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International Journal of Current Research
Vol. 9, Issue, 03, pp.47940-47946, March, 2017

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

ISSN: 0975-833X

RESEARCH ARTICLE

CADMIUM INDUCED BREAST CANCER AND RNA GENE EXPRESSION

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ARTICLE INFO

Article History:
Received 15th December, 2016
Received in revised form
17th January, 2017
Accepted 28th February, 2017
Published online 31st March, 2017

Key words:
Breast cancer,
Cadmium,
Gene expression,
Cell proliferation,
Hormone receptors.

ABSTRACT

Cadmium (Cd) is a heavy metal that is ubiquitous all over the world. Breast cancer is the most widely recognized tumor affecting women. The work aims to study the effect of cadmium toxicity on cancer associated genes expression in a sample of breast cancer Egyptian patients. A case - control study was conducted on 100 female patients attending the Surgical Oncology Center and complaining from breast mass. From each patient 10 gm breast tissue sample was collected for estimation of cadmium by atomic absorption spectrometer and testing RNA gene expression (CycD1; CycE; cdk2; c-myc; and GAPDH as housekeeping gene) using Real Time - Polymerase Chain Reaction (RT – PCR). The results found that the age of breast cancer patients ranged from 37–74 years. The cadmium level and four genes expression levels were significantly higher in tumor tissue as compared to both non - cancerous and healthy tissues ($p < 0.001$). Also no significant difference in Cd and gene expression levels were found when compared between hormone receptors positive or negative cancer patients ($p > 0.05$). It could be concluded that Cd may induce breast cancer through increasing the cancer associated gene expression responsible for cell proliferation.

Citation: Doaa Abdel Wahab A. El Morsi, Rania Hamed Abdel Rahman, Omar Farouk and Sahar M. Hamed, 2017. "Cadmium Induced Breast Cancer and RNA Gene Expression", *International Journal of Current Research*, 9, (03), 47940-47946.

INTRODUCTION

Cadmium (Cd) is widely distributed as environmental pollutant from different sources. Cd exposure occurs through dietary contamination; water and soil. Tobacco smoking is an essential source of Cd exposure (Sirchia and Luparello, 2009). The International Agency for Research on Cancer has classified Cd as category I carcinogen (IARC, 1993). Cd at the cellular level initiated increments in DNA synthesis, cell proliferation, apoptosis, and can bring about various molecular lesions that would be relevant to carcinogenesis (Mahmood *et al*., 2012). It empowers transcription factors expression as well as stress proteins synthesis that are suggested to be the components for the cancer-causing activity of this metal (Shams *et al*., 2012). Breast cancer (BC) is the commonest pervasive cancer influencing women and is causing mortality between the ages of 35 and 45 years (Yeon *et al*., 2011). In Egypt, breast and liver cancers had the top positions representing 45 % of all cancers. Among females, in Lower, Middle, and Upper Egypt, breast cancer had the higher frequency (33.8 %, 26.8 % and 38.7 % respectively). The number of breast cancer patients is expected to increase from 115,000 in 2013 to more than 331,000 patients in 2050, approximately 3- fold increase (Ibrahim *et al*., 2014). Breast cancer is classified either estrogen(ER) positive or ER negative, contingent upon the presence or absence of ERα. Advancement of ER-positive breast cancer was controlled by the action of the ERs and the circulating levels of 17 β estradiol. Heavy metals such as cadmium (Cd) which acts as endocrine disruptor, might be included in ERα modulation of breast tumorigenesis (Siewit *et al*., 2010).Various risk factors were contributed to breast cancer such as hereditary factors, drinking alcohol, smoking, obesity, low physical activity, menstrual history, pregnancy history and environmental pollutants (Strumylaite *et al*., 2008). Cadmium causing impact of breast cancer could be clarified by aberrant gene expression; inhibition of apoptosis; DNA damage and induction of oxidative stress (Joseph, 2009). Previous studies were carried out to assess the risk of breast cancer and Cd exposure in different biological material (Antila *et al*., 1996; McElroy *et al*., 2006; Gallagher *et al*., 2010; Siewit *et al*., 2010; El-Atta *et al*., 2011; El-Harouny *et al*., 2011; Makowska *et al*., 2011; Strumylaite *et al*., 2011; Arooj *et al*., 2012; Mahmood *et al*., 2012; Nagata *et al*., 2013; Rahim *et al*., 2013; Strumylaite *et al*., 2014; Peng *et al*., 2015; Hilal *et al*., 2016; Lin *et al*., 2016; Maele-Fabry *et al*., 2016). Other literatures studied the mechanism of action of Cd either

