

THE RNA EXPRESSION LEVEL OF *SURVIVIN*, *OCT 4* AND *CYCLIN D1* GENES IN TRIPLE NEGATIVE AND NON-TRIPLE NEGATIVE BREAST CANCER

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Abstract

Background: the purpose of the present study to investigate the RNA expression level of *Oct4*, *Survivin* and *Cyclin D1* genes in Triple-negative breast cancer (TNBC) and compared to non-triple negative breast cancer (non-TNBC) group. Also, correlate the results of gene expression with clinicopathological features of patients. **Method,** RNA expression levels of *Oct4*, *Survivin* and *Cyclin D1* was tested for 100 breast cancer (BC) patients [formalin fixed paraffin embedded tissue (FFPE)] diagnosed as invasive duct carcinoma in Pathology Department, National Cancer Institute (NCI), Cairo University. The qRT-PCR technique is use and the results correlated to clinico-pathological characteristics of patients and survival rates. **Result,** in (TNBC) patients, *oct4*, *survivin* and *cyclin D1* showed positive expression in 30 (60%), 32(64%) and 32 (64%) (**p<0.001, p= 0.001 and p= 0.003**; respectively). In non-TNBC patients, the positive expression of *oct4*, *survivin* and *cyclin D1* gene was 11(22%), 15(30%) and 17(34%) (**p<0.001, p= 0.001 and p= 0.003**; respectively). All patients in TNBC have negative *ER*, *PR* and *HER-2* receptor (p=0.001). There was highly statistically significant difference between *oct4* gene expression and all the clinico-pathological features except the family history. A significant difference between *cyclin D1* gene expression and all the clinico-pathological features except the tumor size, tumor grade and lymphnode status. Statistically difference was found between *survivin* gene expression and all the clinico-pathological features except the menopause state, tumor stage and tumor grade. Triple negative breast cancer patients showed significantly decreased DFS (**p< 0.001, log rank**) and OS (**p= 0.002, log rank**) when compared to those with non-TNBC patients. In TNBC group, patients with positive expression of *survivin* was significantly associated with decreased OS (**p= 0.03, log rank**). TNBC tumors with positive *oct4* and *cyclin D1* had reduced OS compared to those negative to *oct4* and *cyclin D1* but without significant difference (**p= 0.8 and p= 0.09, respectively, log rank**). In non-TNBC group, with positive *oct4*, *cyclin D1* and *survivin* did not significantly differ in terms of DFS and OS when compared to those with negative expression.

Conclusion: *Oct4* and *survivin* expression gene are better marker used for diagnosis in TNBC and for molecular targeting therapy of TNBC treatment. *Cyclin D1* expression used as a marker for aggressive TNBC. The TNBC tumor possibly respond to treatment that downregulates *cyclin D1* amplification. More studied and large sample size is needed.

Key words: Triple negative breast cancer, *Oct4*, *CyclinD1*, *Survivin*

1.Introduction

Breast is a heterogenous disease composed of different molecular subtypes that differ in patterns of gene expression, clinical features, response to treatment, and prognosis **Munjal K et al., (2009) and Curtis C et al., (2012)**. Triple negative breast cancer, characterized by lack of expression of *ER- α* , *PgR* and *HER-2*, is its- self a heterogenous disease and accounts for ~15% of all breast cancer cases. In Egypt, TNBC phenotype is highly prevalent compared to US and other Western countries **Danielle Bogan et al., (2017) and El-Hawary et al., (2012)**. Triple negative breast cancer (TNBC) tumors are responsive to a wide range of chemotherapeutic agents, majority of patients are more likely to develop recurrence during the first three years after treatment **Kaplan, H.G. et al., (2009)** with predominance of visceral and central nervous system metastases **Liedtke, C.et al., (2008)**. TNBC is essential, there are incomplete overlap in immunohistochemistry determine it, molecular analysis to basal-like breast cancer and the mutation of *BRCA1* that correlated with breast cancer. In this study, they reported that about 80% of TNBC have a basal-like molecular profile **Weigelt et al., (2010a)**.

The octamer-binding transcription factor 4 (*oct4*) is a transcription factor known as *POU* (Pituitary-specific Pit-1, Octamer transcription factor, and neural Unc-86 transcription factor) domain class 5 Homeobox transcription factor 1 (*POU5F1*). It regulates the pluripotency of pluripotent stem cells. The *POU5F1* gene is located on chromosome 6p21.33 (**Liu et al., 2013**). The expression of *oct4* is high in the most types of cancer cells either solid or hematological. Otherwise, the downregulated of *oct4* inhibits cell proliferation or metastasis in different types. of tumors. The *oct4* over-expression was suppressed the metastasis in breast cancer cells (**Shen et al., 2014**). Thus, the reprogramming factor *oct4* may regulate cancer properties differentially.

Cyclins consisted of about 30 proteins with weight from 35 to 90 kDa. The structures of *cyclins* proteins determined by *cyclin* box which contains approximately one hundred domain amino acid residues that forms a stack of five α -helices. *Cyclins* regulates the activity of *cyclin*-dependent kinases (*Cdks*), so they can control the progression of cell cycle.(**Malumbres and Barbacid, 2005**). The *cyclin*-*CDK* complexes rule a linear progression of events that change the cells from a resting state (G 0) to the growth phase (G 1), through DNA replication (S)in the S phase followed by cell division (M). (**Sarosiek, 2018**). *Cyclin D1* is an oncogene that is directly related to the carcinogenesis. *cyclin D1* gene was high expression many cancers. It was expressed in about 50% in invasive breast cancer patients compared to normal epithelium. Its amplification rate is approximately 13% of the normal epithelium (**Choi et al., 2018**).

Cancer cell proliferation affected by *survivin* overexpression. It inhibits apoptosis protein family that inhibits caspases then stoop cell death. It has a poor outcome survival of patients. *Survivin* expression was correlated significantly with high tumor grade, survival rates and recurrence. *Survivin* gene polymorphisms can help to understand the biology of the disease. It is a prognostic and predictive factor (**Li et al., 2017b and Boullosa et al., 2018**).

2.Methods

Patients and tissues specimens

Tissues specimens (Formalin-fixed paraffin-embedded (FFPE) tissue blocks) for 100 patients who were diagnosed as invasive duct carcinoma. All patients attended in the National Cancer Institute (NCI), Cairo University. All patients have the relevant clinicopathological and follow up data. All cases were diagnosed as stage and grade according to the WHO (Fletcher CDM BJ et al., 2013). Fifty FFPE tissue blocks of breast cancer patients who had negative estrogen (*ER*), progesterone (*PR*) and *Her/2 neu* receptors (TNBC). And another fifty samples who have the positive *ER*, *PR* and *Her/2 neu* receptors (non-TNBC). Twenty tissues block of normal tissue as a control group. The representative paraffin blocks, 5 µm thick sections (7 sections) were cut into a sterile, plastic, 2 ml Eppendorff tube for RNA extraction and quantitative real time polymerase chain reaction (qPCR).

