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Interplay Between Membrane Lipid Peroxidation, Transglutaminase Activity, and Cyclooxygenase 2 Expression in the Tissue Adjoining to Breast Cancer

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Breast cancer, a leading cause of cancer related deaths worldwide, is one of the most common neoplasms in women. The increased generation of reactive oxygen species (ROS) in breast lesion is critically involved in the mutagenic processes that drive to breast carcinoma initiation and progression. To date, the molecular events occurring in the tissue adjoin the cancer lesion have not been elucidated. Here, we investigated the role of excess ROS generation during human breast carcinogenesis by evaluating oxidative stress biomarkers, tissue transglutaminase (t-TGase) activity, and expression levels of ubiquitin and cyclooxygenase-2 (COX-2) in the normal tissue adjoin to fibroadenoma (nFA), atypical ductal hyperplasia (nADH), and invasive ductal carcinoma (nIDC) from 45 breast cancer patients. We found that lipid peroxidation and nitric oxide production significantly increased in nIDC respect to nFA and nADH ($P < 0.005$) whereas the 4-hydroxy-2-nonenal (HNE) protein-adducts increased only in nADH ($P < 0.005$). The increased lipid damage observed in nIDC correlates with estrogen receptor exposure in IDC ($R^2 = 0.89$). Moreover, nIDC and invasive ductal carcinoma (IDC) showed a 10-fold higher t-TGase activity compared to nFA and nADH. Contrary, COX-2 expression levels significantly decreased nIDC and IDC respect to the nFA and nADH ($P < 0.001$). The analysis of the free ubiquitin expression revealed equal levels in nADH and nIDC samples whereas high molecular weight-ubiquitin conjugate increased about fivefold only in nIDC ($P < 0.01$ vs. nADH). These novel findings reveal an interplay between membrane lipid peroxidation, t-TGase activity, and COX-2 expression levels in the tissue adjoining to neoplastic lesion during breast cancer progression.

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Excess generation of oxygen free radicals can cause oxidative damage to biomolecules resulting in lipid peroxidation (LPO), mutagenesis, and then carcinogenesis, a long and multistep process that includes initiation, promotion, and progression as a consequence of an imbalance between cell proliferation and cell death (Hristozov et al., 2001; Kumaraguruparan et al., 2002; Fucito et al., 2008).

Oxygen radicals (O_2^-, H_2O_2, ^-OH, O_2^-) are continuously generated within mammalian cells in endogenous processes, this being a consequence of the use of oxygen in aerobic respiration during electron leakage from transport chains in mitochondria and endoplasmic reticulum (Ziech et al., 2011).

A disturbance in the balance between the production of reactive oxygen species (ROS) and antioxidant defenses, favoring the overproduction of ROS, plays an important role in the initiation of the oxidative stress (Ziech et al., 2011). The interplay between mitochondrial ROS production and the role of oncoproteins and tumor suppressors is critical in the in modulation of mitochondrial function to promote malignant cell transformation and avoid senescence (Ralph et al., 2010).

Abbreviations: AA, arachidonic acid (all-cis-5,8,11,14-eicosatetraenoic acid); COX, cyclooxygenase; LOX, lipoxygenases; t-TGase, transglutaminase; HNE, 4-hydroxy-2-nonenal; FA, fibroadenoma; ADH, atypical ductal hyperplasia; IDC, invasive ductal carcinoma; LPO, lipid peroxidation; ROS, reactive oxygen species; MDA, malondialdehyde; NO, nitric oxide.

Maria Luisa Balestrieri and Alessandra Dicitore contributed equally to this study.

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Indeed, products of oxidative stress can induce cellular metabolic alterations and rearrangements including DNA breaking, interaction with functional cluster of proteins, cellular membranes, and intracellular calcium release pointing out a possible role in cancer initiation and promotion (Tas et al., 2005). In breast tissue, ROS production, mainly due to macrophages infiltration and altered tissue metabolic pathways, is critically involved in the mutagenic processes that drive to breast carcinoma initiation and progression (Dalle-Donne et al., 2006).