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on DNA damage (El-Atta *et al*., 2011; Saleh *et al*., 2011; Ni *et al*., 2014) or oxidative stress (Yeon *et al*., 2011; Shams *et al*., 2012; El-Deeb *et al*., 2016) or stress response genes and apoptosis (Sirchia and Luparello, 2009), or cytotoxic effect (Gonçalves *et al*., 2012; Luparello *et al*., 2013) or effect on the estrogen receptors (Stoica *et al*., 2000; Siewit *et al*., 2010; Strumylaite *et al*., 2014; Wei *et al*., 2015) in breast cancer. Few studies were concerned with the Cd effect on cell regulation genes and were done mostly in cell lines (Siewit *et al*., 2010; Wei *et al*., 2015). This work to the best of our knowledge is one of few clinical researches that aimed to study the effect of cadmium toxicity on the expression of cancer associated genes involved in cell proliferation in a sample of Egyptian women.

Patients & Methods

Study Design

A case – control study was conducted on 100 female patients suffering from breast mass. They were randomly chosen from those attending to the Surgical Oncology Center, Mansoura University, Egypt.

Exclusion criteria

Patients with positive family history of breast cancer or had previous surgery were excluded from the study.

Inclusion Criteria

Patients recruited in the study were non – smokers and not drinking alcohol.

Patients were classified into two groups

Control group "non-cancerous group"

It comprised 50 female patients with histologically confirmed non - risky non - proliferative benign fibroadenoma (i.e. no risk of cancer development). They were matched with the cancer patients in age and residence.

Test group "Cancerous group"

It included 50 female patients with new histologically confirmed breast cancer lesion. This group was classified as follows: ***Tumor group*** (samples were taken from the cancer tissue) and **healthy group** (samples were obtained from the adjacent histologically healthy tissue six cm distant from the lesion).

Ethical consideration

A written informed consent was obtained from all patients of the studied groups before performance of all procedures in the present work besides approval from the Ethical Committee of Mansoura Faculty of Medicine.

Methods

All patients underwent:

- **Data collection:** Age; history of smoking; residence and alcohol consumption.
- **Clinical examination:** Clinical stage; grading and TNM staging (tumor – lymph node – metastasis) and metastasis.
- **Investigations:**
- Fine needle aspiration cytology (FNAC) or true cut biopsy of the breast mass.
- Postoperative histopathological examination of breast mass and hormone receptors [estrogen receptors (ER), progesterone receptors (PR) and Human Epidermal Receptor 2 (HER2)].
- Radiological examination by Sono-mammography.

Surgery and Sampling

From each patient ten gm tissue sample was taken during the surgical procedure:

Each sample was divided into two parts

- One part (nine gm) was collected in Epindorff tubes and put in liquid nitrogen to be preserved at – 70 ^{o}C for RNA gene expression.
- The remaining part (one gm) was collected in polyethylene tubes and stored at – 20 ^{0}C for measuring the cadmium level (µg / g) by atomic absorption spectrometer.

