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Original Research

Calcineurin level and activity in breast cancer: Relation to apoptosis

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ABSTRACT

Background: Calcineurin (CN) is a Ca^{2+}/calmodulin- dependent phosphatase and has been implicated in both transcription-dependent and transcription-independent apoptosis. **Objectives:** We aim to interpret the correlation between CN and apoptosis in relation to pathogenesis of breast cancer. **Materials and Methods:** Both CN level and activity, as well as caspase-3 activity, were evaluated in tissue homogenate of 50 breast cancer patients, 20 patients with fibroadenoma and 15 healthy women. **Results:** CN activity was significantly decreased in malignant breast tissues compared with fibroadenoma and normal breast tissue ($P < 0.001$) without significant changes in its level ($P > 0.05$). While caspase-3 showed a significant higher activity in a malignant group compared with other groups ($P < 0.05$). In addition, there was a significant negative correlation between CN activity with grade, stage ($P < 0.001$) and a significant positive correlation with its level ($P = 0.039$). **Conclusion:** CN activity, but not its level might have a role in breast neoplasia; restoration of its normal activity may act as an adjuvant factor to control breast cancer pathogenesis.

KEY WORDS: Breast cancer, calcineurin, caspase-3

INTRODUCTION

Breast cancer is the most significant worldwide health problem in women >35-40 years of age. In addition, breast cancer accounts for ~1.35 million new cases and >450,000 cancer-related mortalities annually worldwide [1]. Despite improved early detection, treatment options, and survival, the morbidity and mortality continue to increase, and numerous patients with invasive breast cancer develop metastatic diseases. Thus, there is an urgent requirement to search for and identify novel biomarkers to predict tumor recurrence and metastasis and to develop more novel treatment strategies to effectively control aggressive breast cancers [2].

An important feature of the signal transduction pathway in cancer is reversible serine/threonine phosphorylation of proteins and any aberrant activity in the kinases or phosphatases can disturb the phosphorylation/dephosphorylation cycle leading to uncontrolled proliferation. Therefore, identification of key signaling molecules (kinases and phosphatases) responsible for neoplastic development can have potential clinical relevance allowing for diagnosis, early detection and intervention [3]. Calcineurin (CN) is a Ca^{2+} and calmodulin dependent serine/threonine protein phosphatase [4]. CN is a heterodimer with a 61 kDa catalytic A subunit (CnA) and a 19 kDa regulatory B subunit (CnB). The B subunit has four Ca^{2+} binding sites and has generally been thought of as only regulating CnA [5].

CN plays an important role in cancer by regulating a number of transcription factors and ion channels. The active dephosphorylated form of Na^+, K^+-ATPase that is maintained by CN is directly involved in the migration of cancer cells [6]. In addition, CN activation by calcium-dependent signaling pathways [7] mediates dephosphorylation of the nuclear factor of activated T-cell (NFAT) transcription factors and their

subsequent translocation from the cytoplasm to the nucleus [8]. Once in the nucleus, NFATs bind to specific sequences located in the regulatory regions of target genes and switch on their expression [9]. Interestingly, several reports have implicated the CN/NFAT pathway as a mediator of cellular apoptosis via upregulation of the expression of the pro-apoptotic molecule Fas Ligand [10]. Similarly, previous study on neonatal rat brain by Saeki *et al.* [11] suggested that CnB itself had no effect on apoptosis but promotes apoptotic signal transduction system through the presentation of scaffold for the activation of caspase-3 by caspase-9. Therefore, CnB may be useful for therapies to induce and promote apoptosis. On the other hand, other studies reported that CN might play a critical role in promoting tumor cell metastasis in breast cancer by transcriptional factor NFAT [12]. A peptide that interferes with the CN-NFAT interaction might attenuate breast cancer cell invasion [13].

It seems difficult to draw substantial conclusions from these previous reports due to the diversities of the disease and different evaluation criteria. Therefore, we evaluate the possible interplay of CN and apoptosis in the pathogenesis of breast cancer patients.