Oxidative stress also leads to tissue transglutaminase (t-TGase) accumulation on the cell surface and in the extracellular matrix (Ientile et al., 2007). This enzyme plays a key role in cancer cell motility and invasiveness (Antonyak et al., 2009; Fésüs and Szondy, 2005) and its expression in breast cancer cells plays an important role in the development of the metastatic phenotype (Singer et al., 2006; Mangala et al., 2007). Moreover, the t-TGase2 expression inversely correlates with the level of the inhibitory subunit of NF-kB in breast tumors, implying that this enzyme is responsible for the constitutive activation of NF-kB in breast tumors (Park et al., 2009).

LPO, a ROS induced process involving the oxidation of polyunsaturated fatty acids in the plasma membranes, is a critical mechanism leading to growth inhibition and cell death (Ray et al., 2000). Clinical studies have provided evidence of the potential role of oxidative stress and LPO in breast cancer etiology (Kumaraguruparan et al., 2002). However, the role of LPO in breast cancer is controversial. Indeed, elevated levels of LPO products have been detected in breast cancer patients and in women at high risk for breast cancer as opposed to controls (Ray et al., 2000; Gonenc et al., 2001; Tas et al., 2005; Kedzierska et al., 2010), although other studies have found them to be significantly decreased (Gago-Dominguez et al., 2007). More in detail, in mammary gland tissues LPO promotes the production of linoleic acid-derived arachidonic acid (AA), a fatty acid susceptible to lipid oxidation with a final formation of aldehydes, such ad malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE) (Dalle-Donne et al., 2006). A lower antioxidant capacity and higher MDA levels are strictly related to breast cancer (Sener et al., 2007; do Val Carneiro et al., 2009; Kedzierska et al., 2010). Similarly, HNE, which binds to proteins with high affinity, acts as a cytotoxic or growth regulating signaling molecule (Dalle-Donne et al., 2006) and takes part to the mechanism contributing to the genotoxic injury in the etiology of breast cancer (Albright et al., 2005; Steiner et al., 2007). Mildly cross-linked, HNE-modified proteins are preferentially degraded by the ubiquitin-proteasome pathway, but extensive modification with this cross-linking aldehyde leads to the formation of protein aggregates that can be inhibit the proteasome (Nikseresht et al., 2010; Zhao et al., 2010; Mittal et al., 2011). The ubiquitin-proteasome pathway, which facilitates the degradation of damaged proteins and regulates growth and stress response, is activated in breast cancer and can be determinant in the progression of metastatic breast cancer (Nikseresht et al., 2010; Zhao et al., 2010; Mittal et al., 2011).

ROS and LPO mediators exert their influences largely by modulating transcription factor activities that affect the expression of cyclooxygenases (COX) and lipoxygenases (LOX) (Kumagai et al., 2000; Davies et al., 2002; You et al., 2009). Analysis of COX and LOX expression in a cohort of breast cancer patients revealed that these enzymes are aberrantly expressed. Specifically, compared with normal breast tissues, tumor tissues exhibited significantly higher levels of 12-LOX and COX-2, and significantly lower level of 15-LOX (Jiang et al., 2003).

Nitric oxide (NO) also shows varied effects on human breast cancer cells (Pervin et al., 2010). This pleiotropic ancestral molecule is particularly harmful in adipogenic milieu of the breast, where it initiates and promotes tumorigenesis (Pervin et al., 2010). Epidemiological studies have associated populations at a greater risk for developing breast cancer to express specific polymorphic forms of endothelial nitric oxide synthase, that produce sustained low levels of NO (Pervin et al., 2010). At low concentration NO generates oxidative stress and inflammatory conditions at susceptible sites in the heterogeneous microenvironment of the breast, where it promotes cancer related events in specific cell types (Pervin et al., 2010).

