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ORIGINAL ARTICLE

Taxane-based adjuvant chemotherapy reduces endothelin-1 and symmetric dimethylarginine levels in patients with breast cancer

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Summary

Purpose: *To evaluate the levels of asymmetric dimethylarginine (ADMA), an endothelin and nitric oxide (NO) synthase inhibitor, in patients with node-positive breast cancer who had undergone surgery and in a control group including healthy individuals. The effects of taxane-based chemotherapy on endothelin-1 (ET-1) and ADMA levels in the patient group were also studied.*

Methods: *Body mass index (BMI), serum lipids (total cholesterol, LDL-cholesterol, HDL-cholesterol and triglycerides), ADMA and ET-1 were studied in 19 healthy individuals and in 19 patients with stage II and III, lymph node-positive breast cancer receiving taxane-based chemotherapy.*

Results: *ET-1 (34.3±12.8 vs. 13.8±4.5 pg/mL; p<0.001) and ADMA (0.87±0.18 vs. 0.68±0.24 μmol/L; p=0.024) levels were significantly higher in the breast cancer group compared to the control group. A significant reduction was noted in ET-1 (34.3±12.8 vs. 27.3±4.3 pg/mL; p=0.021) and ADMA (0.87±0.18 vs. 0.73±0.15 μmol/L; p=0.014) levels in patients following 6 cycles of adjuvant chemotherapy to baseline values.*

Conclusion: *The present study demonstrated significantly higher levels of ET-1 and ADMA in the breast cancer group compared to the control group, which were reduced significantly with adjuvant taxane-based chemotherapy. It is apparent that prospective studies are needed to understand the effect of reducing ET-1 and ADMA levels on patient survival. We believe that the present study will provide guidance to relevant future studies.*

Key words: adjuvant chemotherapy, asymmetric dimethylarginine, breast cancer, endothelin-1, taxanes

Introduction

Breast cancer is the most common cancer in women and the second leading cause of cancer-related deaths. This situation highlights the need of developing further treatment strategies against breast cancer. Understanding the pathogenesis of this disease will contribute to the development of novel treatment approaches. Several molecular mechanisms are involved in the pathogenesis of breast cancer. In particular, endothelins of endothelial origin and NO are current topics of research.

Endothelin (ET) family is made up of 3 isoforms: ET1, ET2 and ET3. They exert their activity via endothelin receptors A and B. Basically, they act as endogenous vasoconstrictors, as well as mediators in renal and cardiovascular disorders [1]. Evidence regarding the involvement of ET in carcinogenesis, apoptosis, angiogenesis, tumor invasion and development of metastases, is being reported in recent investigations [2,3]. Increased secretion of ET-1 has been demonstrated in human ovarian, prostate, colorectal and breast cancer. Besides, higher expression of ET-1 has also been reported in breast cancer compared to normal breast tissue [4].

NO is an important mediator synthesized from L-arginine by the NO synthase which is involved in many biological events including vasodilatation, platelet adhesion, cell growth, apoptosis, neurotransmission and macrophage-associated immunity [5]. ADMA is an en-

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dogenous inhibitor of NO synthesis. Dimethylarginine dimethylaminohydrolase plays a pivotal role in tumor growth and the development of the tumor vasculature by regulating the concentration of NO and altering vascular endothelial cell growth factor (VEGF) production [6]. In addition, NO has been shown to increase proliferation of breast cancer cells at low physiological concentrations [7].

In this up-to-date study, the levels of ADMA, an endothelin and NO synthase inhibitor, were evaluated in patients with node-positive breast cancer who had undergone surgery and in a control group consisting of healthy women. The effects of taxane-based adjuvant chemotherapy on ET-1 and ADMA levels in the patient group were also studied.

Methods

Patient selection

Nineteen women with breast cancer who applied to our clinic between January 2008- January 2009 were included in this study. Also, 19 healthy women were enrolled as the control group. The study was approved by the local ethics committee. Written informed consent was obtained from each patient and from the healthy women.