The extraction of RNA

The RNA was extracted from the tumor as well as the normal tissues using the RNeasy Mini Kit (Qiagen, Milan, Italy) and by spectrophotometry the quality of RNA was estimated. Dissolve RNA in diethyl pyrocarbonate-treated water containing 10 mmol/l of MgCl₂. Incubation for 30 min at 37 °C with 100 µg/ml of RNase-free DNaseI to remove the contaminating DNA. Add EDTA to a final concentration of 30 mmol/l, the reaction was stopped at 95 °C for 5. RNA was retro transcribed using iScript™ cDNA Synthesis Kit (Bio-Rad, Milano, Italy). The qRT-PCR analysis was performed in a final volume of 25 µl with a SYBR Green PCR Master Mix using 1 µl cDNA and 400 nM of predesigned.

Amplification of *Oct4*, *Cyclin D1*, *Survivin* and *β-actin* mRNA in the samples were assessed in triplicates using the primers

- 1) *Oct4* Forward: 5'-GATGGCGTACTGTGGGCCC-3'
Reverse: 5'-TGGGACTCCTCCGGGTTTTG-3'
- 2) *Cyclin D1* Forward: 5'-CTGGGTGTCCTACAAATG-3'
Reverse: 5'-AGCGGTCCAGGT AGTTCAT-3'
- 3) *Survivin* Forward: 5'-TCCACTGCCCCACTGAGAAC-3'
Reverse: 5'-TGGCTCCCAGCCTTCCA-3'
- 4) *β-actin* Forward: 5'-ACAGAGCCTCGCCTTTGC-3'
Reverse: 5'- GCGGCGATATCATCATCC-3'

Interpretation of the qPCR results

Calculate the mean cycle threshold (CT value) to 1- determine the delta CT (ΔCT) for each sample as follows: $\Delta CT = CT$ for the gene of interest-CT of the internal control gene (β -actin). 2- the delta delta CT ($\Delta\Delta CT$) was calculated as follows: $\Delta\Delta CT = (\Delta CT$ for sample A - ΔCT for sample B), where sample A is the tumor and sample B is the calibrator (normal breast). For the statistical analysis, the $\Delta\Delta CT$ was used and then the data were expressed as relative expression units **Schmittgen TD and Livak KJ. (2008)**.

Statistical method:

Mann-Whitney test (non-parametric t-test) method was used to comparison the quantitative data between TNBC ana non-TNBC group. Kaplan-Meier method, and the log-rank test was done to calculate the survival functions and to compare the survival curves. Cox proportional hazards model analyze the prognostic factors of disease-free survival and overall survival. The hazard ratios (HRs) were calculated with a 95% confidence interval (CI). All tests were two-tailed. The p value, $p < 0.05$, was considered significant.

3.Results

The study involved hundred breast cancer patients diagnosed as invasive duct carcinoma of the breast. The patients were divided into two groups (TNBC and non-TNBC). The relation of the studied markers (*Oct4*, *Cyclin D1* and *Survivin*) and the two groups was assessed. The results were correlated with the relevant clinic-pathological features of patients as well as to demonstrate the response to treatment and outcome of patients.

▪ Clinico-pathological patient's characteristics

The features of all breast cancer patients (100 patients) demonstrated in Table 1, all patients had negative estrogen (ER), progesterone (PR) and Her-2/neu receptors. There were not statistically significant difference of the relevant clinico-pathological features of patients and age, Tumor size and Menopausal status. The age ≤ 50 year was in 54% and age >50 year was in 64% ($p=0.554$). The tumor size was detected in 48% with the diameter ≤ 5 cm versus to 52% with tumor size >5 cm ($p=0.770$). Moreover, 54% of patients had postmenopausal status versus to 46% of patients had premenopausal status ($p=0.334$). There was a significant association between clinico-pathological features of patients and the lymph node status, tumor grade, family history, stage, margin and chemotherapy treatment. Family history was found in 11% of all patient and absent in 89% of patients ($p=0.001$). The patients which had grade I-II were in 81% of patients compared to 19% with grade III-IV ($p=0.017$). Regrade to tumor stage, 80% of patients were in early stage and 20% with late stage of tumor ($p=0.01$). Lymphnode status, positive lymphnode was in 73% of patients and negative lymphnode was in 27% ($p=0.01$). There was 93% of patients

had free margin from tumor and 7% of patients had positive margin ($p=0.001$). Finally, 91% of patients were responded to the treatment versus 9% were non responder ($p=0.001$).

Table 1: Clinico-pathological feature of patients

Patient's characteristics	Frequency (%)	P value
Age		
≤50	54 %	0.554
>50	46 %	
Tumor size		
≤5	48%	0.770
>5	52%	
Family history		
Negative	89 %	0.001
Positive	11 %	
Menopausal status		
Post	54 %	0.334
Pre	46 %	
Grade		
I-II	81 %	0.017
III-IV	19 %	
Stage		
Early	80 %	0.01
Late	20 %	
Lymph node		
Negative	27 %	0.01
Positive	73 %	
Margin		
Free	93 %	0.001
Positive	7 %	
ER, PR and Her2/new		
Negative	100 %	
Positive	0%	
Chemotherapy		
No	9 %	0.001
Yes	91 %	

ER: estrogen receptor; *PR*: progesterone receptor and *HER2*: human epidermal growth factor receptor

▪ Clinico-pathological features of TNBC and non-TNBC patients.