Molecular mechanisms of breast cancer have been widely described at the cancer lesion levels. However, the events that occur in the normal tissue around the lesion have not been fully elucidated yet. We hypothesized that possible changes occurring in normal tissue adjoin the benign and malignant lesions could affect the breast cancer evolution. To this end, this study was designed to investigate molecular mechanism(s) occurring in the normal tissues adjoin the fibroadenoma (nFA), as benign breast diseases, the atypical ductal hyperplasia (nADH), as a "borderline" category associated with a significant risk for the development of invasive breast cancer, and the invasive ductal carcinoma (nIDC), a very common type of breast cancer. Specifically, we investigated the LPO, t-TGase activity, and COX-2 expression and evaluated ubiquitin expression, NO levels, MDA, and HNE-protein adducts, in order to determine possible relationships between their production and the progression of breast cancer.

Materials and Methods

Materials

Solvents and reagents used for hypotonic buffer, urea, SDS and thiobarbituric acid (TBA) were obtained from Sigma (St. Louis, MO). Trifluoroacetic acid (TFA) was from Fluka while all reagents used for t-TGase assay and NO measurements were purchased from Cayman Chemical (Ann Arbor, MI, USA). The tissues were homogenized by an Ultradurax T25 basic while the membrane samples were sonified with a Sonicator Ultrasonic Processor XL Heat Systems. The absorbance data were obtained by Jenway Genova spectrophotometer. Proteins were determined using the Biorad proteins assay procedure, with albumin as standard. For Western blotting antibody against COX-2 antibody (rabbit) was purchased by Vinci-Biochem and anti-Ubiquitin antibody was obtained by Sigma.

Patients and samples preparation

This study was conducted on breast tissues of patients affected by fibroadenoma (FA), atypical ductal hyperplasia (ADH), and invasive ductal carcinoma (IDC). All the patients included in the investigation were non-smoking with mean age $\pm$SD of 52 ± 9 years for all patients. The modified Bloom and Richardson tumor grades I, II, and III (indicating low, moderate, and high grade) were used in the analysis. The ethical committee of the institution approved the study, and informed consent of all the subjects was obtained. Fifteen women for each group were operated on Geriatrics Surgery Service, Second University of Naples. The normal tissue samples adjacent to fibroadenoma (nFA) ($n = 15$), atypical ductal hyperplasia (nADH) ($n = 15$), and invasive ductal carcinoma (nIDC) ($n = 15$) were obtained during surgical resection at 2 cm from the lesions directly frozen in liquid nitrogen and stored at -80°C until use. The tissue specimens were weighted and suspended in hypotonic buffer (Tris–HCl 10 mM, EDTA 0.1 mM and $MgCl_2$ 5 mM, PMSF 90 μM, 2-β-mercaptoethanol 0.1 mM (pH 7.5).The suspensions were homogenized and then they were centrifuged at 13,000 rpm for 10 min in order to separate citosol (supernatants) from membranes (pellets).

Analysis of tumor grade and biomarkers

The tumor grade and biomarker status were examined using the standard clinical protocol. The estrogen receptor (ER) and progesterone receptor (PR) status was considered to be negative if immunoperoxidase staining of tumor cell nuclei in the biopsy specimen was less than 5%. Expression of c-erbB-2 was evaluated semi-quantitatively on paraffin slices. The material was immunohistochemically stained for c-erbB-2 (polyclonal antibody Dakopatts-Dania, Code Nr. K-353) and analyzed under light microscopy (Olympus BX 5).

Phospholipid extraction

The membranes (0.004–0.015 g) were added of 500 μl urea 6M, SDS 0.1% buffer. After mixing, membranes were sonified while unbroken cells and large debris were removed by centrifugation at 15,000 rpm for 15 min. Supernatants (100 μl) were used for extraction of phospholipids. The phospholipids were extracted from total membranes isolated from each tissue by Bligh and Dyer method (1959). Glassware was rinsed with chloroform, and then air-dried and Teflon-lined caps were used to prevent contamination with plastics. The lower phase was extracted, transferred to a glass vial and the solvent evaporated under a stream of nitrogen. The dried phospholipids were stored at −70°C under a nitrogen atmosphere and used for TBARS assay.