Women were included in this study if they had: 1) histologically confirmed operable stage II or III breast adenocarcinoma; 2) had primary surgical resection within one month from the beginning chemotherapy; 3) were older than 18 years; and 4) were scheduled for receiving adjuvant chemotherapy regimens. Women with breast cancer were excluded from the study if they were already receiving neoadjuvant chemotherapy. Other exclusion criteria included patients and controls receiving antihyperlipidemic and antihypertensive drugs, and patients with a history of diabetes mellitus, coronary artery disease or hypo- or hyperthyroidism and smoking.

Study design

The adjuvant chemotherapy regimen used in this study was docetaxel (75 mg/m^2), epirubicin (100 mg/m^2) and cyclophosphamide (500 mg/m^2) (TEC), all given intravenously (i.v.) on day 1. TEC was administered every 3 weeks for 6 cycles. Methylprednisolone 16 mg/day i.v. for 3 days was given during treatment to prevent docetaxel reactions. The patients were also administered 5 mg/kg filgrastim for prophylaxis of neutropenia on days 2-7 after the end of each treatment cycle.

Patients with breast cancer were evaluated on two occasions: at baseline (on day -1 of the first chemotherapy cycle), and 1 week after the completion of the 6th cycle.

Laboratory evaluation

After overnight fasting, 20 cm^3 of venous blood were taken on 8-9 a.m. for the lab tests. Serum lipids (total cholesterol, HDL cholesterol, LDL cholesterol and triglycerides) were assayed on the same day. Serum and plasma samples for the measurement of ADMA and ET-1 were preserved at -80°C until analysis. Analyses of ADMA and ET-1 were performed using commercially available ELISA kits in accordance with the supplier's instructions. Serum lipids (total cholesterol, HDL and LDL, and triglycerides) were measured according to standard procedures.

ADMA

Serum ADMA concentration in supernatants was determined using a competitive 96-well plate ADMA-ELISA kit (DLD, Diagnostika GMBH, Hamburg, Germany) according to the supplier's protocol. This immunometric assay is based on the competition of acylated ADMA in samples with solid phase bound ADMA for fixed number of rabbit anti-ADMA antiserum binding sites. ADMA shows negligible cross reactivities with L-arginine (<0.02%) and other endogenous derivatives of L-arginine. Coefficients of variation are 8.3-10.3% and 4.5 – 7.5% for inter-assay and intra-assay, respectively. The ELISA assay can accurately measure ADMA concentrations over the full range of physiologically-relevant concentrations (i.e. 0.05 – 2 μmol/L). The specificity of ADMA ELISA kit is 100% and the sensitivity 0.05 μmol/l).

Endothelin-1

Plasma ET-1 concentration in supernatants was determined using 100 μl of supernatant per well of a 96-well plate with an endothelin EIA kit (Cayman Chemical Company, Michigan, USA) according to the supplier's protocol. This immunometric assay is based on a double-antibody "sandwich" technique and permits endothelin measurements within the range of 0-250 pg/ml, typically with a limit of detection of 1.5 pg/ml. Monoclonal anti ET-1 antibody in this kit had a cross reactivity of 100% with both ET-2 and ET-3. Samples were assessed with no prior purification. In this method the monoclonal antibody specific to endothelin and acetylcholinesterase Fab' conjugate (AchE:Fab') binds to

different epitopes on the ET-1 molecule, forming sandwich. This sandwich is immobilized on the plate so the excess reagents are washed away. The concentration of the analyte is detected by measuring the enzymatic activity of the AchE by adding Ellman's Reagent which contains the substrate for AchE. Addition of Ellman's Reagent produces a yellow-colored product which can be measured spectrophotometrically. The intensity of the color is directly proportional to the amount of bound conjugate which in turn is proportional to the concentration of ET-1. The intra- and inter-assay coefficients of variation of the method were 5 and 6%, respectively.