TNBC patients have negative *ER*, *PR* and *HER-2* receptor ($p=0.001$). At tumor size, 48 patients had tumor size $\leq 5\text{cm}$ [25(52.1%) in TNBC vs 23(47.9%) in non-TNBC] and fifty-two patients had tumor size $>5\text{cm}$ [25(48.1%) in TNBC vs 27(51.9%) in non-TNBC]. There was not Statistically difference between the tumor size in TNBC or non-TNBC group. ($p=0.69$) (Table2). The early stage (I&II) was common in TNBC group than those in non-TNBC group, in TNBC 50 (62.5%) vs 30 (37.5%) were in non-TNBC. As for in stage III & IV, 0/20(0 %) were in TNBC compared to 20/20 (100%) non-TNBC cases ($p=0.0001$). Stage (III-IV) was more significant in non-TNBC than those in TNBC (Table2). Grade I was found in 3 patients [2(66.7%)] were in the TNBC group and only one (33.3%) was in non-TNBC, seventy-eight patients had the tumor grade II [43(55.1%) in TNBC compared to 35(44.9%) in non-TNBC]. Finally, grade III reported in 19 cases [5(26.3%) in TNBC and 14(73.7%) in non-TNBC] ($p=0.12$) (Table2). In TNBC group, the mean \pm SD of the age was 51.2 \pm 12.5 years old, in the TNBC 22 out of 46 were <50 year-old vs. 28/54 were ≥ 50 year-old. In the non-TNBC, the mean \pm SD for the age is 53.3 \pm 12.3 years old “24 out of the 46 were less than 50year-old compared to 18/54 with more than 50year-old ($p=0.68$) (Table2). In premenopause, 23/46(50%) were in TNBC group vs 23/46(50%) in non-TNBC one. In postmenopause 27/54(50%) were in TNBC group compared to 27/54(50%) ($p=0.33$) (Table2). A higher proportion of patients had positive family history. Eleven patients have a family history, 10/11(90.9%) in TNBC group and 1/11(9.1%) in non-TNBC ($p=0.05$) (Table2). Lymph node, 43 (58.9%) out of the 73 patients were positive in the TNBC compared to 30(41.1%) cases in non-TNBC. In TNBC, the negative lymph node was in 7(25.9%) vs 20(74.1%) in non-TNBC cases ($p=0.003$) (Table 2). As for surgical margin, it was positive in 7 (100%) patients in TNBC patients but none in the non-TNBC patients ($p=0.014$) (Table 2). Response to treatment given good outcome in non-TNBC [35/55(63.6%)] than those in TNBC [20/55 (36.4%)]. Whereas 45 patients did not respond to treatment [33(73.3%) in TNBC and 12(26.7%) in non-TNBC group. A significantly higher proportion of patients in the non-TNBC group responded to the given treatment (chemotherapy and/or radiotherapy) compared to the TNBC patients (35 verses 20 patients: respectively ($p = 0.001$) (Table2).

Table 2: The clinico-pathological features of TNBC and non-TNBC patients

Parameters	TNBC N (%)	non-TNBC N (%)	P value
Age (yr): < 50 (n= 46) ≥ 50 (n= 54)	22/46 (28/54)	(24/46) (26/54)	0.68
Menopause: Pre (n=46) Post (n=54)	(23/46) (27/54)	(23/46) (27/54)	0.33
Family History Absent (n= 89) Present (n= 11)	(40/89) (10/11)	(49/89) (1/11)	0.05
Tumor size (cm): ≤ 5 (n= 48) >5 (n=52)	(25/48) (25/52)	(23/48) (27/52)	0.69
Tumor stage: Low (0-I) (n=21) Early (II) (n= 59) Late (III) (n= 20)	(21/21) (29/59) (0/20)	(0/21) (30/59) (20/20)	0.0001
Tumor Grade: 1 (n= 3) 2 (n= 78) 3 (n= 19)	(2/3) (43/78) (5/19)	(1/3) (35/78) (14/19)	0.12
LN status: Positive (n=73) Negative (n=27)	(43/73) (7/27)	(30/73) (20/27)	0.003
Margin Free (n= 93) Positive (n=7)	(43/93) (7/7)	(50/93) 0 (0.0%)	0.014
Chemotherapy Yes (n=91) No (9)	(48/91) (2/9)	(43/91) (7/9)	0.160
Ungrouped Response Yes (55) No (45)	(20/55) (33/45)	(35/55) (12/45)	0.001
ER Positive (37) Negative (63)	(0/37) (50/63)	(37/37) (13/63)	0.001
PR Positive (29) Negative (71)	(0/29) (50/71)	(29/29) (21/71)	0.001
HER-2 Positive (18) Negative (82)	(0/18) (50/82)	(18/18) (32/18)	0.001

LN: lymph node; ER: estrogen receptor; PR: progesterone receptor and HER2: human epidermal growth factor receptor

▪ **mRNA expression levels of *Oct4*, *Survivin* and *Cyclin D1* genes in the two groups (TNBC and non-TNBC).**

There was significant association between RNA expression level of *oct4*, *survivin* and *cyclin D1* genes and TNBC or non-TNBC group ($p < 0.001$, $p = 0.001$ and $p = 0.003$; respectively). RNA expression level of *oct4*, *survivin* and *cyclin D1* was 60%, 32% and 32%; respectively in TNBC patients. In non-TNBC patients, RNA expression level of *oct4*, *survivin* and *cyclin D1* genes was 22%, 15% and 17% of patients (Table 3).

Table 3. RNA expression level of *oct4*, *survivin* and *cyclin D1* in TNBC and non-TNBC patients

Gene marker	TNBC n=50 (%)	non-TNBC n=50 (%)	P value
<i>Oct4</i>			
High	30(60%)	11(22%)	<0.0001
Low	20(40%)	39(78%)	
<i>Survivin</i>			
High	32(64%)	15(30%)	0.001
Low	18(36%)	35(70%)	
<i>Cyclin D1</i>			
High	32(64%)	17(34%)	0.003
Low	18(36%)	33(66%)	

TNBC:Triple negative breast cancer, non-TNBC:Non Triple negative breast cancer, *Oct4*:biomarker gene, *Survivin*: biomarker gene, *CyclinD1*: biomarker gene

▪ **Correlation between *Oct4*, *Cyclin D1* and *Survivin* gene expression and patient's feature in TNBC**

In the TNBC group. The *oct4* gene, the mean fold expression was 2.0-fold with the age <50year and 2.7fold with the age ≥ 50 year, ($p = 0.03$). In premenopause status, the mean fold expression was 2.3-fold vs 2.5fold in postmenopause, ($p = 0.031$). The mean fold expression was 1.44-fold in tumor size ≤ 5 cm compare to 1.0fold in tumor size > 5 cm, ($p = 0.01$). The mean fold expression was 2.4fold in the early tumor stage compared to 2.6fold in late tumor stage, ($p = 0.03$). The mean fold expression was 2.7fold with tumor grade (I, II) compared to 1.7fold in tumor grade (III), ($p = 0.02$). Similarly, the mean fold expression was 2.3-fold with positive lymphnode vs 3.0fold in negative cases, ($p = 0.01$).

Finally, the mean fold expression was 2.8fold in metastasis state compared to 1.6fold in non- metastatic state, ($p=0.003$) (Table4). There was a high significant difference between the expression of *oct4* and the clinic-pathological features except the family history.