Thiobarbituric acid (TBA) assay

The TBA test was conduced on phospholipids extracted using 1.5 ml of the buffer TCA 15%, TBA 0.3% in HCl 0.12 N, as previously described (Stiuso et al., 2010; Caraglia et al., 2011). The mixtures were vortexed, heated at 100°C for 30 min, cooled in a cold water bath for 10 min, and centrifuged at 7,000*g* for 10 min. The absorbance of supernatants was measured at wavelength 532 nm.

t-TGase assay

t-TGase activity was assayed by measuring the incorporation of [^{3}H] spermidine trihydrochloride into *N*,*N*-dimethylated casein. The assay mixtures (100 μl) containing 125 mM Tris/HCl buffer, pH 8.0, 10 mM dithiothreitol, 2.5 mM Ca^{2+}, 50 nM [^{3}H] spermidine and 0.2 mg of dimethylated casein were incubated at 37°C for 1 h in the presence of various amounts of samples. The reactions were stopped by adding 1.0 ml of 10% trichloroacetic acid containing 2 mM unlabeled spermidine and the samples were then centrifuged (15,000*g*, 8 min). The resulting precipitates were dissolved in 1.0 ml of 0.1 M NaOH, and finally radioactivity was counted following the addition of 5 ml Pico-Fluor 40 scintillation mixture Packard (Milan, Italy).

NO measurement

The NO produced by the NOS-catalyzed reaction was evaluated on pellets which were centrifuged at 1,600*g* × 5 min. Five hundred microliters of supernatants were mixed with an equal volume of Griess reagent (sulfanilamide 1% and naphthylethylenediamide dihydrochloride 0.1% in H_3PO_4 5%). After incubation at room temperature for 10 min the absorbance was measured at 550 nm. The NO concentration was calculated considering the mean of the data obtained by tests in double and referring it to standard curve obtained by the measurement of the absorbance of buffer added of a NaNO solution at various concentrations.

HNE-protein adduct measurement

Fluorescence measurements were performed on a LS55 Perkin Elmer luminescence spectrofluorometer equipped with 1 cm quartz cuvettes. All spectra were corrected for blank emission and the data were referred to protein concentration of the samples. The quantitation of the fluorescence intensity (360 nm excitation and 430 nm emission) was taken as an indirect measure of the HNE-protein adducts (AFU/mg protein).

Immunoprecipitation

Antibody against COX-2 or Ubiquitin (10 μl) was added to 200 μl of cytosol and incubated overnight at 4°C. 20 μl of Protein A-sepharose (Sigma) was added and further incubated on a rotating device overnight at 4°C. At the end of incubation the mixtures were centrifuged at 15,000*g* and the supernatants were discarded while aliquots of pellets were resuspended in 20 μl 3× SDS sample buffer (125 mM Tris–HCl pH 6.8), 6% SDS, 30% glycerol, 150 mM DTT, 0.03% bromophenol blue) and heat at 100°C for 5 min. The samples were analyzed by 12% SDS–PAGE as described by NuPAGE kit (Invitrogen, Carlsbad, CA, USA) and then by Western immunoblotting with antibodies against COX-2 or Ubiquitin (1:5,000) as primary antibody (following instruction described in Western Breeze Kit, Invitrogen).

Statistical evaluation

Results are reported as mean ± standard error (SE) unless otherwise stated. Data were analyzed using GraphPad software (San Diego, CA) and the level of significance was accepted at $P < 0.05$.

Results

Beaseline features of patients

The clinical features of the study population (n = 45) are summarized in Table 1. At diagnosis, the women age was between 30 and 60 years. Women were predominantly premenopausal (71%) and the size of the lesion tended to be greater than 2 cm. The patients with IDC were all estrogen receptor (ER) positive (+) and resulted of tumor grade II (Table 2). Moreover, in the majority of cases (73%) were lymphnode negative (−). All biochemistry evaluations were conducted on nFA, nADH, and nIDC obtained during surgical resection at 2 cm from the breast lesion.

