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Breast fine-needle aspiration malondialdehyde deoxyguanosine adduct in breast cancer

MARCO PELUSO[1], ARMELLE MUNNIA[1], GABRIELLA G. RISSO[2], SANDRA CATARZI[2], SARA PIRO[1], MARCELLO CEPPI[3], ROGER W. GIESE[4] & BENIAMINO BRANCATO[2]

[1]*Cancer Risk Factor Branch, ISPO-Cancer Prevention and Research Institute; Florence, Italy,* [2]*ISPO-Cancer Prevention and Research Institute, Florence, Italy,* [3]*Molecular Epidemiology Unit, National Cancer Institute, Genoa, Italy, and* [4]*Department of Pharmaceutical Sciences in the Bouve College of Health Sciences, Barnett Institute, Northeastern University, Boston, MA, USA*

Abstract

This study has analysed the generation of 3-(2-deoxy-β-D-erythro-pentafuranosyl)pyrimido[1,2-a]purin-10(3H)-one deoxyguanosine adduct [M_1dG], a biomarker of oxidative stress and lipid peroxidation, in breast fine-needle aspirate samples of 22 patients with breast cancer, at different clinical stages, in respect to 13 controls. The multivariate analysis show that M_1dG adduct was higher in cases than in controls (Mean Ratio (MR) = 5.26, 95% CI = 3.16–8.77). Increased M_1dG was observed in women with a tumour grade 3 and a pathological diameter 2 (MR = 7.61, 95% CI = 3.91–14.80 and MR = 5.75, 95% CI = 3.13–10.59, respectively). A trend with increasing tumour grade and pathological diameter was present (MR = 1.98, 95% CI = 1.57–2.50 and MR = 2.44, 95% CI = 1.71–3.48, respectively). Not significant effects of age and smoking habit were found (MR = 1.58, 95% CI = 0.92–2.72 and MR = 1.68, 95% CI 0.88–3.20, respectively). An increment over the background frequency of M_1dG can contribute to breast cancer development. Increasing severity of breast tumour can influence DNA damage level.

Keywords: *Breast cancer, free radicals, M_1dG, fine-needle aspirate, tumour grade, pathological diameter*

Abbreviations: *CT, calf-thymus; FNA, fine-needle aspirate; LPO, lipid peroxidation; MDA, malondialdehyde; M_1dG, 3-(2-deoxy-β-D-erythro-pentafuranosyl)pyrimido[1,2-α]purin-10(3H)-one deoxyguanosine; MALDI TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MR, Mean Ratio; nn, normal nucleotides; G, tumour grade; pT, pathological diameter; RAL, relative adduct labelling; ROS, reactive oxygen species.*

Introduction

Breast cancer is a leading cause of cancer death among Western country women and, at the same time, it is a disease difficult to prevent because its aetiology is not well understood [1]. An association between oestrogen exposure, chronic inflammation and increased risk of developing cancer of breast has been suggested [2–4]. Knowledge of how such risk factors induce tumourogenesis in breast tissues is not well defined, but it is possible that an excessive generation of free radicals can be an important mechanism of breast carcinogenesis for oestrogens and

inflammation. These reactive intermediates have been shown to cause lipid peroxidation (LPO) and a number of mutagenic DNA adducts, that, if unrepaired, can induce mutations and ultimately cancer [5].

Malondialdehyde (MDA) is a natural product of LPO, which is also formed during prostaglandin E_2 biosynthesis via cyclooxygenase [5]. MDA is a reactive by-product capable of interacting with DNA to form different endogenous adducts, including 3-(2-deoxy-β-D-erythro-pentafuranosyl)pyrimido[1,2-a]purin-10(3H)-one deoxyguanosine (M_1dG). In addition to these pathways, M_1dG adduct can be formed

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from reactive oxygen species (ROS) through the formation of base propenal intermediates in DNA [6]. The relevance of M_1dG adduct in carcinogenesis is emphasized by their ability to induce base pair mutations and cause frameshift mutations in reiterated sequences [7]. Physiological levels of endogenous DNA damage have been detected in a number of tissues, including larynx, bronchi and peripheral leukocytes [8–13]. Nature and nurture susceptibilities have been reported to modulate M_1dG production [8–13]. In addition, some authors, including us, have suggested that enhanced level of M_1dG adduct can be related to cancer development and tumour progression [10–12].