Cadmium analysis (AOAC, 1990): In Atomic absorption Lab; Unit of Genetic Engineering and Biotechnology; Faculty of science; one gm of breast tissue was poured into 50-mL beakers, followed by the addition of 10 mL mixture of analytical grade acids HNO_3: $HClO_4$ (5:1). The digestion was performed at a temperature of about 190 °C for 2 - 3 hrs. After cooling, the solution was made up to a final volume (25 mL) with deionized water acidified with 3 % nitric acid. The metal concentrations were determined using "Buck Scientific Accusys 211 atomic absorption spectrophotometer". The results and the data were reported in µg/g.

Gene Expression Analysis

The genetic analysis was done in the Genome Unit, Faculty of Medicine, Cairo University. From each tissue sample, total RNA was extracted using the Trizol reagent according to the manufacturer's protocol. Three micrograms of total RNA, Moloney murine leukemia virus retention time (RT) (Invitrogen, Carlsbad, CA), and oligo-dT18 primers were used in the RT reaction, followed by cDNA synthesis for each extracted sample by semiquantitative PCR (Bio-Rad). PCR products were analyzed by agarose gel electrophoresis, and bands were visualized with a ChemiDoc Imager (Bio-Rad). The primer sequences of four genes used for RT-PCR were listed below:
Gene expression was measured using SYBER Green based real-time PCR, and consequent relative quantification analysis with the aid of GAPDH as a house keeping gene on LightCycler 2.0 instrument (Roche applied Science, Germany). The 20-uL PCR reaction mixture for each of the four genes and the corresponding housekeeping gene for each sample, included 15 uL of master mix with the following components: nine uL of PCR grade water, one uL of forward primer for each parameter & the housekeeping gene (20 picomol/uL), one uL of reverse primer for each parameter & the housekeeping gene (20 picomol/uL), four uL of ready to use MasterPLUS

SYBER Green I (Roche Applied Science, Germany) and 50 ng of cDNA. The thermal profile was as follows: initial denaturation at 95 ^{0}C for 10 min, followed by 45 cycles of amplification, starting by denaturation at 95 ^{0}C for 10 sec, annealing at 61^{0}C for 20 sec and extension at 72 ^{0}C for 25 sec. Following amplification, an extra cycle of melting curve analysis was done for product characterization by heating the reaction mixture from 65°C to 95°C at a rate of 0.2 ^{0}C/Sec. Light Cycler 2.0 real time PCR systems software automatically calculates the gene expression values by relative quantitative-monocolour analysis.

Statistical Analysis

Data was collected, processed and analyzed using SPSS v.20. Quantitative variables were described as median (minimum and maximum) for non-parametric data after testing for normality using Kolmogorov-Smirnov test. Categorical variables were described as number and percentages. Mann - Whitney test was used to compare two groups while Kruskal Wallis test was used to compare more than two groups of non-parametric data. Statistical significance was realized at probability $p < 0.05$.

RESULTS

The age of the studied cancer patients ranged from 37 – 74 years (median age: 52 years), while the age of the non-cancerous group ranged from 20 to 74 years with no significant difference between both groups ($p = 0.056$). Fig. (1) showed the thermal profile overview for housekeeping and target genes. The frequency of some pathological characters among cancer patients were represented in Table (1). There was significant increase in cadmium level and all cancer associated genes' expression in the tumor tissues in comparison with other tissue samples ($p < 0.001$) (Table 2). Cadmium level and all cancer associated genes expression increased in negative hormone receptor patients' more than positive patients. However no significant difference was found regarding receptor positive or negative patients (Table 3).