Oxidative markers evaluation in nFA, nADH, and nIDC

Increasing evidence from animal and human studies highlight the key role of oxidative stress and lipid peroxidation in breast cancer development. Here, the evaluation of thiobarbituric acid reactive substances (TBARS), NO, and HNE-protein adducts was performed in nFA, nADH, and nIDC patients. Results indicated that LPO and NO production were significantly increased in nIDC respect to nFA and nADH, with values of TBARS ranged from 50 to 170 nmol/μg protein ($P < 0.005$ vs. nFA and nADH) and NO between 14 and 20 nmol/μg protein ($P < 0.007$ vs. nFA and nADH) (Table 3). The HNE-adducts increased only in nADH and the values are 3.75- and 23.3-fold higher than those detected in nFA and nIDC, respectively (Table 3). The relationship between estrogen-induced breast cancer and oxidative stress it is well established (Felty et al., 2005). Furthermore, oxidative DNA damage is reportedly increased in breast cancer tissues relative to normal breast, with a strong correlation to ER status (Mobley and Brueggemeier, 2004). When LPO and ER exposure were evaluated in nIDC and IDC, an increased lipid damage with a

TABLE 1. Clinical characteristics of patients enrolled in the study

	FA (n = 15)	ADH (n = 15)	IDC (n = 15)
Age (years)[a]	40 ± 10	45 ± 5	52 ± 11
Size (cm)[a]	3 ± 0.5	4 ± 0.8	3 ± 0.6
Premenopusal	80%	66%	66%

This study was conducted on breast tissues from patients affected by fibroadenoma (FA), atypical ductal hyperplasia (ADH), and invasive ductal carcinoma (IDC).
[a]The value represented the mean ± SD.

TABLE 2. Characteristics of patients with IDC

Patients	Lymph node status	Tumor grade	%ER+	%PgR	%C-erb-2
1	Neg.	II	50	70	70
2	Pos.	II	50	Neg.	Neg.
3	Pos.	II	30	Neg.	Neg.
4	Neg.	II	85	40	60
5	Neg.	II	30	Neg.	Neg.
6	Neg.	II	70	25	75
7	Neg.	II	20	Neg.	Neg.
8	Neg.	II	90	90	90
9	Neg.	II	90	90	Neg.
10	Neg.	II	70	60	80
11	Neg.	II	80	20	25
12	Neg.	II	90	20	90
13	Pos.	II	90	90	60
14	Pos.	II	70	Neg.	30
15	Neg.	II	40	Neg.	Neg.

neg. negative; pos., positive.

TABLE 3. Evaluation of oxidative stress markers in nFA, nADH, and nIDC

	TBARS (nmol/μg protein)	NO (nmol/μg protein)	HNE-protein[a] (arbitrary units)
nFA	$8.5 \times 10^{-6} \pm 3.5$	2.15 ± 0.85	14 ± 4
nADH	$20 \times 10^{-6} \pm 5^{*}$	$9.7 \pm 2.47^{*}$	$52.5 \pm 14^{*}$
nIDC	$115 \times 10^{-6} \pm 55^{*}$	$17 \pm 1.5^{*}$	$2.25 \pm 0.38^{*}$

The results are expressed as the mean ± SEM of n = 15 determinations performed in triplicate with $^{*}P < 0.005$ versus nFA.
[a]The value represented the ratio between emission fluorescence intensity at 430 nm (excitation 360 nm) and μg protein.

strong correlation to ER status was observed ($R^2 = 0.89$ and $P < 0.0001$; Fig. 1). In particular, the mean of TBARS values was 6×10^{-6} and 160×10^{-6} nmol/μg proteins when the exposure of ER was between 25–30 and 90-95%, respectively.

Evaluation of t-TGase activity, COX-2, and ubiquitin expression

t-TGase and COX-2 have been reported to play an important role in breast cancer risk and development (Shim et al., 2003; Antonyak et al., 2009; Kumar et al., 2010). Here, we evaluated the t-TGase activity and COX-2 expression levels in nFA,