The mean fold expression of *cyclin D1* gene was 4.3fold with the age of patients <50year and 3.5fold with age ≥ 50 year ($p=0.02$). In premenopause status, the mean fold expression was 3.8fold compared to 4.0fold in postmenopause ($p=0.01$). The mean fold expression was 3.9fold in cases who have no family history compared to 0.33fold in cases who have family history, ($p=0.001$). The mean fold expression was 3.7fold in the early tumor stage compared to 4.3fold expression in late tumor stage, ($p=0.03$). Finally, in metastatic status, the mean fold expression was 4.3fold in metastasis state compared to 2.9fold in no metastatic state, ($p=0.003$) (Table4). There was a significant difference between *cyclin D1* expression and age, menopause, family history, tumor stage and metastasis status.

The data detected that the mean fold expression of *survivin* gene was 2.8fold in age <50 year and 3.7fold in age ≥ 50 year, ($p=0.001$). the mean fold expression was 3.3fold with the patients who have no family history compared to 0.32fold in the patients who have family history, ($p=0.002$). Similarly, in tumor size ≤ 5 cm, the mean fold expression was 2.5fold and 2.8fold with size >5 cm, ($p=0.01$). In negative lymphnode status the mean fold expression was 2.7fold compared to 3.4fold in positive lymphnode, ($p=0.04$). The mean fold expression was 2.4fold in patients who have no metastatic status compared to 3.7fold in in metastatic status, ($p=0.03$) (Table 4). There was statistically difference between *survivin* and age, family history, tumor size, lymphnode and metastatic status.

▪ **The relation between *Oct4*, *Cyclin D1* and *Survivin* gene expression and patient's features in non-TNBC**

In the non-TNBC group, a highly significant association was found between *oct4* gene expression and family history, tumor size, tumor grade and metastasis. The mean fold expression was 1.0fold in patients who have no family history compared to 1.44fold in patients who have family history ($p=0.05$). The mean fold expression levels were 1.4fold with the tumor size ≤ 5 cm and 0.89fold in tumor size >5 cm, ($p=0.002$). Regard to tumor grade, the mean fold expression was 1.0fold in grade I, II compared to 1.9fold in grade III, ($p=0.003$). Finally, the mean fold expression was 1.0fold in the patients who have no metastasis compared to 1.4fold in the patients who have metastasis status, ($p=0.02$) (Table 5).

There was a significant association between *cyclin D1* gene expression and tumor size, tumor grade and lymphnode. The mean fold expression was 3.2fold with the tumor size ≤ 5 cm and 2.6fold in tumor size >5 cm, ($p=0.05$). As for tumor grade, the mean fold expression was 2.7fold in grade I, II compared to 2.3fold in grade III, ($p=0.02$). And the mean fold expression was 2.3fold in negative lymphnode compared to 3.2fold in positive one, ($p=0.002$) (Table 5).

The mean fold expression of *survivin* gene was 2.9fold with the age<50year compared to 1.09fold with the age \geq 50year, (p=0.03). The mean fold expression was 2.7fold in absence of family history vs 2.28fold with the patients who have family history, (p=0.03). At tumor size \leq 5cm, the mean fold expression was 2.49fold and 2.82fold in the tumor size>5cm, (p=0.4). As for, the mean fold expression was 2.8fold for early stage of tumor compared to 2.4fold for late tumor stage, (p=0.002) (Table 4). With the tumor grade (I, II), the mean fold expression was 2.69fold while in cases with tumor grade (III, IV) the mean fold expression was 2.31fold, (p=0.04). Finally, with positive lymphnode, the mean fold expression was 2.2fold and it was 2.96fold with negative lymphnode cases, (p=0.04) (Table5). There was a significant correlation between the *survivin* gene expression and all clinic-pathological characteristic of patients except the menopause and metastasis

Table 4: The association between *Oct*, *Cyclin D1* and *Survivin* genes mRNA-expression levels with the clinico-pathological features in TNBC patients

Parameters	<i>Oct</i> Exp.		<i>Oct 4</i> Mean fold expression	<i>P</i> <i>value</i>	<i>Cyclin D1</i> Exp.		<i>Cyclin D1</i> Mean fold expression	<i>P</i> <i>value</i>	<i>Survivin</i> Exp.		<i>Survivin</i> Mean fold expression	<i>P</i> <i>value</i>
	High	Low			High	Low			High	Low		
Age (yr): < 50(n=24) ≥ 50(n= 26)	15 17	9 9	2.0±1.4 2.7±2.1	0.03	20 19	4 7	4.3±2 3.5±2.1	0.02	16 22	8 4	2.8±2 3.7±1.9	0.001
Menopause: Pre (n= 23) Post (n= 27)	16 16	7 11	2.3±1.5 2.5±2	0.031	17 22	6 5	3.8±2.3 4.0±1.9	0.01	19 19	4 8	3.5±1.9 3.0±2.1	0.62
Family History Absent (n= 49) Present (n= 1)	32 0	17 1	2.45 ±1.85 0.35±00	0.062	39 0	10 1	3.9±2 0.33±00	0.001	38 0	11 1	3.3±2 0.32±00	0.002
Tumor size (cm) ≤ 5(n=23) > 5(n=27)	13 19	10 8	1.44±1.2 1.0±1.1	0.01	15 24	8 3	3.2±1.9 2.5±1.6	0.06	16 22	7 5	2.5±1.8 2.8±1.8	0.01
Tumor stage Early(n=30) Late(n=20)	22 17	8 3	2.4±1.6 2.6±2.1	0.03	22 17	8 3	3.7±2.1 4.3±2.0	0.03	22 16	8 4	3.2±1.8 3.5±2.2	0.13
Tumor Grade I-II(n=36) III(n=14)	25 7	11 7	2.7±1.9 1.7± 1.4	0.02	29 10	7 4	4±2.1 3.5±2.2	0.07	27 11	9 3	3.0±1.9 3.8±2.2	0.25
LN status: Negative(n=7) Positive(n=43)	4 28	3 15	3.0±3 2.3±1.6	0.01	5 34	2 9	3.8±2.5 3.9±2.0	0.31	5 33	2 10	2.7±1.9 3.4±2.0	0.04
Metastasis: M0(n=16) M1(n=34)	7 25	9 9	1.6±1.4 2.8±1.9	0.003	9 30	7 4	2.9±2.4 4.3±1.8	0.003	8 30	8 4	2.4±2.2 3.7±1.7)	0.03

Table 5: The association between OCT, Cyclin D1 and Survivin genes mRNA-expression levels with the clinico-pathological features in non-TNBC patients