Here, we have undertaken a study to analyse the production of M_1dG adduct in breast fine-needle aspirate (FNA) samples of women undergoing breast FNA for diagnostic purpose using ^{32}P-DNA post-labelling and mass spectrometry techniques [13]. We have compared the frequency of M_1dG adduct in the breast lesions of 22 patients suffering from breast cancer, at different clinical stages, in respect to 13 controls. Our aim was to evaluate the role of DNA damage induced from free radicals in the target tissue of breast carcinogenesis.

Material and methods

Study women and sampling

During 35 consecutive FNA for histological exams, additional samples have been obtained for this study

(250 µl). Breast sample collection was performed in women at the ISPO-Cancer Prevention and Research Institute, Florence, Italy. This study was conducted according to the guidelines of the Declaration of Helsinki. All women signed an informed consent after being informed of the purpose of the study. Ethical clearance for this study was obtained from the institutional ethic committee. Detailed information including demographic data, tobacco smoking and clinical parameters, including histologic tumour grade (G), pathological diameter (pT) and radiological diameter of benign and tumour breast lesions, were collected. FNA samples were obtained from 35 women (Table I). Twenty-two women who underwent a surgical excision were confirmed as having a cancer of breast after histopatological analysis. FNA samples were shortly frozen after collection, sent to the laboratory and kept frozen until laboratory analysis. Histopathological diagnosis of the tissue was performed in the laboratory of the Unit of Senology, ISPO, Florence. Thirteen women with benign breast lesions were classified such as the control group. Thirty women were classified as non-smokers, five as current smokers (in the last week before examination). The mean age of breast cancer and benign breast disease groups were 66.2 years ± 15 (SD) and 56.1 years ± 15, respectively.

M_1dG adduct standards

Calf-thymus (CT)-DNA or leukocyte DNA from a blood-donor was treated with 10 mM MDA as

Table I. Median of malondialdehyde deoxyguanosine adduct and interquartile range for different variables considered in the study plus the parameter estimates of the multivariate regression mode adjusted by age (continuous), smoking habit, beast cancer case-control status, tumour grade and pathological diameter, as appropriate.

	n^a	Median^b (25–75) percentiles	Means ratio	95% CI^c	p
Age					
< 50	12	2.5 (1.8–8.4)	1		
50–76	11	7.1 (4.3–11.3)	1.01	0.59–1.72	0.971
> 76	11	12.1 (6.4–23.5)	1.58	0.92–2.72	0.095
Smoking habit					
Never smokers^d	30	7.0 (2.3–12.1)	1		
Current smokers	5	2.9 (1.9–5)	1.68	0.88–3.20	0.111
Status					
Controls^d	13	1.8 (1.4–2.6)	1		
Breast cancer cases	22	9.2 (6.9–15.2)	5.26	3.16–8.77	< 0.001
Tumour grade					
Controls^d	13	1.8 (1.4–2.6)	1		
G1	4	8.2 (6.9–10.7)	4.58	2.31–9.09	< 0.001
G2	6	8.0 (7.1–11.3)	5.38	2.82–10.25	< 0.001
G3	4	15.2 (12.2–15.4)	7.61	3.91–14.80	< 0.001
Pathological diameter					
Controls^d	13	1.8 (1.4–2.6)	1		
pT1	12	8.8 (6.9–13.6)	5.07	2.92–8.79	< 0.001
pT2	8	13.2 (7.0–20.5)	5.75	3.13–10.59	< 0.001

^{a}Some figures do not add up to the total because of missing values.
^{b}Level of M_1dG per 10^8 normal nucleotides.
^{c}Confidence interval.
^{d}Reference level.

previously described [14]. MDA treated CT-DNA was diluted with untreated DNA to obtain decreasing levels of reference standard. Then, we decided to expose human epithelial lung carcinoma A459 cells to 100 mM H_2O_2, a known source of ROS and LPO, to evaluate the formation of M_1dG adduct not only in naked DNA but also in experimental *in vitro* cells, a system that should reflect the physiological conditions of *in vivo* organisms.