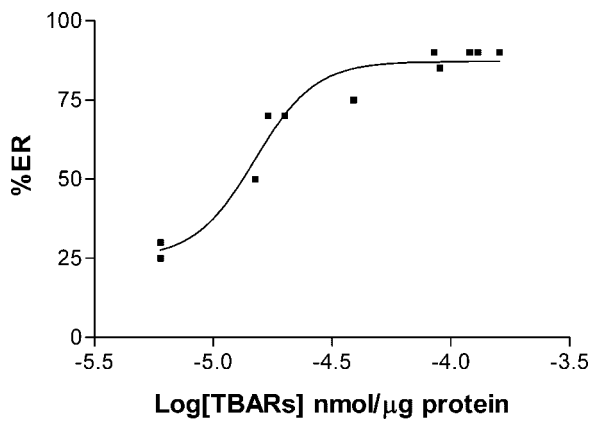

Fig. 1. Correlation between ER (%) exposure and TBARS in IDC and nIDC. The TBARS value was expressed as logarithm of the molar concentration. Nonlinear regression fit (sigmoid dose–response) to the data shows an $R^2 = 0.957$.

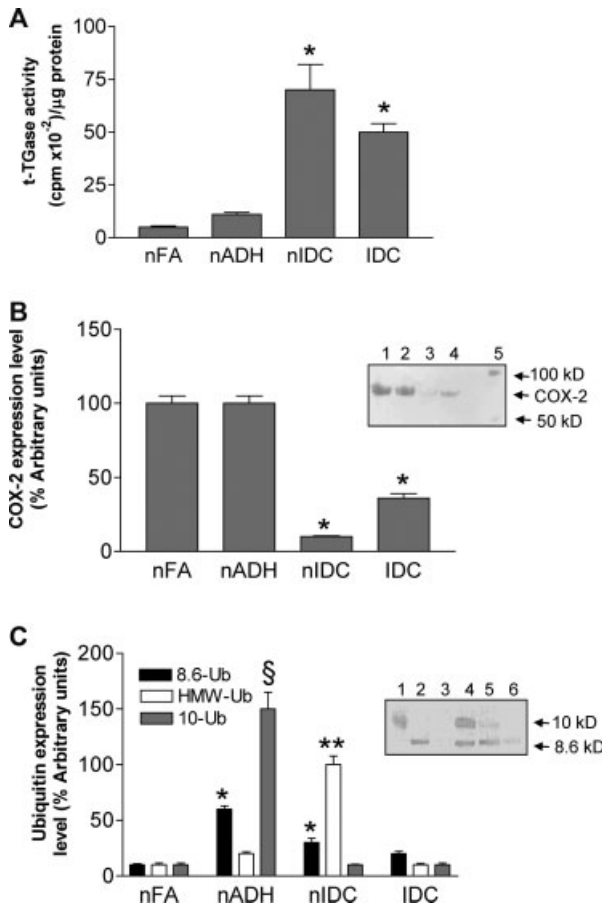

Fig. 2. Evaluation of t-TGase activity, COX-2, and ubiquitin expression in nFA, nADH, nIDC, and IDC. A: t-TGase activity was assayed by measuring the incorporation of [^{3}H] spermidine trihydrochloride into *N,N*-dimethylated casein ($^{*}P < 0.01$ vs. nFA and nADH). B: COX-2 expression was evaluated by Western blot analysis after immunoprecipitation with protein A from cytosolic tissues extract, as described in Materials and Methods Section. The expression levels are reported as percentage of nFA. Insert; lane 1, nFA; lane 2, nADH; lane 3, nIDC; lane 4, IDC; lane 5, molecular weight markers ($^{*}P < 0.01$ vs. nFA and nADH). C: Ubiquitin expression was evaluated by Western blot analysis after immunoprecipitation. The arrows indicate the ubiquitin at 8.5 and 10 kDa. Insert; lane molecular weight; lane 2, ubiquitin standard; lane 3, nFA, lane 4, nADH, lane 5, nIDC, lane 6, IDC. The expression levels of the proteins are reported as intensities quantified with Image J software (NIH) ($^{*}P < 0.05$ vs. nFA, $^{**}P < 0.01$ vs. nADH and nFA; $^{\S}P < 0.01$ vs. nFA).

nADH, nIDC, and IDC lesion which was used as control since it is the most studied breast cancer (Fig. 2). Results revealed that the t-TGase activity was significantly enhanced (about 10-fold) in the nIDC and IDC compared to the nFA and nADH ($P < 0.01$; Fig. 2, part A). On the contrary, the COX-2 expression levels were consistently decreased in the nIDC and IDC compared to nFA and nADH ($P < 0.01$) (Fig. 2, part B).