Parameters	OCT Exp.		OCT 4 Mean fold expression	P value	Cyclin D1Exp.		Cyclin D1 Mean fold expression	P value	Survivin Exp.		Survivin Mean fold expression	P value
	High	Low			High	Low			High	Low		
Age (yr): < 50(n=22) ≥ 50(n= 28)	8 7	14 21	1.25±1.2 1.07 ±1.27	0.65	16 21	6 7	2.96±1.95 2.76±1.67	0.66	16 16	6 12	2.97±1.74 1.09±1.2	0.03
Menopause: Pre (n= 23) Post(n= 27)	8 7	15 20	1.22±1.2 1.09±1.2	0.70	17 20	6 7	2.86±1.8 2.83±1.7	0.75	15 17	8 10	2.7±1.8 4.0±0.58	0.61
Family History Absent(n= 40) Present(n= 10)	11 4	29 6	1.08±1.16 1.44±1.52	0.05	11 8	29 2	2.78±1. 80 3.14±1.77	0.83	27 5	13 5	2.7±1.7 2.28±2.17	0.03
Tumor size (cm) ≤ 5(n=25) > 5(n=25)	10 5	15 20	0.46±0.17 0.32±0.20	0.002	19 18	6 7	0.344±0.18 0.29±0.11	0.05	25 17	10 8	2.49±1.8 2.82±1.8	0.04
Tumor stage Early(n=29) Late(n=21)	23 14	6 7	3.0±1.78 2.64±1.8	0.03	23 14	6 7	3.0±1.7 2.64±1.8	0.06	20 12	9 9	2.8±1.7 2.45±1.9	0.002
Tumor Grade I-II(n=45) III(n=5)	12 3	33 2	1.09±1.21 1.81±1.3	0.003	32 3	13 2	2.72±1.8 3.98±1.1	0.02	29 3	16 2	2.69±1.84 2.31±2.0	0.04
LN status: Negative(n=20) Positive(n=30)	6 9	14 21	1.12±1.14 1.17±1.30	0.91	12 25	8 5	2.29±1.81 3.21±1.69	0.002	10 22	10 8	2.20±1.99 2.96±1.7	0.03
Metastasis: M0(n=40) M1(n=10)	11 4	29 6	1.08±1.19 1.42±1.40	0.02	29 8	11 2	2.83±1.87 2.91±1.44	0.32	26 6	14 4	2.66±1.71 2.65±2.12	0.33

4. Survival analysis.

The average follow-up period was 43 months. The median overall survival of the 100 breast cancer patients was 43 months (range, 2–68 months), and the median time of disease-free interval was 42 months (range, 6–43 months). The triple negative breast cancer patients showed significantly decreased disease-free interval (DFS) ($p < 0.001$, log rank) and overall survival (OS) ($p = 0.002$, log rank) when compared to those with non-TNBC patients (Figure 1). In TNBC group, patients with positive expression of *survivin* was significantly associated with decreased OS ($p = 0.03$, log rank).

On the other hand, TNBC tumors with positive *oct4* and *cyclin D1* had reduced OS compared to those negative to *oct4* and *cyclin D1* but without significant difference ($p = 0.8$ and $p = 0.09$, respectively, log rank) (Figure 2). However, in non-TNBC group, with positive *oct4*, *cyclin D1* and *survivin* did not significantly differ in terms of DFS and OS when compared to those with negative expression (Figure 3).

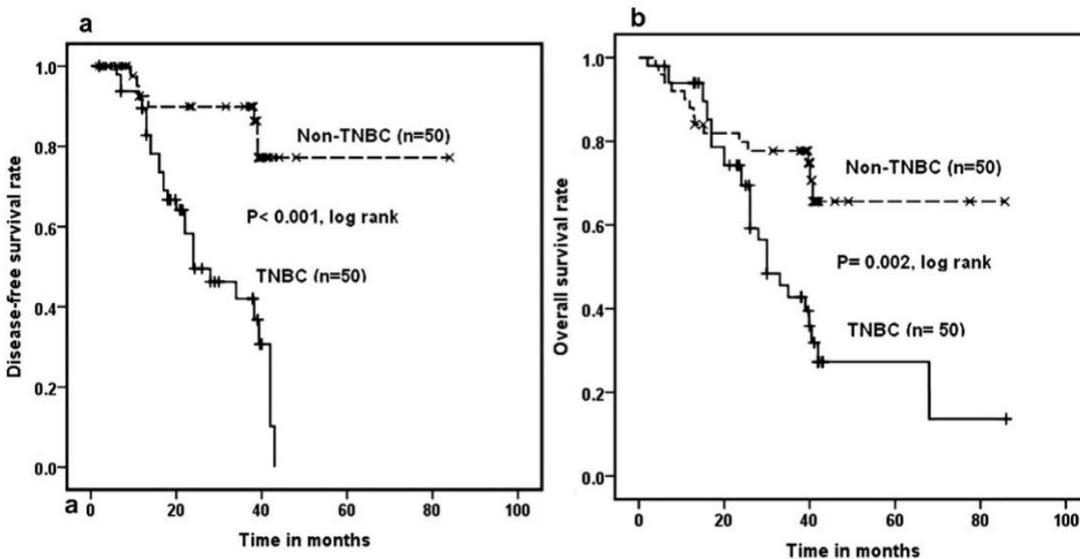


Figure 1: The disease-free survival and overall survival in TNBC and non-TNBC patients.