DISCUSSION

Breast cancer is one of the most prevalent types of malignancy that affects women. Cd is considered one of the most important environmental risk factors causing this disease. To the best of our knowledge; this work is one of few clinical researches that aimed to study the effect of cadmium toxicity on the expression of cancer associated genes involved in cell proliferation in a sample of Egyptian women. By studying the frequency of some pathological data in cancer patients; the present study reveals that 70 % of patients are in stage T2; 80 % are grade II; 60.5 % are ER positive; 67.4 % are PR negative; 63.3 % are HER2 negative and 88 % with no metastasis. These findings are in accordance with the studies done by Rhodes and Jasani (2009) in England (54.8 % was ER+/ PR+; 22.1 % was ER- / PR-; 19.8 % was ER+/ PR- and 3.2 % was ER- / PR+); Ng *et al*. (2012) in Malaysia (ER+/ PR+ was 46.8 %; ER- / PR- was 37 %; ER+ / PR- was 11.6 % and 4.6 % was ER- / PR+); Mohammadizadeh *et al*. (2013) in Iran [ER, PR and HER2-neu were positive in 60.7%, 58.4% and 36% of the cases, respectively]; Strumylaite *et al*. (2014) in Lithuania (ER+ and ER- were 65.3 % and 32 % respectively; HER2+ / HER2- were 13.5 % and 81.7 %) and El Deeb *et al*. (2016) in Egypt (ER+/ PR+ were 93.3 % and HER2 – in 60 % of patients). Moreover the results of the current work showed that cadmium level is significantly higher ($p < 0.001$) in tumor tissue samples compared to control (0.97 & 0.15 µg/g respectively) and healthy tissues (0.08 µg/g). In addition; Cd level is significantly higher in benign than healthy tissues. While there is no significant difference in Cd levels in cancer tissues classified as positive or negative for ER, PR and HER2 receptors with higher Cd levels in ER-; PR- and HER2-patients.Many studies were done on blood (Saleh *et al*., 2011; Arooj *et al*., 2012; Peng *et al*., 2015; El-Deeb *et al*., 2016)and urinary Cd levels(Strumylaite *et al.,* 2008; McElroy *et al.,* 2006; Nagata *et al.,* 2013; Gallagher *et al*., 2010; Lin *et al.,* 2016) in BC patients and all revealed the Cd levels were higher in cancer patients than control.

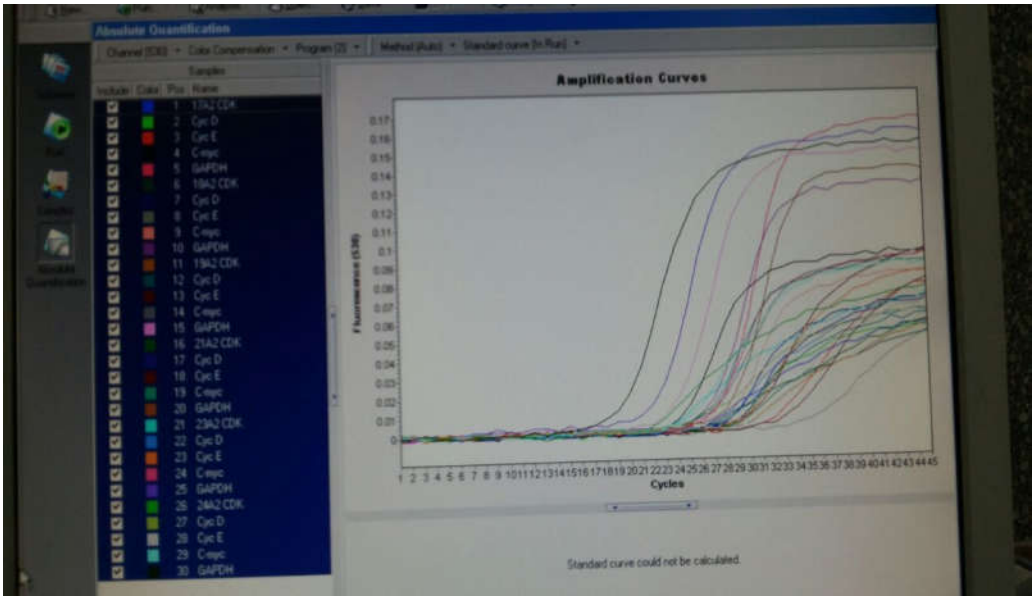

Fig. 1. The thermal profile overview for reference and target genes

Table 1. Frequency of some pathological data in the cancer patients (n = 50)

