0.261
	p	0.196	0.004	0.136
PR	r_s	0.099	0.473*	0.197
	p	0.576	0.005	0.265
Tumor Size	r_s	0.070	0.196	-0.165
	p	0.694	0.267	0.352
Lymph nodes	r_s	0.179	0.328	-0.005
	p	0.319	0.062	0.976
Grade	r_s	0.284	0.183	-0.123
	p	0.103	0.299	0.488
Stage	r_s	0.161	0.331	-0.128
	p	0.362	0.056	0.470

r_s: Spearman coefficient, *: Statistically significant at $p \leq 0.05$.

Table 4. Univariate and multivariable analysis of disease-free survival (DFS) in patients with breast cancer

	Univariate			
	HR	**95% C.I.**		**p**
		LL	**UL**	
OCT4	1.022	1.008	1.036	0.002*
NANOG	1.015	1.002	1.029	0.021*
ER (-)	18.722	2.576	136.065	0.004*
PR (-)	2.109	0.351	12.677	0.415
Tumor size				
T1(1-5) (R)	1.000	-	-	-
T2(5-9)	1.000	0	7×10^{10}	1.000
T3(>9)	42.714	0	9×10^{11}	0.757
Grade				
I (R)	1.000	-	-	-
II	5844	0	9.7×10^{159}	0.962
III	126348	0	2.1×10^{161}	0.949
Stage				
II (R)	1.000	-	-	-
III	8376.98	0	2.5×10^{110}	0.942
IV	180558	0	5.4×10^{111}	0.923
	#Multivariate			
	HR	**95% C.I.**		**p**
		LL	**UL**	
OCT4	1.073	0.964	1.195	0.197
NANOG	0.998	0.955	1.043	0.922
ER (-)	145747.2	0.001	2×10^{13}	0.211

HR: hazard ratio, *: Statistically significant at $p \leq 0.05$.

Univariate and multivariate analysis were performed on BC cases to identify factors that correlate with prognosis using Cox proportional hazards regression model analysis. The results showed that OCT4 ($p = 0.002$), NANOG ($p = 0.021$), and ERα status ($p = 0.004$) were associated with worse DFS. However, the multivariate analysis did not show that any of them can be an independent prognostic factors for DSF Table 4.

4. Discussion

The discovery of CSC has revolutionized the understanding of tumor behavior especially in terms of tumor relapse and metastasis [13]. Studying markers associated with stem-like characteristics of tumor cells paves the way for new prognostic markers that can predict patients' outcome. Among these markers, OCT4 and NANOG are transcription factors that maintain stem cell phenotypes. There's a growing evidence that OCT4 and NANOG may be implicated to the process of tumorigenesis, metastasis, and distant recurrence after treatment [14].

In the current study, we quantitatively measured the genetic expression of OCT4 and NANOG in BC tissues compared to normal adjacent noncancerous tissues. Our results revealed that both OCT4 and NANOG expression have been upregulated in the majority of BC patients with multiple fold increase.

The aberrant expression of these genes supports the fact that tumor cells exhibit stem cell-like properties that contribute to maintaining tumor progremssion and sustainability [15]. Like their role in embryonic stem cells, pluripotency genes possess the ability to activate downstream target genes that regulate the processes of self-renewal and differentiation in cancer stem cells [16,17].

Increasing evidence supports the regulatory mechanisms and functional importance of OCT4 especially in cancer cells with stem-like properties [18]. OCT4 has previously been reported to be a potential biomarker for the initiation, progression, and differentiation of many types of cancer including BC [19]. Furthermore, OCT4 expression induced the acquisition of CSC phenotypes by mediating cancer cell dedifferentiation [20]. OCT4 has been also linked to tamoxifen-acquired resistance in BC cells and in xenograft tumor models [21].