Other measurements

BMI was calculated according to the Quetelet's index [8] as the ratio of weight (kg) to height (m) squared (kg/m^2).

Statistical analysis

All data were analyzed using SPSS for Windows version 10.0. Descriptive statistics summarized frequencies and percentages for categorical, mean and standard deviation for continuous variables. To compare the continuous variables, the Mann Whitney's U-test was used to compare independent variables between groups. The Wilcoxon's signed rank test was used for comparison of the treatment effect on variables. Spearman's rank correlation was calculated for the estimation of the level of association between variables in breast cancer and control subjects. A p-value of <0.05 was considered as statistically significant. Results were expressed as mean ± SD.

Results

The characteristics of the 19 female breast cancer patients and the 19 healthy controls are shown in Table 1. The mean age of breast cancer patients was 50.5±8.8 years (range 32-69). Ten (53%) patients were postmenopausal. All patients had non-metastatic, node-positive breast cancer. Of the patients, 14 (73.7%) had stage III disease.

All patients completed the planned 6 cycles of adjuvant TEC chemotherapy. A one-week delay of treatment was experienced in one patient due to leucopenia. Grade 1-2 nausea-vomiting was noted in 3 patients.

Demographic features and lab findings of patients and controls are presented in Table 2. There were no significant differences between the patient and control group in terms of BMI and lipid levels ($p>0.05$). ET-1 (34.3±12.8 vs. 13.8±4.5 pg/mL, $p<0.0001$) and ADMA (0.87±0.18 vs. 0.68±0.24 μmol/L, $p=0.024$) levels were significantly higher in the breast cancer group compared to the control group.

Patients' pre- and post-chemotherapy demographic features and lab findings are depicted in Table 3. No statistically significant differences were noted in patients' BMI and blood lipid measurements taken at the baseline and after 6 cycles of chemotherapy ($p>0.05$). However, a significant reduction was noted

Table 1. Demographic characteristics of breast cancer patients and control group

Characteristics	*Patient group (n=19) n (%)*	*Control group (n=19) n (%)*
Age, years (mean±SD)	50.5±8.8	50.1±7.5
Range	32-69	35-59
Menopausal status		
Premenopausal	9 (47)	9 (45)
Postmenopausal	10 (53)	11 (55)
Surgical treatment		
Modified radical mastectomy	13 (68.4)	–
Local excision and axillary dissection	6 (31.6)	–
Disease stage (TNM)		
II	5 (26.3)	–
III	14 (73.7)	–

Table 2. Clinical and laboratory characteristics of patients ad controls. Values are means±standard deviation

Characteristics	*Patient group (+=19)*	*Control group (n=19)*	*p-value*
Body mass index (kg/m^2)	28.3±3.4	28.1±4.2	0.98
Blood lipids (mg/dl)			
Total cholesterol	217.7±50.4	208.6±42.6	0.60
Triglycerides	152.1±68	115.8±47.4	0.14
LDL	134.5±40.4	131.8±34.8	0.93
HDL	48.4±10	54.2±11.8	0.10
ADMA (μmol/L)	0.87±0.18	0.68±0.24	0.024
Endothelin-1 (pg/mL)	34.3±12.8	13.8±4.5	<0.001

Table 3. Clinical and laboratory features at baseline and after 6 cycles of adjuvant chemotherapy in breast cancer patients (n=19) and control subjects (n=19). Values are means±standard deviation