Moreover, the analysis of the free ubiquitin (ub) expression (8.6 kDa), involved in the removal of oxidized proteins (Marques et al., 2004), revealed that equal levels of unbound ubiquitin are present in nADH and nIDC samples, and that is not detectable in nFA tissue (Fig. 2, part C). In addition, only in nIDC, high molecular weight (HMW)-ubiquitin conjugate increased about fivefold respect to nADH. Finally, another band with a slightly higher molecular weight (about 10 kDa) was revealed only in nADH samples. This band, probably, might

derive by deubiquitination process for regenerating the free ubiquitin.

Discussion

The results of the present study reveal for the first time a strict association between membrane lipid peroxidation and t-TGase activation in the tissue adjoining to breast cancer lesions. Findings suggest that in these breast tissues the oxidative stress markers (MDA, NO, and HNE-protein adducts), t-TGase, and COX-2 enzyme regulation might take part to the process responsible for of cancer progression (Fig. 3). Indeed, a moderate oxidative stress associated with a high COX-2 and a low t-TGase and ubiquitin expression levels was detected in the normal tissue adjoin the benign breast disease and a "borderline" category of breast disease (Fig. 3). On the contrary, both the invasive ductal carcinoma and the normal tissue adjoin it show higher oxidative stress markers as well as an increased t-TGase activity (Fig. 3).

Several evidence from animal and human studies describe a role of oxidative stress and lipid peroxidation in the initiation and promotion of events that lead to the development of breast cancer (Hristozov et al., 2001; Ziech et al., 2011). The current model of human breast cancer progression proposes a linear multi-step process which initiates as flat epithelial atypia, progresses to ADH, evolves into IDC and culminates in the potentially lethal stage of invasive ductal carcinoma. Initiation results when a normal cell sustains and fixes a DNA mutation that can occur with physical and chemical carcinogens as ROS and reactive nitrogen species, producing an initiated mutated cell. Free radicals have been known for causing oxidative damages and could attack lipids of the cellular membranes initiating lipid peroxidation (Hristozov et al., 2001; Ziech et al., 2011).

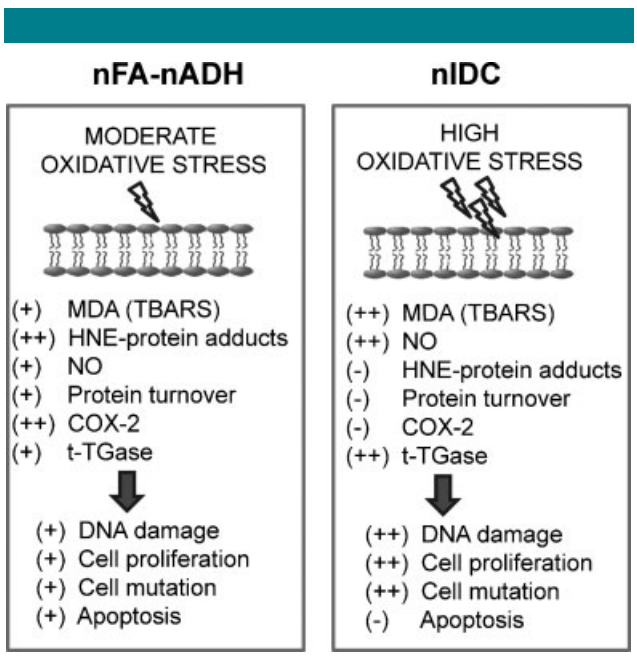

Fig. 3. Schematic diagram of the possible of oxidative stress-dependent mechanism(s) in nFA, nADH, and nIDC. A moderate oxidative stress in nFA and nADH is associated with a high COX-2 and a low t-TGase activity. High oxidative stress in nIDC is linked to an increased t-TGase activity and a decreased COX-2 expression level. (+) Moderate increase, (++) high increase, and (−) decrease.