Data	n = 50 (%)
Operation type	
•LT MRM	29 (58 %)
•RT MRM	20 (40 %)
•LT LDF	1 (2 %)
TNM	
•T1	6 (12 %)
•T2	35 (70 %)
•T3	8 (16 %)
•T4	1 (2 %)
Lymph node	
•N0	17 (34 %)
•N1	12 (24 %)
•N2	12 (24 %)
•N3	9 (18 %)
Metastasis	
•No	44 (88 %)
•Bone	2 (4 %)
•Lung	4 (8 %)
Pathological type	
•IDC	42 (84 %)
•ILC	6 (12 %)
•Mucinous	1 (2 %)
•Medullary	1 (2 %)
Grade	
•I	2 (4 %)
•II	40 (80 %)
•III	8 (16 %)
Hormone Receptors	
ER*	
•Negative	17 (39.5 %)
•Positive	26 (60.5 %)
PR*	
•Negative	14 (32.6 %)
•Positive	29 (67.4 %)
HER2**	
•Negative	19 (63.3 %)
•Positive	11 (36.7 %)
Loco-regional recurrence	
•No	50 (100 %)

Lf = Left; MRM= modified radical mastectomy; Rt= right;
IDC= invasive ductal carcinoma; ILC= invasive lobular carcinoma;
LDF = Latissimus Dorsi Flap; ER = estrogen; PR= progesterone;
HER2 = Human Epidermal Receptor2; n= number; *Done only for 43 cases;
**Done only for 30 cases.

Table 2. Comparison between Cd Level and Cancer Associated Gene expression in Cancer Patients and Control Group (n = 100)

Parameters	Cancer patients (Tumor tissue)n= 50	Control group (Non – cancerous)n= 50	Cancer patients (Healthy tissue)n = 50
	Median (Min-Max)	Median (Min-Max)	Median (Min - Max)
Cd (µg / g)	0.97 (0.09 – 1.76)	0.15 (0.05 – 1.29)	0.08 (0.05-1.23)
Test of sig.		Z= 6.152 , P < 0.001**	Z= 7.14 , P= 0.08; Z1= 1.76, P1< 0.001**
cdk2	4.695 (2.01 - 9.45)	0.0047 (0.0 - 0.06)	2.17 (0.1-12.17)
Test of sig.		Z= 8.62, P < 0.001**	Z= 5.88 , P < 0.001** ; Z1= 8.62 , P1< 0.001**
CycD1	3.135 (1.07 - 9.8)	0.28 (0.02-4.4)	2.63 (0.01 - 9.14)
Test of sig.		Z= 2.19 , P= 0.029*	Z= 8.25 , P <0.001**; Z1= 4.00 , P1<0.001**
CycE	3.48 (1.01 - 9.76)	0.0013 (0.0 - 0.35)	0.165 (0.0-5.74)
Test of sig.		Z= 8.62, P < 0.001**	Z= 7.46 ,P <0.001**; Z1= 6.54, P1< 0.001**
c-myc	4.26 (1.01 - 8.52)	0.165 (0.0 - 5.74)	0.61 (0.08-5.31)
Test of sig.		Z= 5.82 , P < 0.001**	Z= 7.76 , P <0.001** ; Z1= 4.59 , P1 <0.001**

n = number; Cd = cadmium; µg= microgram; g= gram; Min = minimum; Max = maximum; Cdk = cyclin dependent kinase; Cyc = cyclin;
Z,p comparison of each group with Cancer tissues;Z1,p1 healthy tissues compared to control group; $p < 0.05$ is significant.