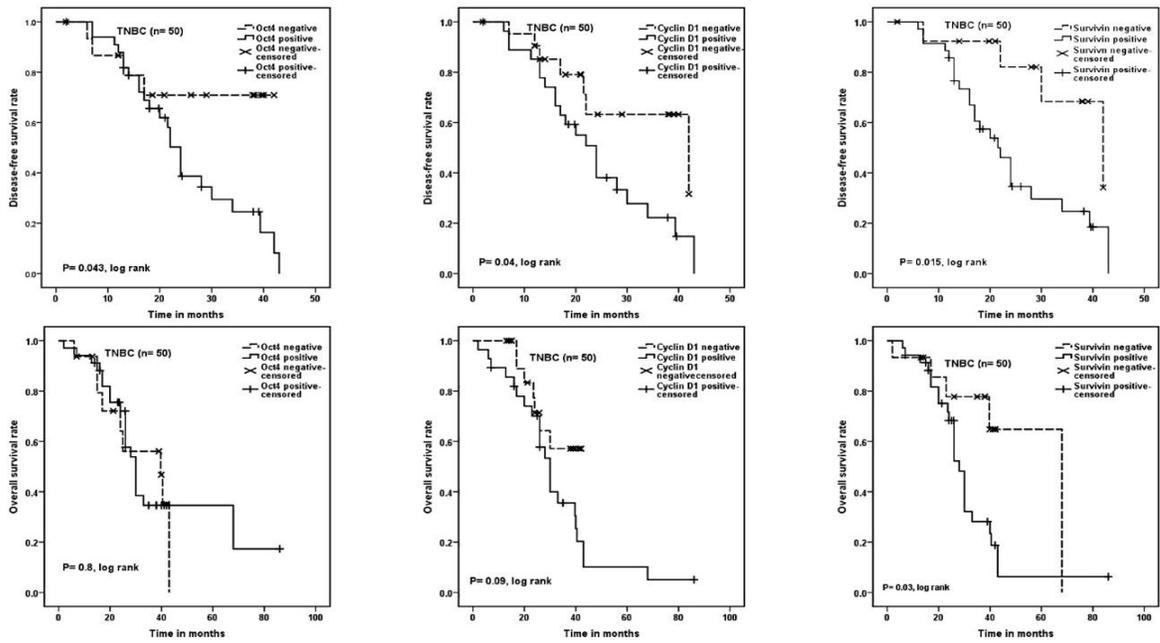


Figure 2: The gene expression in association to disease free survival and overall survival in TNBC patients.

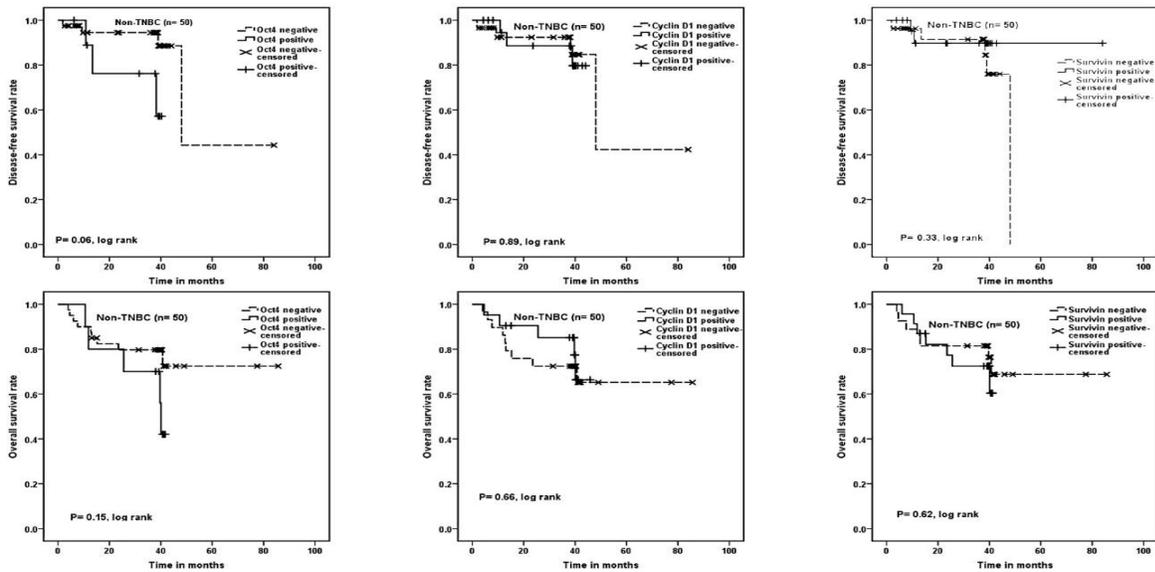


Figure 3: The gene expression in association to disease free survival and overall survival in non-TNBC patients

DISCUSSION

Breast cancer is the most common type of cancer in women worldwide, represents 32% of the diagnostic cases in female and considered the second cause of cancer related death **Houssami, N. and Cho, N. (2018)**.

Triple-negative breast cancer (TNBC) accounts for 12-17% of breast cancer cases and it is considered a discrete breast cancer subgroup. However, TNBC had been shown to constitute a vastly heterogeneous disease encompassing a wide spectrum of entities with marked genetic, transcriptional, histological type and clinical differences. Although most TNBCs are high-grade tumors, there are well-characterized group with low-grade tumors that usually have an indolent clinical course, better natural history; and molecular features as well as optimal the response to therapy vastly differ from those of high-grade TNBC **Fresia Pareja et al., (2016)**.

In the current study, one-hundred tested patients were classified according to their age to two groups: 46 less than 50 years and 54 more than 50 years old. Twenty-two out 46 patients (47.8%) which less than 50 years have TNBC and 24 out of them (52.2%) with non-TNBC. Regarding to 54 patients with age more than 50 years, 28 of them (51.9%) have TNBC and 26 out of them (48.1%) with non-TNBC. No significant difference was reported between the two groups regarding to the age. This could be attributed to the small number of the patients assessed in the study.

In a previous study from Iran, the authors reported that 30 out of 214 patients (14%) were TNBC and 184 (86%) patients were non-TNBC. A significant difference was reported in this study where the mean age (\pm SD) of the TNBC patients was 43 (\pm 12) years whereas in the non-TNBC, the mean age of the patients was 50 (\pm 12) years ($p=0.03$). This study declared that TNBC is correlated with young age and therefore it is more aggressive **Abdollahi, A. and Etemadi, M., (2016)**. Similarly, another study from Taiwan, reported a highly significant difference in the age of the patients between TNBC and non-TNBC groups **Yao-Lung, K. et al., (2011)**. In another study by **Li et al., (2013)** they reported the statistically significant difference between the mean age of younger patients with TNBC group (49 years) compared to 54 years in nonon-TNBC group cases ($p<0.003$) **Li, D. et al., (2017a)**. The discrepancy between results of the present study and previously reported ones may be due to the difference in genetic background and environmental factors. The epidemiological approaches may have also contributed to this discrepancy.

As for tumor size, 23 (47.9%) out of the 48 cases were ≤ 5 cm in the non-TNBC patients compared to 25 (52.1%) in the TNBC ones. As for, in patients with tumor size >5 cm 27/52 (51.9%) cases were non-TNBC and 25/52(48.1%) were TNBC ($p=0.69$). The results reported by **Yao-Lung et al.(2011)** and **Abdollahi & Etemadi (2016)** agree with the current results. They found that there were no significant differences between the tumor size in the two groups. The mean size of the tumor was greater in TNBC than in non-TNBC group. The mean size in TNBC was 3.83 ± 1.88 (1-10 cm range) and was 2.98 ± 2.22 in

non-TNBC group (0.2-13 cm range). Similarly, **Li et al. (2013)** found the statistical difference between tumor size and the two groups ($p=0.000$). The TNBC patients had a bigger tumor more than the non-TNBC cases **Li, D. et al., (2017a)**.