NANOG, the pluripotency factor promotes tumors' migration and invasion according to previous reports [22]. The ectopic expression of NANOG was found to deregulate the expression of genes responsible for tumor formation and invasion [23] forcing the expression of NANOG has also resulted in expansion of self-renewal in $CD44^{positive}$/ $CD24^{negative}$ MCF7 cells [24]. In addition, suppressing the expression of NANOG in BC cells resulted in decreases in cell-growth, colony-forming, and metastatic capacities [25]. The co-expression of OCT4 and NANOG was also consistent with the fact that both genes interact with each other to exert their regulatory function. Previous studies have shown that OCT4 has a binding site on the 5' terminal promoter region of NANOG which initiates and regulates NANOG expression [26].

Our results indicated that higher levels of OCT4 and NANOG expression are correlated with higher histological grade and advanced clinical stage. Moreover, NANOG expression was positively correlated with hormonal receptor status (ERα and PR), advanced disease stage and poor clinical outcome. This observation is supported by previous clinical studies. For example, OCT4 was found to be upregulated and correlated with histological grade, stage, and lymph node metastasis in renal carcinoma patients [27]. Also, increased expressions of OCT4 and NANOG were significantly associated with aggressive behaviors of nasopharyngeal carcinoma [28]. OCT4 and NANOG positive expressions were also correlated with poor differentiation and advanced disease stage of Her2/neu positive BC [29,30]. These results might be attributed to the OCT4/NANOG-based promotion of cell proliferation, migration, and invasion.