Table 3. Relation between Cadmium Level and Cancer Associated Gene Expression in Cancer Patients Regarding Hormone Receptors (n = 50)

Hormone Receptors		Cd (µg/g)	cdk2	CycD1	CycE	c-myc
		Median (Min-Max)	Median (Min-Max)	Median (Min – Max)	Median (Min – Max)	Median (Min-Max)
ER	Negative	1.11	4.8100	3.09000	2.9200	3.2700
	n=17	(0.09-1.76)	(2.01 - 9.45)	1.160 - 9.800	1.17 - 8.48	1.01 - 6.78
	Positive	0.97	4.5900	3.44000	3.8050	4.1000
	n=26	(0.09-1.76)	(2.08 - 8.93)	(1.070 - 7.110)	1.01 - 9.76	1.22 - 8.52
	Z	0.5	0.099	0.236	0.584	0.994
	P	0.62	0.921	0.813	0.559	0.320
PR	Negative	1.11	5.8300	3.33500	4.1550	3.8600
	n=14	(0.09-1.76)	(2.01 - 9.45)	(1.120 - 9.800)	(1.45 - 9.76)	(1.01 - 6.78)
	Positive	0.97	4.2100	3.04000	3.0000	4.0200
	n=29	(0.09-1.76)	(2.08 - 8.93)	(1.070 - 6.520)	(1.01 - 7.20)	(1.22 - 8.52)
	Z	0.352	1.762	0.920	1.128	0.674
	P	0.725	0.078	0.358	0.260	0.500
HER2	Negative	1.11	4.2000	3.04000	3.4400	2.9200
	n=19	(0.09-1.76)	(2.08 - 8.91)	(1.070 - 7.480)	(1.04 - 9.76)	(1.29 - 7.09)
	Positive	0.88	4.8100	3.80000	3.3800	4.4500
	n=11	(0.09-1.35)	(2.92 - 9.45)	(1.520 - 9.800)	(1.45 - 8.48)	(1.67 - 6.62)
	Z	0.89	1.054	0.538	0.710	1.571
	P	0.374	0.292	0.591	0.478	0.116

n= number; Cd= cadmium; Min = minimum; Max = maximum; Cdk = cyclin dependent kinase; Cyc= cyclin; µg=microgram; g= gram; $p < 0.05$ is significant; Z= Mann Whitney test; ER = estrogen; PR=progesterone; HER2 = Human Epidermal Receptor 2.

Moreover, Peng *et al*. (2015) in China revealed that there was no significant difference in blood Cd levels between ER or PR positive or negative patients.

Similarly; in Egypt; El Deeb *et al*. (2016) stated that there was no significant difference in Cd levels between positive or negative patients regarding ER, PR and HER2 receptors. Furthermore, Strumylaite *et al*. (2008) in Lithuania; found that cancer patients had 2.7 fold increases in Cd level in breast cancer compared to healthy tissue and 1.5 fold higher in cancer tissues than begnin tissues but there was no difference between healthy and benign tissues. On the contrary; they demonstrated significant difference between Cd levels in tumor tissues of ER +ve and ER –ve patients (0.052 and 0.035 µg/g respectively). Again Ionescu *et al*. (2006) proved the same findings. In agreement with the current findings, Makowska *et al*. (2011) observed that Cd levels were higher in breast tissues compared to control tissues (0.86 and 0.61 µg/g respectively). Moreover, Hilal *et al*. (2016) in India mentioned that Cd level was increased in the tumor tissues more than healthy tissues (6.48 and 1.58 ug/g respectively). Also, the serum Cd was significantly higher in cancer patients than the control (1.22 and 0.4 ug/L respectively).