DNA extraction and purification

Before FNA DNA extraction, 250 μl of TE-buffer (10 mM Tris, 1 mM EDTA, pH 7.4) containing two anti-oxidants: 6.8 mM 8-hydroxyquinoline and 500 μM N-tert-butyl-*a*- phenylnitrone were added to FNA samples. DNA was then isolated and purified using a method that requires digestion with ribonuclease A, ribonuclease T1 and proteinase K, extraction with saturated phenol, phenol/chloroform/isoamyl alcohol (25:24:1), chloroform/isoamyl alcohol (24:1) and ethanol precipitation, according to Reddy and Randerath [15]. DNA was gently dissolved in sterile distilled water and its concentration and purity was determined using a Beckman DU 800 spectrophotometer (the total amount of FNA DNA recovered ranged from 6–10 μg). Coded DNA was stored at –80°C until laboratory analysis.

^{32}P-DNA post-labelling

DNA (2 μg) were digested by micrococcal nuclease and spleen phosphodiesterase. After nuclease P1 treatment, M_1dG adduct were labelled with [γ-^{32}P]-ATP. The analysis of M_1dG adduct was performed using multidirectional chromatography [13]. Detection and quantification of M_1dG adduct and normal nucleotides was performed as previously described [13]. M_1dG adduct levels were expressed such as relative adduct labelling (RAL) = screen pixel in adducted nucleotides/screen pixel in total nucleotides. M_1dG adduct values were corrected across experiments based on the recovery of the internal standard. The coefficient of variation of the assay was 0.22 and the mean recovery of internal standard was 16.7%.
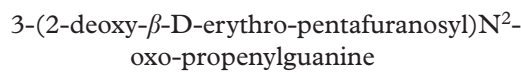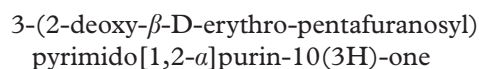
Mass spectrometry

DNA adducts were also detected in MDA treated CT-DNA by mass spectrometry through the following sequence of steps: (1) react DNA with $NaBH_4$ [16] followed by precipitation with isopropanol; (2) digest with snake venom phosphodiesterase and nuclease P1; (3) extract DNA adducts that are less polar than normal nucleotides (nn) on an OASIS cartridge (Waters Corporation); (4) tag with an isotopologue pair of benzoylhistamines (d_0 and d_4) in a phosphate-specific labelling reaction in the presence of carbodiimide [17]; (5) remove residual reagents by ion exchange solid-phase extraction; (6) resolve tagged adducts by capillary reversed-phase HPLC with collection of drops onto a MALDI plate; (7) add matrix (*a*-cyano-4-hydroxycinnamic acid); and (8) analyse by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS).

Alkaline hydrolysis

Since previous studies have shown the hydrolytic ring-opening of M_1dG at alkaline pH [18,19], we have evaluated whether the alkaline hydrolysis can induce the autoradiography disappearance of M_1dG adduct spots [20]. M_1dG adduct spots, that have been detected in the chromatograms of the study women, were excised and incubated with and without increased concentrations of ammonium hydroxide 0.1, 0.5, 1.0 and 2.0 M, pH 11.7 for 10' to induce the following ring-opening reaction:

3-(2-deoxy-β-D-erythro-pentafuranosyl) pyrimido[1,2-*a*]purin-10(3H)-one

$$\downarrow OH^-$$

3-(2-deoxy-β-D-erythro-pentafuranosyl)N^2-oxo-propenylguanine

After alkaline hydrolysis, the samples were transferred to the same chromatogram and run side-by-side with 0.24 M sodium phosphate, 2.4 M urea, pH 6.4.

Statistical analysis

All statistical analyses were performed on log transformed data to stabilize the variance and normalize the distribution of M_1dG adduct. A descriptive analysis was initially performed to explore the relationship between individual variables and M_1dG adduct. The multivariate analysis was performed using log-normal regression models including terms for age, smoking habit and breast cancer case-control status to estimate the effect of each variable on the outcome adjusting for the concomitant effect of the other variables included in the model. Women were also grouped according to tertiles for age before statistical analyses. The regression parameters estimated from the model are interpreted as ratios between the means of M_1dG adduct of each level of the study variables with respect to the reference level, adjusted by age (continuous), smoking habit, breast cancer case-control status, tumour grade and pathological diameter, as appropriate. A p-value of < 0.05 (two-tailed) was considered significant for all of the tests. As stated in Greenland and Poole [21], when a trend on the regression coefficients is tested, the inclusion of the

category of controls improves the precision of the estimated trend, unless a selection bias is suspected. Since we do not assume that there is a selection bias among our controls, we have included the category of controls in the test for trend. Therefore, to test whether the MR's of tumour grade and pT were distributed according to a linear trend, we have fitted categorical variables as if they were continuous. In this way, we have obtained the mean MR moving from one level to another of each variable. The data were analysed using SPSS 13.0 (SPSS, Chigago, IL, USA).