MATERIALS AND METHODS

Patients' Database

A prospective analysis was performed on tissue samples of 85 female Egyptian subjects, selected from The General Surgery department, Ain Shams University Hospitals, Egypt, between June 2011 and June 2013. The malignant group included 50 primary breast cancer patients (median age 46 years old, range from 37 to 73), pathologically diagnosed as infiltrating ductal carcinoma. They were staged according to TNM classification of the American Joint Committee on cancer [14] and graded by the Nottingham grading system [15]. The benign group included 20 patients (median age 46 years old, range from 38 to 58), pathologically negative for malignant hyperplasia, and was diagnosed as a fibroadenoma. None of the patients were using oral contraceptives, hormones or vitamins. The third control group was represented by 15 healthy volunteers who underwent plastic breast surgery (median age 42 years old, range from 32 to 46). A written consent was taken from all participates. The pathological reports, estrogen, and progesterone hormonal status were obtained from hospital records.

Samples Collection

Specimens from breast tissues were obtained from all subjects immediately after surgery, washed and transported in ice cold saline, then stored at −80°C until subsequent processing and measurements.

Preparation of the Cytosolic Fractions

Processing of the frozen tissue was done according to Mabrouk *et al.* [16]. Briefly, homogenization was performed on ice in 1:10 (w/v) ice cold homogenization buffer (10 mM Tris buffer pH 7.5 containing 10% glycerol, 0.4 M KCl, 10 mM K_2EDTA, 5 mM benzamidine, 10 mM β-mereaptoethanol, 0.39 mM phenylmethylsulfonyl fluoride (PMSF) and 5 mg/L aprotinin). The tissues were weighted and homogenized for 3 bursts 55 s each with one minute pause on ice. The homogenate was filtered, and the cytosolic fractions were obtained by centrifugation at 100.000 rpm for 15 min at 4°C using Beckman centrifuge L7 (Brae, USA).

Measurement of Protein Concentration

The protein concentration in the cytosol fraction was measured by colorimetric method of Bradford [17].

Determination of Caspase-3 Activity

The activity of caspase-3 was determined in the cytosol according to manufacturer instructions of ApoTaget colorimetric assay reagents (Invitrogen Corporation Camarillo, California, USA). It was measured with the fluorescent tetrapeptide substrate Ac-DEVD-MCA (Peptides Institute, Osaka, Japan). Crude lysates were prepared by sonicating cells in 200 μl of lysis buffer (10 mM Hepes-KOH, pH 7.5, 2 mM EDTA, 0.1% CHAPS, 1 mM phenylmethylsulfonyl fluoride, and 5 mM DTT) followed by centrifugation. Each lysate (20 μl) was incubated with 1 μM peptide-MCA substrates in 0.5 ml of reaction buffer (100 mM Hepes-KOH, pH 7.5, 10 mM DTT, 0.1% CHAPS, and 10% glycerol) for 30 min at 37°C. Before and after the reaction, the fluorescence intensity of free 7-amino-4-methylcoumarin released from the substrates was measured with a spectrofluorometer set at an excitation wavelength of 380 nm and an emission wavelength of 460 nm. The fluorescence intensity of purified control 7-amino-4-methylcoumarin in an amount equal to that of peptide-MCA added to the reaction mixture was used as a reference (fluorescence intensity/nmol). The amount (nmol) of free 7-amino-4-methylcoumarin released in the reaction mixture was calculated by dividing the increase into fluorescence intensity by the reference. The enzyme activity (nmol/min/mg of protein) of each cell lysate was determined by dividing the amount (nmol) of released 7-amino-4-methylcoumarin by the reaction time (30 min) and the amount of protein (mg) in each lysate.

Determination of CN Activity

Ca^{2+}-calmodulin dependent phosphatase activity of CnA present in the samples was assayed modified according to Padma and Subramanyam [18] in presence and in absence of 150 μM trifluoperazine. Assays were conducted in a reaction mixture (100 μl volume) containing 25 mM Tris (pH 7.2), 25 mM MES (pH 7.0), 2.4 μM p-nitrophenyl phosphate, and 1 mM $MnCl_2$ and 30 μl of tissue cytosol (≈30μg protein). Reactions were initiated by addition of p-nitrophenyl phosphate, followed by incubation at 37°C for intervals 3 min and initial velocity of CN was determined. Samples were then transferred to microtiter plates, and absorbance was measured at 405 nm (Spectra II, SLT Instruments, Salzburg, Austria). The difference between the amounts of p-nitrophenol released in absence and in the

presence of trifluoperazine indicated the activity of CN. One unit is defined as the nmoles of p-nitrophenol formed per min/dl.