In consonance with the present results, an Egyptian study conducted by El-Harouny *et al*. (2011) concluded that there was significant increase in Cd levels in cancer tissue samples compared to non-cancerous cases (0.0109 mg/g and 0.0665 mg/g respectively). This finding is contradicted by Antila *et al*. (1996) who reported that there was no significant difference in Cd level between cancer and control patients (20.4 and 31.7 µg/g respectively). They accounted this to the tight bound of Cd to adipose tissues. The higher Cd level in breast tissue could be related to its metallo-estrogenic effect and its ability to mimic the estrogenic action with consequent binding to ER forming a complex. The higher Cd level in ER- and PR- cancer patients in this study could be clarified by the Cd induced malignant transformation of normal breast epithelial tissues into basal like tumors which are characterized by negativity of Erα and HER2 (Strumylaite *et al*., 2008; Tallaa *et al*., 2009). In addition to a strong relation between Cd exposure and epidermal growth factor (EGFR) which promoted the cell cycle progression and proliferation that develop breast cancer in of triple-negative receptor patients, suggesting that Cd is an endocrine disruptor (Wei *et al*., 2015). In a trial to understand the mechanism by which cadmium; in particular; takes part in the development of breast cancer.

The findings of the present work point out that the expression of four genes (cdk2; CycD1; CycE and c-myc) involved in cell proliferation is significantly increased in cancer tissue compared with healthy and non-cancerous tissues ($p < 0.001$). As the studied groups were matched in age; alcohol intake and residence; hence, the variable here is Cd as a risk factor for breast cancer and the elevation of its level is associated with significant increase in the expression of all the studied genes and this is in line with the hypothesis which claims that the cancer associated gene expression is a mechanism through which Cd could induce breast cancer. A previous study on cell line done by Siewit *et al*. (Siewit *et al*., 2010) proved that activation of c-*myc* is trailed by the activation of both cyclin E-cyclin-dependent kinase 2 (cdk2) cyclin D1-Cdk4.

The activation of CycE – cdk2 is imperative for G1- S phase progression and these genes were involved in the development and progression of breast cancer. Additionally, the authors reported increment in cyclin E and cycline dependent kinase (cdk2) after Cd treatment. Over expression of any of these cell cycle regulators brings about the deregulation of growth with induction of cancer. Filipic (2012) concluded that cadmium did not cause direct DNA damage; however it leads to increased in reactive oxygen species (ROS) formation, which in turn induces DNA damage and can also interfere with cell signaling. Cdks are seen to drive cell cycle movement though cyclins are thought to be the riggings that are changed to help the move between cycle phases. The kinase activity of Cdk/cyclin complexes is firmly managed by Cdk inhibitors (CKIs), which serve as brakes to stop cell cycle progression under unfavorable conditions (Lim, 2013). Cyclins E1 and E2 (collectively referred to as cyclin E) drive expansion by advancing the start of DNA replication and by activating cdk2. Cyclin E/CDK2, alongside cyclin D/CDK4, stimulate E2F-responsive genes and enhance progression into S phase (Caldon *et al*., 2012). Moreover by studying the relation

between gene expression and hormone receptor state (ER; PR and HER2), the present work shows no significant difference in gene expression between receptor positive and negative patients. Mohammadizadeh *et al*. (2013) mentioned that there is a significant relationship between cyclin D1 and ER and PR (p = 0.0001). The higher level were mostly seen in patients with ER and PR positive while the negative status was most frequent in patients with ER and PR negative. Niu *et al*. (2015) stated that there was over expression of cyclin E in breast cancer compared to benign tumors and the expression of cyclin E was strongly associated with estrogen receptors expression. The discrepancy between the current work and other studies may be due to different sample size and variable stages of cell proliferation. From the previous findings, it could be concluded that Cd is strongly associated with the development of breast cancer. It plays its role through promotion of cancer associated gene expression which is responsible for cell proliferation and differentiation. In addition, it has metallo-estrogenic action and this might explain the development of breast cancer in ER negative patients. Further studies on large population size are recommended with more stratification of patients according to hormone receptor and investigating other genes involved in Cd-induced BC.

Conflict of interest: Nothing to be declared**.**

Acknowledgment

We would like to thank Dr. Lobna Abdelsalam, Genome Unit, Faculty of Medicine, Cairo University for her great support and effort in performance of gene analysis.

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