Results

Standard M_1dG adduct by ^{32}P-DNA post-labelling

A statistically significant increased formation of M_1dG adduct was found in MDA treated CT-DNA in respect to untreated CT-DNA ($p < 0.001$) by ^{32}P-DNA post-labelling assay. The RAL values per 10^6 were 0.32 ± 0.11 (SE), 1.6 ± 0.38 (SE) and 5.0 ± 0.6 (SE) in 1, 4 and 10 mM MDA treated CT-DNA, while a RAL of 0.06 ± 0.01 (SE) was detected in untreated DNA. Figure 1 shows a typical autoradiogram of M_1dG adduct in 10 mM MDA treated CT-DNA (Figure 1A). A calibration curve was obtained by the analysis of decreasing level of MDA treated CT-DNA, $r^2 = 0.99$. A statistically significant production of M_1dG adduct was also observed in MDA treated leukocyte DNA in respect to control leukocyte DNA ($p < 0.001$). The RAL values per 10^6 were 2.2 ± 0.6 (SE) and 0.02 ± 0.01 (SE) in MDA treated and untreated DNA. Then, our results show that the treatment with 100 mM H_2O_2 induces a statistically significant formation of M_1dG adduct in lung carcinoma cells in respect to the unexposed cells ($p < 0.001$). The RAL values per 10^6 were 0.25 ± 0.09 (SE) and 0.07 ± 0.02 (SE) in H_2O_2 treated and untreated lung carcinoma cells.

Standard M_1dG adduct by mass spectrometry

The presence of M_1dG adduct in the 10 mM MDA treated CT-DNA was also confirmed by us subjecting it to analysis by mass spectrometry. M_1dG adduct was identified after mass tagging by MALDI-TOF-MS in MDA treated, $NaBH_4$-reduced CT-DNA. Relative to the accurate masses that are seen in the spectrum from one spot, the exact mass of M_1dG is as follows using the M nomenclature of Goda and Marnett [16]: M_1dG (581.166).

Breast M_1dG adduct

The level of M_1dG adduct was analysed in 35 women. The intensity of M_1dG spots was stronger in the chromatograms of the breast cancer cases, in respect to that observed in benign breast disease patients. Figure 1 shows typical autoradiograms of M_1dG adduct in fine needle aspirate of breast cancer case and control (B and C, respectively).

Furthermore, the alkaline hydrolysis experiments showed that the autoradiography disappearance of M_1dG adduct was associated to increased OH^- concentrations, supporting the identity of breast adduct spots such as M_1dG adduct.

The results of the multivariate analysis show that the level of M_1dG adduct was significantly higher among breast cancer cases in respect to controls (Mean Ratio (MR) = 5.26, 95% confidence interval (CI) = 3.16–8.77, $p < 0.001$). In addition, when the clinical parameters were analysed, a statistically significant increment of M_1dG adduct with increased histological tumour grade was observed. The women with a G3 grade presented the highest MR value (MR = 7.61, 95% CI = 3.91–14.80, $p < 0.001$). A significant trend with increasing tumour grade was found (MR = 1.98, 95% CI = 1.57–2.50, $p < 0.001$). An enhanced formation of M_1dG adduct was also observed with increased pT levels. The breast cancer cases with a pT2 level presented the highest level of DNA damage (MR = 5.75, 95% CI = 3.13–10.59, $p < 0.001$). A significant trend with increasing pT was also found (MR = 2.44, 95% CI = 1.71–3.48, $p < 0.001$). When women were grouped according to tertiles for age, a not statistically significant increment was found (MR = 1.58, 95% CI = 0.92–2.72). A not significant effect of smoking habit was also found (MR = 1.68, 95% CI = 0.88–3.20). When the mammography analyses were considered, any significant associations were not observed between M_1dG adduct and diameters of benign and tumour breast lesions, $p = 0.404$ and $p = 0.486$, respectively.