CN Level Assay

CN levels were determined in the cytosol fraction using ELISA kit (R and D Systems, Minneapolis, USA) according to the instruction manual [19].

Statistical Analysis

The analysis was performed using the Statistical Package for the Social Sciences (SPSS software version 15). Statistical comparisons were made using Mann–Whitney U (to compare two groups) and Kruskal–Wallis tests (to compare three groups). Chi-square test was used to compare quantitative parameters between groups. Correlation between different variables was performed by Pearson`s correlation coefficient. Statistical significant was set at a value $P \leq 0.05$.

RESULTS

Demographic and clinical data are shown in Table 1, menopausal status, and parity showed no significant difference between the studied groups, $P > 0.05$.

Breast Tissue CN Level and Activity

CN level showed no significant difference between different groups, while CN activity was significantly low in the malignant group (Mean rank 25.8) as compared to benign (Mean rank 64.07) and normal control groups (Mean rank 77.5), $P < 0.001$. In addition, the benign group had a significant lower CN activity compared with the normal group, $P = 0.022$ [Figure 1]. No significant correlation was found between CN level and any of the studied clinicopathological factors except grade and stage, the mean rank of CN activity was significantly low in grade 3 and Stage III compared with lower grades and stages, $P < 0.001$ [Table 2]. Using Pearson's correlation, CN activity showed a significant negative correlation with grade, stage ($P < 0.001$), lymph node positivity ($P = 0.024$) and a significant positive correlation with CN level ($P = 0.039$) and estrogen positive tumors ($P = 0.002$), [Table 3].

Breast Tissue Caspase-3 Activity

Caspase-3 activity was significantly high in malignant group (Mean rank 51.33) compared with benign (Mean rank 40.75, $P = 0.024$) and control group (Mean rank 18.23, $P < 0.001$), with significant higher level in benign compared with normal group ($P = 0.014$), [Figure 1]. The Mean rank of caspase-3 activity was significantly higher in Stage I compared with other stages ($P = 0.029$), [Table 2].

Cancer is caused by defects in the signaling mechanisms that govern cell proliferation and apoptosis [7]. CN has been implicated in both transcription-dependent and transcription-independent apoptosis. The former is attributed to dephosphorylation of NF-AT by CN and subsequent trans-activation of apoptosis genes such as Fas-Ligand and p53 [20]. The latter may result from dephosphorylation of Bcl-2-associated death promoter (BAD) by CN [21].

Many previous studies were conducted on the potential role of CN in mediating apoptosis in experimental animals and tissue culture but, to our knowledge, none of them correlate CN level and activity with caspase-3 in breast cancer tissues. Therefore, this study was designed to interpret the correlation between CN as a serine-threonine phosphatase and apoptosis in relation to pathogenesis of breast cancer. We found a significant lower CN activity in malignant breast tissues compared to benign

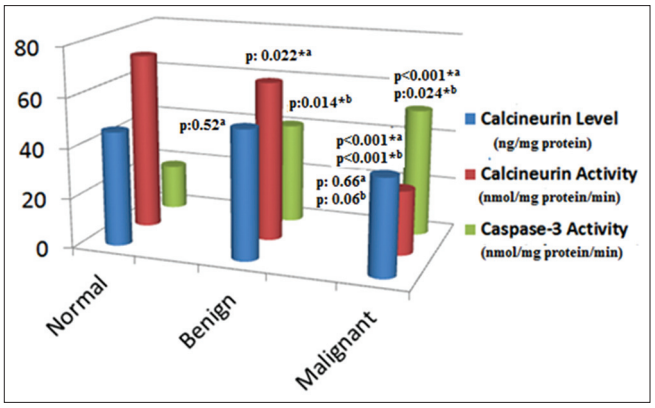

Figure 1: Mean Rank of calcineurin (level and activity) and Caspase-3 activity in the malignant group compared with benign and normal control groups, [a]Significance versus control group. [b]Significance versus Benign group (Mann–Whitney and Kruskal–Wallis Tests were used to compare main ranks between groups; *$P < 0.05$ is significant)