In the current study, three patients were with tumor grade I, two of them (66.7%) from TNBC group and only one (33.3%) from non-TNBC patients. Seventy-eight patients had the tumor grade II, 43 (55.1%) were with TNBC compared to 35/78 (44.9%) with non-TNBC. Finally, grade III was found in 19 cases, 5 of them 5/19 (26.3%) were TNBC patients and 14 (73.7%) in non-TNBC ones ($p=0.12$). In contrary to current data, studies by **Abdollahi and Etemadi (2016)**, **Yao-Lung et al.(2011)** and **Li et al.(2013)** showed that grade III was significantly in TNBC and non-TNBC group ($p=0.02$, $p=0.0001$, $p=0.008$ and $p=0.00$; respectively). **Yao-Lung et al. (2011)** reported that the TNBC patients were with grade III (74 of 152, 48.7%) ($p=0.0001$). In TNBC group, majority 35 (56.4%) patients had modified Scarff Bloom–Richardson grade III tumors, whereas 24 (38.7%) had grade II and 3 (4.8%) had grade I tumors. In non-TNBC group, 22 (18.6%) patients had grade I tumors, 53 (44.9%) had grade II and 43 (36.4%) had grade III tumors. The Chi-square test showed a significant difference in the distribution of histologic grade of the diagnosed TNBC and non-TNBC patients ($P= 0.008$). **Li et al. (2013)** compared the histologic grade of TNBC and non-TNBC patients. In TNBC cases, there were 618 (23.33%) with grade 1 tumors, 1118 (42.20%) with grade 2 tumors and 913 (34.47%) with grade 3 tumors. In non-TNBC cases, there were 4154 (21.75%) Grade 1 tumor, 8721 (45.66%) Grade 2 tumors and 6225 (32.59%) Grade 3 tumors. The Chi-square test showed a significant difference in the distribution of histologic grade of the diagnosed TNBC and non-TNBC ($P=0.001$).

According to tumor stages, early stage was significantly more common in the TNBC group compared to the non-TNBC groups. Tumor stage I was recorded in 21 cases [21(100%) was in TNBC]. The patients which had tumor stage II were in 59 patients [29(49.2%) in TNBC versus 30(50.8%)]. As for late stage III and IV, all 20 (100%) patients with non-TNBC tumor compared to 0/20 (0%) were in TNBC patients. Stage (I-II) was detected more frequently in the TNBC compared to non-TNBC where stage (III-IV) was more significant in non-TNBC than those in TNBC. **Nabi, et al. (2015)**, they study the clinicopathological features of 180 cases of TNBC and non-TNBC tumors, 5(8.0%) cases at stage I, 28 (45.1%) cases at stage II and III, 1 (1.6%) case at stage IV in TNBC tumor. In non-TNBC group, 22 (18.6%) cases were diagnosed with disease at stage I, 52 (44.0%) cases at stage II, 42 (35.5%) cases at stage III and 2 (1.6%) cases at stage IV. **Li et al. (2013)**, they reported that no significant association between tumor stage and TNBC or non-TNBC. The results of the previous studies were contrary with current data.

The lymph nodes were positive in 43(58.9%) out of the 73 patients in the TNBC group compared to 30 (41.1%) patients in the non-TNBC. lymph nodes were negative in 7 (25.9%) TNBC patients and 20 (74.1%) non-TNBC ($p=0.003$). Surgical margins of the lumens were positive in 7 patients, all of them (100%) were TNBC patients compared to none in the non-TNBC group. The study of **Nabi et al. (2015)** included 180 breast cancer patients, 62 out of them (34.4%) were NBC patients. They reported that the lymphovascular

involvement and axillary lymph node metastasis was more in TNBC group than those in non-TNBC group. Also, the extra nodal spread increased in non-TNBC cases than in TNBC patients. No statistically insignificant were recorded between the lymph node status and the two group ($p=0.246$). **Yao-Lung et al. (2011)** found that the lymph node not significant between TNBC and non-TNBC group ($p=0.307$). These results are on the contrary with the current results, there was a significant association between the lymph node status in the two groups, whereas 43 (58.9%) out of the 73 patients with positive in the TNBC compared to only 30(41.1%) cases in non-TNBC. Also, the negative lymph node was detected in 7(25.9%) TNBC patients compared to 20(74.1%) non-TNBC patients ($p=0.003$) **Yao-Lung et al.(2011)**. However, our data agree with **Li et al. (2013)**, they compared the positive and negative lymph node in TNBC and non-TNBC patients. The number of lymph node was high in TNBC (71.39%) patients than those of non-TNBC (36.78%) patients. A significant difference was found between positive lymph node in the two groups ($p=0.000$) **Li et al.(2013b)**.

In the current study, as for the presence of family history, a higher proportion of patients had positive family history in the TNBC group. Patients that have a family history were eleven; 10 of them (90.9%) in TNBC group and only one (9.1%) in non-TNBC ($p=0.05$). A study by **Li et al. (2013)** which was carried out on 21,749 breast cancer patients, declared that TNBC which is related to family history of breast cancer may be increased metastatic risk and have a poor prognosis. They found that family history was in one member for 4227 cases, two members for 1607 cases and 3 or more members for 444 cases members had breast cancer family members in non-TNBC patients. A significantly propagation of breast cancer reported with the members who had family history in TNBC cases compared to non-TNBC cases ($p=0.000$) **Li et al.(2013b)**.

According to the response to treatment, **Yin et al. (2009)** recorded that 87.30% of TNBC patients respond to treatment compared to 13.70% not responders. In subgroups (*ERBB2+* and *HR+/ERBB2-*), 88.33% and 85.61%; respectively were responders and 11.67% and 14.39% did not respond to treatment; there was no statistically difference between respond to treatment between these two groups **Yin et al. (2009)**. This result is corresponded with the present data, where in fifty-five tested patients were responders to treatment, 35 out of 55 (63.6%) were non-TNBC patients and 20 of them (36.4%) were TNBC patients. Out of 45 non-responder patients, 33 (73.3%) in TNBC patients and 12 (26.7%) in non-TNBC patients.