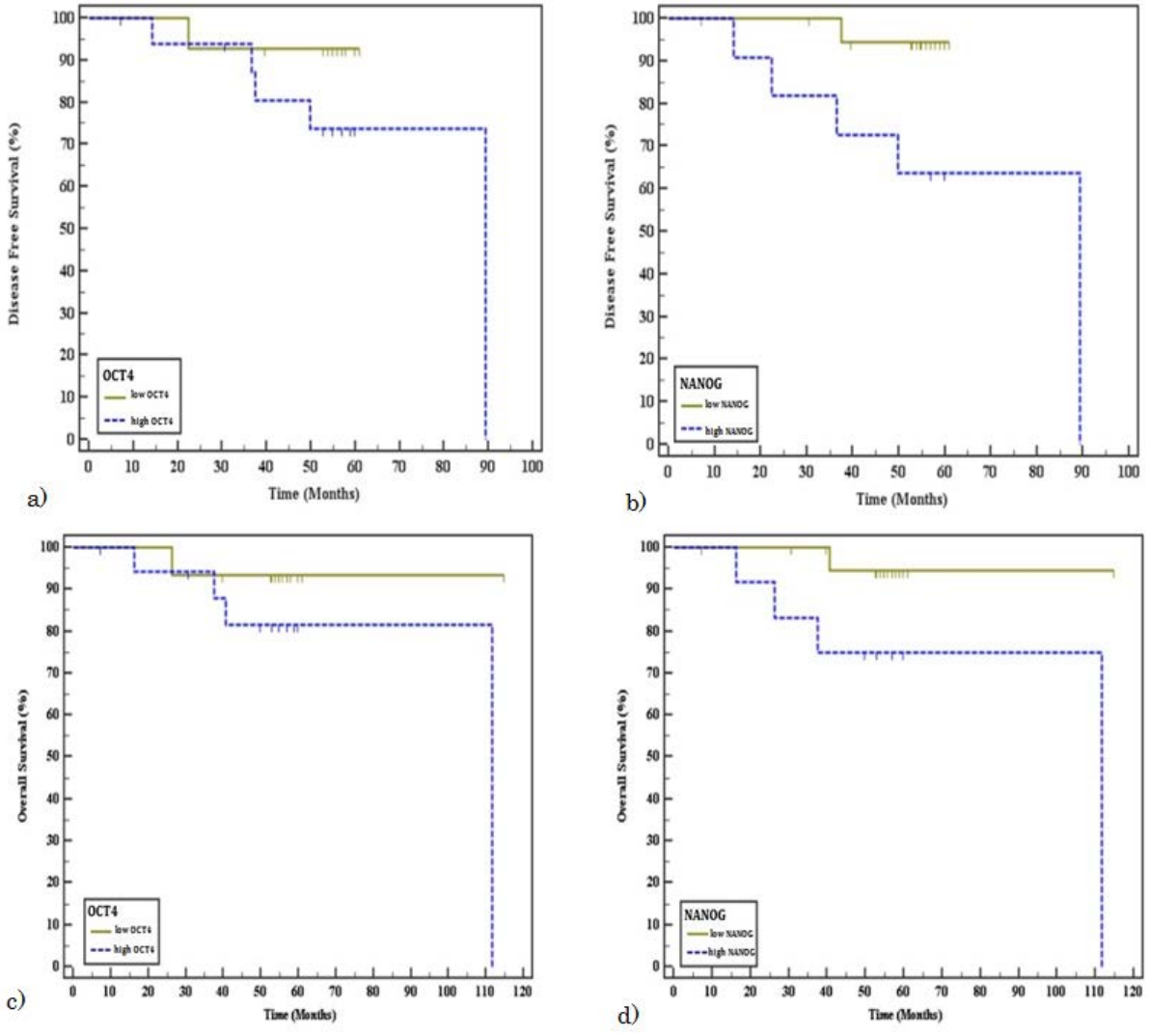

Figure 2. Kaplan-Meier survival curves for the association of disease-free survival (DFS) with OCT4 (a) and NANOG (b) expressions and overall survival (OS) with OCT4 (c) and NANOG (d) expressions grouped into low vs high-expression levels

In the present study, there was a significant association between elevated NANOG expression and DFS and OS, OCT4 expression was associated with poor OS and DFS but without statistical significance. Previous reports have suggested that the overexpression of OCT4 either alone or in association with NANOG was significantly associated with reduced cumulative survival in BC patients [31,32,33].

Wang et al reported that upregulation of OCT4 and NANOG positively affects the expression of epithelial mesenchymal transition-related genes in CSCs, and promoted CSCs invasiveness [33]. The findings from these studies further suggest that OCT4 and NANOG co-expression may be a valuable biomarker to predict the outcome of patients with BC.

Univariate and multivariate analyses included OCT4, NANOG and clinicopathological parameters. Most notable are the significant associations of OCT4 and NANOG with DFS which further support the fact that both OCT4 and NANOG are involved in cancer metastasis. CSCs have played a critical role in cancer recurrence and metastasis due to their resistance to radiotherapy [34] and chemotherapeutic agents [35] Concerning the predictive value for patients' outcomes, several previous studies have demonstrated that the over-expression of OCT4 and or NANOG have been associated with poor patients' prognosis and poor survival in other types of cancers including lung, brain and hepatocellular carcinomas [36,37,38]. Moreover, co-expression of OCT4 and NANOG was reported to be a strong independentpredictor of tumor recurrence an unfavorable outcome in hepatocellular carcinoma patients [39].

5. Conclusions

In conclusion, the upregulations of CSCs markers OCT4 and NANOG expressions in BC patients were correlated with poor prognosis and advanced disease stages. Also, OCT4 and NANOG could be used as predictive markers for poor DFS in BC patients.

6. Limitations

Our study experienced some limitations including the small sample size, which might have caused less statistical power. Another limitation is that we didn't investigate in this study the underlying mechanisms by which OCT4 and NANOG exert their effects on BC patients. Therefore, further study with a larger sample size and through investigation of underlying mechanisms is needed.

Conflict of Interest

Authors declare no conflicts of interest related to this work.

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Authors declare that no sources of financial assistance were used to conduct the study described in this manuscript, or used to assist with the preparation of the manuscript.

Ethical Approval

This study was conducted in accordance with the principles of the Declaration of Helsinki. Ethical approval for patients' recruitment was obtained from the local Ethics Committee of Medical Research Institute, University of Alexandria.

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