Features	*Patients*		*Controls*		
	Before therapy	*After therapy*		*p-value; before vs. after therapy*	*p-value; control vs. after therapy*
BMI (kg/m^2)	28.3±3.4	28.6±3.4	28.1±4.2	0.37	0.42
Total cholesterol (mg/dl)	217.7±50.4	226.4±49.6	208.6±42.6	0.19	0.37
Triglycerides (mg/dl)	152.1±68	213.4±102.4	115.8±47.4	0.06	<0.001
LDL (mg/dl)	134.5±40.4	145.4±38.7	131.8±34.8	0.10	0.35
HDL (mg/dl)	48.4±10	44.8±9.8	54.2±11.8	0.07	0.015
ADMA (μmol/L)	0.87±0.18	0.73±0.15	0.68±0.24	0.014	0.544
Endothelin-1 (pg/mL)	34.3±12.8	27.3±4.3	13.8±4.5	0.021	<0.001

in ET-1 (34.3±12.8 vs. 27.3±4.3 pg/mL, p=0.021) and ADMA (0.87±0.18 vs. 0.73±0.15 μmol/L, p=0.014) levels in patients following 6 courses of chemotherapy compared to baseline. Patients' ADMA levels were not statistically different than those of the control group following treatment, while ET-1 levels were still higher in patients with breast cancer.

Discussion

In this trial, we demonstrated that: 1) ET-1 and ADMA levels are significantly higher in patients with breast cancer compared to the control group of healthy individuals; and 2) that adjuvant chemotherapy significantly decreased the baseline ET-1 and ADMA levels in patients with breast cancer.

Increased expression of the endothelin axis has been determined in invasive breast carcinoma compared to normal breast tissue or noninvasive carcinoma *in situ* [9]. Higher levels of Big-ET-1, precursor of ET-1, have been reported in sera of patients with breast cancer compared to controls [10]. In breast cancer patients with lymph node metastasis, increased ET-1 serum levels have been reported to coexist with tumors of large diameter, high histological grade and lymphovascular invasion [11]. Similarly, in our study, high serum ET-1 levels were observed in patients with breast cancer, which may be explained by the lymph node positivity in our patients based on the findings of a previous study [12].

In our study, we demonstrated that taxane-based adjuvant chemotherapy significantly reduced the levels of ET-1 in lymph node-positive breast cancer. In a previous study association of increased expression of peptides of the endothelin group with decreased disease-free and overall survival was found [9]. Reductions in ET-1 levels may contribute to disease-free and overall survival benefit achieved with adjuvant chemotherapy in breast cancer.

ADMA is an endogenous inhibitor of NO and increased levels of this molecule are associated with reduced NO synthesis [13,14]. A negative correlation exists between the levels of ADMA and NO. Low levels of NO, in particular, result in proliferation of breast cancer cells and disease progression [7]. ADMA contributes to this effect by decreasing the levels of NO. The only study evaluating the levels of ADMA in cancer patients has investigated plasma ADMA concentrations in patients with hematological malignancies [12]. In that study significantly higher levels of ADMA were identified in patients compared to the control group. Similarly, ADMA levels were significantly higher in patients with breast cancer in our study. Free ADMA is found in the plasma of healthy individuals and is synthesized by the post-translational methylation of protein arginine residues and liberated upon their hydrolysis [6]. Our observation could be that tumors have an enhanced protein turnover; release of ADMA from methylated proteins during their degradation may thus produce elevated circulating ADMA levels. Besides, our study was the first to demonstrate that adjuvant chemotherapy significantly decreased the levels of ADMA. Based on this finding, it may be speculated that a decline in tumor progression may be achieved with adjuvant chemotherapy.

Endothelin and ADMA, besides being biomarkers revealing endothelial dysfunction, are important molecules with considerable direct or indirect activity in the pathogenesis of cancer. This study confirmed increased levels of ET-1 and ADMA in patients with breast cancer and also demonstrated significant reductions in the levels of these molecules with adjuvant chemotherapy. ADMA levels in patients with breast cancer, however, declined to those of the healthy individuals' levels following chemotherapy, while ET-1 levels remained high. Although comparable levels of ADMA

observed in patients and in the subjects of the control group suggest a decline in tumor progression, higher levels of ET-1 compared to normal individuals -despite significant reductions- may indicate persistent activity of the disease. Based on these findings, it is apparent that prospective studies are needed to understand and elucidate the effect of reducing ET-1 and ADMA levels on patient survival. We believe that the present study will provide guidance for relevant future studies.

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