In the current study, the RNA expression levels of *oct4*, *cyclin D1* and *survivin* genes in all studied patients were 39(78%), 33(66%) and 35(70%); respectively in non-TNBC tumor compared to 20(40%), 18(36%) and 18(36%); respectively in TNBC tumor. The data showed statistical significance in the distribution of the RNA expression level of all genes and the two groups ($p<0.001$, $p=0.003$ and $p=0.001$; respectively. **Ezeh et al.(2005)** also found that normal breast tissues do not express detectable levels of *oct4* expression, and breast carcinoma in advanced stage reveals *oct4* expression along with other stem cell markers **Ezeh et al.(2005)**. In addition, **Chang et al.,2011)** demonstrated that *oct4* promotes tumorigenesis of colorectal cancer cells in both autocrine and paracrine

way. **Saigusa et al., (2009)** showed that *oct4* expression is usually associated with the recurrence of rectal cancer after chemo-radiotherapy **Saigusa et al., (2009)** and **Liu et al., (2014)** reported that the *oct4* expression is positively correlated to breast cancer as it has a role in angiogenesis vasculogenic mimicry formation by increasing cancer stem cells subpopulation, thereby potentiating breast cancer metastasis **Liu et al., (2014)**. Similarly, tumor tissue *oct4* expression was positively correlated with histologic grade, pathological tumor size, N stage and TNM stage, and it could be served as an independent biomarker to predict worse prognosis in surgical patients with TNBC **Zhang et al.(2018)**.

The median overall survival of the 100 breast cancer patients was 43 months (range 2 - 68 months), and the median time of disease-free interval was 42 months (range 6 - 43 months). Triple negative breast cancer patients showed significantly decreased disease-free interval ($p < 0.001$, log rank) and overall survival ($p = 0.002$, log rank) when compared to those with non-TNBC patients. In TNBC group, patients with positive expression of *survivin* were significantly associated with decreased OS in TNBC patients. On the other hand, TNBC tumors with positive *oct4* and *cyclin D1* had reduced OS compared to those negative to *oct4* and *cyclin D1* but without significant difference. However, in non-TNBC group with positive *oct4*, *cyclin D1* and surviving, there was not significantly difference in terms of DFS and OS when compared to those with negative expression. These results showed decreased OS among TNBC patients compared to non-TNBC patients.

Goncalves et al., (2018), recorded that OS and DFS rates in 5-year were 62.1% and 57.5% for TNBC and 80.8% and 75.3% for non-TNBC, respectively ($P < .001$). There was significantly increased OS and DFS among non-TNBC compared to TNBC patients.

Hernandez-Aya et al., (2011) showed that in a large cohort of TNBC patients, the median age of TNBC patients was 48 years (range, 21 - 87 years). The median follow-up of 53 months (range, 0.7 - 317 months). The 5-year follow-up of patients, OS or relapse free survival (RFS) rates was decreased significant with the number of positive lymph-node ($p < .0001$; each).

Qiu, J. et al., (2016), they reported that there were 38% of TNBC patients were died at 5-year follow up and 19% of non-TNBC cases. Regarding to recurrences, 43% were in TNBC patients versus 25% were in non-TNBC patients. In this study the recurrence rate was high than those in Chinese cohort (2016).

Che Lin, et al., (2009), they found that OS was poor in TNBC cases, otherwise, there was not significant difference between DFS and non-TNBC in a Taiwan cohort study

In conclusion: *Oct4* gene expression is significantly correlated with clinic-pathological features of patients. *Survivin* gene is useful prognostic marker in TNBC and might be useful for molecular targeting therapy of TNBC treatment. *Cyclin D1* may acts as a marker for the aggressive TNBC. These tumors may respond to target therapy that downregulates *cyclin D1* amplification. More research with large samples size is needed.

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مستوى التعبير الجيني للحامض النووي الريبوزي للجينات ال Oct4,Cyclin D1 and survivin في مرضى سرطان الثدي ثلاثى السلبية و غير ثلاثى السلبية

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الملخص :

الهدف من هذه الدراسة هو تقييم مستوى الحامض النووي الريبوزي للجينات التالية: Oct4,Cyclin D1 and survivin في عينات اورام لأنسجة مرضى سرطان الثدي ثلاثى السلبية مقارنة بمرضى سرطان الثدي غير ثلاثى السلبية. وكذلك ايجاد العلاقة بين التعبير الزائد لهذه الجينات و الصفات الأكلينيكية للمرضى وذلك باستخدام ال qRT-PCR . وقد اظهرت النتائج ان التعبير الجيني الزائد لجين Oct4,Cyclin D1 and survivin له دلالة احصائية فى مجموعة مرضى سرطان الثدي ثلاثى السلبية كما فى مجموعة مرضى سرطان الثدي الغير ثلاثى السلبية. وكانت هرمونات ER, PR and Her-2/neu سلبية فى مجموعة سرطان الثدي ثلاثى السلبية. و بالنسبة الجين Oct4 يوجد دلالة احصائية بين التعبير الزائد للجين و الصفات الاكلينيكية للمرضى ما عدا التاريخ العائلى. ووجد ان هناك علاقة احصائية بين التعبير الجيني الزائد لجين Cyclin D1 و العمر ، سن اليأس، مرحلة الورم، مدى الانتشار، التاريخ العائلى و اخيرا الاستجابة للعلاج. يوجد ايضا دلالة احصائية بين التعبير الزائد لجين Survivin و الصفات الأكلينيكية ما عدا سن اليأس، مرحلة و درجة الورم.

وجد ان مرضى سرطان الثدي ثلاثى السلبية له دلالة احصائية تقلل من (DFS) وهى فترة بقاء المريض على قيد الحياة بدون انتشار المرض وذلك عند مقارنة مع هؤلاء المرضى غير ثلاثى السلبية. فى مجموعة مرضى سرطان الثدي ثلاثى السلبية فى وجود التعبير الزائد لجين ال survivin كان هناك دلالة احصائية مرتبطة بتقليل مدة بقاء المريض على قيد الحياة (OS) مع امكانية رجوع المرض. ومن ناحية اخرى ورم مرضى سرطان الثدي ثلاثى السلبية مع وجود التعبير الزائد لجينات Oct4 and cyclin D1 يقلل من فترة بقاء المريض (OS) مع امكانية رجوع المرض بالمقارنة ب مرضى سرطان الثدي ثلاثى السلبية مه عدم وجود التعبير الزائد لهذين الجينات و لكن بدون دلالة احصائية. و كما فى هؤلاء المرضى غير ثلاثى السلبية مع وجود التعبير الزائد لجينات Oct4, cyclin D1 and survivin ليس لهم دلالة احصائية سواء مع DFS او OS وذلك بالمقارنة مع هؤلاء المرضى فى عدم وجود التعبير الزائد لهذه الجينات.

و من هذه الدراسة نجد ان تقييم التعبير الزائد للثلاث جينات قد يكون عامل مؤثر فى التشخيص والعلاج لمرضى سرطان الثدي ثلاثى السلبية.

الكلمات المفتاحية :- سرطان الثدي ثلاثى السلبية ، سرطان الثدي الغير ثلاثى السلبية، جين الاكتوفور، جين السيكلين، جين السرفيفين