Table 1: Clinicopathological factors in different groups of the study

Clinicopathological factors	*n* (%)			Statistics
	Normal	Benign	Malignant	
Age			46 (37-73)	-
Median (rang)	42 (32-46)	46 (38-58)		
Menopausal status; *n* (%)				χ^2: 5.08
Premenopause	11 (73.3)	9 (45)	36 (72)	*P*=NS
Postmenopause	4 (26.7)	11 (55)	14 (28)	
Parity; *n* (%)				
0	3 (20)	1 (5.0)	4 (8)	χ^2: 8.6
1-2 children	8 (53.3)	13 (65)	18 (32)	*P*=NS
≥3 children	4 (26.7)	6 (30)	28 (56)	
Grade; *n* (%)				
Grade 1		-	2 (4)	
Grade 2		-	36 (72)	
Grade 3		-	12 (24)	
Stages; *n* (%)				
Stage I		-	3 (6)	
Stage II		-	28 (56)	
Stage III		-	19 (38)	
Tumor size:				
<2 cm			3 (6)	
2-5 cm			29 (58)	
>5 cm			18 (36)	
+ve Estrogen receptor			37 (74)	
+ve Progesterone receptor			42 (84)	

NS: Non-significance (*P*>0.05)

Table 2: Mean rank of CN (level and activity) and Caspase-3 in relation to clinicopathological factors in the malignant patients

Factors	CN Level (ng/mg protein)	CN Activity (nmol/mg protein/min)	Caspase-3 Activity (nmol/mg protein/min)
Menopausal status			
Premenopause	40.2	40.2	42.7
Postmenopause	48.3	46.7	40.1
Parity			
No	36.1	35.1	28.8
1-2 children	25.7	33.1	35.9
≥3 children	31.4	30.8	35.01
Grade			
Grade 1	25.5	47.0	29.5
Grade 2	23.5	29.5	26.9
Grade 3	31.5	9.9	20.63
		χ^2: 20.89	
		$P<0.001$	
Stages			
Stage I	34.0	48	46.0
Stage II	26.1	32.3	25.7
Stage III	23.6	11.8	21.97
		χ^2: 30.2	χ^2: 7.06
		$P<0.001$	$P=0.029$
Estrogen receptor			
Positive	32.5	44.7	50.6
Negative	42.05	15.9	38.54
		Z: 4.15*	
		$P=0.001$	
Progesterone receptor			
Positive	35.6	30.9	35.6
Negative	40.8	41.2	40.8

*$P\leq0.05$: is significant, (Mann–Whitney and Kruskal–Wallis tests were used to compare main ranks between groups), CN: Calcineurin

Table 3: Correlation between the investigated parameters and clinicopathological factors

	CN Level	CN Activity	Caspase-3 Activity
Age	$r=0.13$	$r=-0.324$	$r=0.21$
	$P=0.25$	$P=0.003$*	$P=0.06$
Grade	$r=0.096$	$r=-0.625$	$r=-0.18$
	$P=0.5$	$P<0.001$*	$P=0.2$
Stage	$r=-0.188$	$r=-0.753$	$r=-0.31$
	$P=0.192$	$P=0.00$*	$P=0.028$
+ve estrogen receptor	$r=0.162$	$r=0.35$	$r=-0.569$
	$P=0.15$	$P=0.002$*	$P=0.61$
+ve progesterone receptor	$r=0.1$	$r=0.108$	$r=0.005$
	$P=0.37$	$P=0.35$	$P=0.96$
Lymph node positivity	$r=-0.27$	$r=-0.49$	$r=0.027$
	$P=0.24$	$P=0.024$	$P=0.91$
Caspase-3	$r=-0.75$	$r=-0.035$	-
	$P=0.48$	$P=0.807$	
CN activity	$r=0.225$	-	-
	$P=0.039$*		