An increased level of TBARS has been found in the plasma of patients with invasive breast cancer in comparison with a control group (Kedzierska et al., 2010). In our study we observed a link between oxidative stress/lipid peroxidation-related biomarkers and the evolution of breast carcinogenic stages from fibroadenoma to IDC. In fact, the progressive increased levels of TBARs and NO that starts in nFA suggest that ROS production sustains an initial moderate oxidative damage of the membrane lipids that occurs in the initial process of the breast disease.

Inflammation plays an important role in the initiation of the cancer development (Davies et al., 2002; Balestrieri et al., 2008). COX, the key enzyme required for the conversion of AA to prostaglandin, is inappropriately expressed in various cancers and, hence, recognized as one of the hallmarks of chronic inflammation-associated malignancies. COX-2 has been suggested as a marker of precursors and risk of breast cancer (Shim et al., 2003). COX-2-positive foci have also been observed in healthy mammary tissues (Crawford et al., 2004). To date, the literature described many reports on the levels of COX-2 expression and breast cancer progression and its use as a marker for disease-free survival or overall survival (Witton et al., 2004; Lari and Kuerer, 2011). In this study, we observed an increased expression of COX-2 in nFA and nADH but not in nIDC. This results could explain how an initial moderate oxidative damage can be involved in the initiation of an inflammation process (Servillo et al., 2006).

Oxidative stress and estrogen receptor-associated proliferative changes are suggested to play important roles in estrogen-induced breast carcinogenesis. The direct role of estrogens as tumor initiators has been supported by the evidence that some of their catabolites like 17β-estradiol, catechol estrogens (CEs) and their quinone derivatives exert oxidative stress that lead to various types of DNA damage, including single strand breaks as well as stable and depurinating DNA adducts. In our study, we observed a increased levels of TBARs in nIDC linearly correlated with percentage ER exposure in IDC. This evidence could be function of high estrogen exposure and may occur prior to tumor progression though a possible cross-talk between the lipid peroxidation pathway and the estrogen one.

Much emphasis has been placed on the enzymatic activity displayed by t-TGase, which is often associated with adaptive protein modifications following the stress response. Moreover, attention has been paid to elucidate the potential role(s) played by t-TGase in oxidative stress-induced cell which include regulation of apoptosis and maintenance of balance of cell death and survival intracellular survival protein (Piacentini et al., 2005).

Oxidative stress increases t-TGase activity that appears to be responsible for the inappropriate formation and/or stabilization of protein aggregates that may be cytotoxic (Ientile et al., 2007). In addition to removing damaged/unnecessary proteins, the ubiquitin–proteasome pathway is also a crucial mechanism in control of many cellular processes, including activation of transcription factors, cell cycle progression, and apoptosis (Hicke et al., 2005). Here, the increased expression of the HMW-ubiquitin in nIDC could be linked with the marked increased activity of t-TGase. This could explain how protein misfolding related to oxidative damages may also make proteins better substrates for t-TGase-catalyzed cross linking, leading to polymer formation and inhibition of protein turnover. Shabek et al. (2007) showed that free monomeric ubiquitin (8.6 kDa) does not serve as a proteolytic substrate and it must have some form of C-terminal extension, that is, a peptide or an intact substrate, for degradation. One prediction of the "extended ubiquitin" hypothesis is that increased proteolysis of cellular proteins that occurs under stress, would result in concomitant accelerated degradation of ubiquitin (Shabek et al., 2007).

Therefore, the increased expression of ubiquitin (10 kDa) observed in nADH could be ascribed to the over production HNE-protein adduct.

Our results may have give rise to further studies to validate lipid peroxidation as prediagnostic marker for breast cancer. Detection of its levels in women at high risk for breast cancer through non-invasive methods such as nipple fluid aspirate sampling could be a promising tool in breast cancer prevention biomarker. Finally, a better understanding of the relationship between breast cancer risk factors, oxidative stress/lipid peroxidation-related biomarkers, and genes are necessary in identifying the dietary or non-dietary exposure genotype combinations that put women at the lowest risk.

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