CN: Calcineurin, *$P\leq0.05$: is significant

and control ones ($P < 0.001$). In addition, this significant lower level was also observed in benign tumors compared to the normal group ($P = 0.002$). Although the presence of a significant positive correlation between CN level and activity ($P = 0.039$), its level showed no significant difference between studied groups. In contrast to Sanli *et al.* [22] who found that decrease in CN activity in MCF-7 cells following retinoic acid treatment has been linked to down regulation in the expression level of the catalytic subunit of CN. However, Padma and Subramanyam [18] reported that, the activity of CN was decreased by 75% and 85% in sera of patients diagnosed either for acute lymphoid leukemia and acute myeloid leukemia, respectively, without apparent changes in calmodulin or CN contents. Padma *et al.* [3] speculated that a change in redox state of the cancer cell due to oxidative stress contributes to the alteration in the activity of CN without altering its content. In this study, CN activity was low in breast cancer tissues with negative estrogen receptor and lymph node metastasis, revealing its association with an aggressive phenotype of breast neoplasia. Moreover, we found a significant negative correlation between CN activity with grade and stage. This reducing CN activity in a malignant group especially in undifferentiated and advanced stage tumors suggests its putative prognostic role in cancer. Padma *et al.* [3] showed down regulation of CN activity in invasive cervical cancer could help in cellular survival, thereby promoting neoplastic progression.

Many researchers found that inhibition of CN has been linked to apoptosis protection in neuronal cells [23]. Baggott *et al.* [7] reported that activation of the CN/NFAT pathway results in upregulation in the expression of the pro-apoptotic protein Fas Ligand that control cell death and in a concomitant loss of cell viability. Therefore, so far, phosphatase activity of CN has been responsible for the regulatory role in cell death [11]. Interestingly, our malignant patients showed a significant lower CN activity with a significant higher caspase-3 activity compared with benign and normal groups. The increase apoptotic activity in a malignant group may be explained by tumor over proliferation that could not support the balance and resulted in malignant transformation. As cell proliferation and apoptosis are two opposed mechanisms and play an important role in tumorigenesis and tumor progression. Nassar *et al.* [24] reported that caspase-3 immunointensity was generally higher in invasive cancers of mammary epithelium compared to normal mammary epithelium. In this study, caspase-3 was significantly lower in advanced stage than early ones. This indicates that the apoptotic process is stimulated to a greater extent with increasing tumor proliferation and stimulation of proapoptotic factors and inhibited in poorly differentiated tumors. The low apoptotic status in poorly differentiated breast cancer can be explained by the disturbed balance between apoptosis and proliferation due to the upregulation of the anti-apoptotic proteins that antagonized the apoptotic process [25]. Moreover, Huang *et al.* [26] discovered that caspase 3 might be involved in regulating the growth-promoting properties of dying cells and serves as a direct link between cell death and tumor repopulation.

Despite the direct and indirect evidence that CN mediates apoptosis [27], we could not find any significant correlation between CN level or activity with caspase-3. We did not find any previous literatures correlating these parameters in breast cancer tissues. However, Springer *et al.* [28] reported that activation of the caspase-3 apoptotic cascade in spinal cord injury is regulated, in part, by CN mediated BAD dephosphorylation. In the current study, the increased apoptotic activity in malignant

cells might indicate the presence of other proapoptotic factors that stimulate apoptosis even in lower CN activity. Although, Caspase-3 activity is often used as a definite marker for apoptosis; however it alone cannot reflect the apoptotic status of the cell [29]. Correlation of CN activity with other apoptotic factors as plasma membrane integrity or cell fragmentations is recommended in further study to evaluate its role on apoptotic status in breast cancer tissues.

In conclusion, our results revealed that decrease CN activity, but not of its content, might be involved in the pathogenesis of breast cancer. Measuring Ca^{++} as activator molecule and H_2O_2 as an indicator of the oxidative stress might be useful to explain the lower activity of CN in malignant tissues. Moreover, the concomitant increase of caspase-3 activity might indicate the presence of many other proapoptotic factors in breast cancer. However, approaches attempting to restore normal CN activity in patients may act as an adjuvant therapy to control cancer development and progression.

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