Discussion

The present study shows an increased frequency of M_1dG adduct, a biomarker of oxidative stress and LPO, in the breast tissues of women suffering with breast cancer in respect to controls. This is in keeping with previous studies reporting that oxidative stress-related DNA damage is associated with cancer
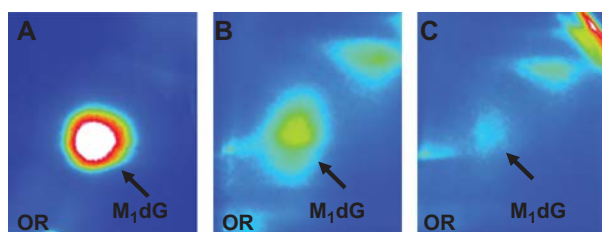

Figure 1. Typical autoradiograms of malondialdehyde deoxyguanosine adduct in 10 mM malondialdehyde-treated calf thymus DNA (A), in fine needle aspirate of breast cancer case (B) and control (C).

of the breast [22–26]. It is possible that an increment over the physiological level of M_1dG adduct generated during physiological processes can contribute to cancer development. Increased levels of M_1dG adduct in breast cancer cases can be reflective of impairments in the metabolic detoxification of oxidative by-products or in the DNA repair activity. In fact, the reduced activity of the enzyme 8-oxoguanine DNA N-glycosylase, an enzyme that repairs oxidative DNA lesions, has been shown to be a cancer risk factor [27].

Different mechanisms can be involved in the increased production of M_1dG adduct in women with breast cancer. Endocrine factors have been reported to have an important role in hormonal carcinogenesis [28]. Oestrogens can be metabolically activated into catechol oestrogens by cytochrome P450 enzymes [2,3]. Then, metabolic redox cycling between catechol oestrogens and their corresponding quinones can generate oxidative stress and ROS. Inflammation can be also involved in the endogenous induction of breast M_1dG adduct by activation of macrophages and neutrophilic response, two processes that can increase the production of hydrogen peroxide and hypochlorite acid. Interestingly, we have recently shown that ROS generated by activated inflammatory neutrophils are involved in M_1dG adduct formation [29].

Our next result shows an increasing production of M_1dG adduct with increasing the degree of differentiation and severity of cancer. The highest frequency of M_1dG adduct was detected in the group of women with the highest pT and G values, parameters that generally identify a type of cancerous breast tumour characterized from poorly differentiated cells. The mechanism by which elevated M_1dG can be related to breast cancer progression is unclear. Oxidative stress has been recently associated to an increasing severity of breast tumour [30]. Elevated circulating markers of inflammation have also been associated to reduced breast cancer survival [31]. The relationship between M_1dG adduct and severity of cancer has been previously examined by us in a hospital-based study [12], where elevated levels of DNA damage were associated to worse survival of lung cancer patients, after adjusting for age, gender and packyear (Hazard Ratio = 2.48; 95% CI = 0.65–9.44). The level of oxidative stress could increase in advance stages of cancer as a result of chronic inflammation, and could enhance the generation of M_1dG adduct, that, if left unrepaired, can induce additional mutations including in key oncogene and tumour suppressor genes necessary for cancer progression. The small sample size is a limitation of the study, which, however, suggests hypotheses for future investigations. For instance, the evaluation of changes of M_1dG adduct level in breast tissues after diminishing breast tumour by surgery and/or chemotherapy would be interesting to pursue this study and could serve to improve our understanding of the mechanisms of tumour progression in the breast.

Tobacco smoke contains a variety of carcinogens and ROS derived from tobacco pyrolysis products that can induce M_1dG adduct. Thus, we have analysed the association between smoking and M_1dG adduct, but no effect was found, probably due to the small number of smokers. Association of M_1dG with smoking has been examined by us in two previous hospital-based studies and higher amount of DNA endogenous lesions have been found in bronchi and larynx of smokers [11,12]. Similar relationship were observed in the leukocytes and oral mucosa of smokers [13,32], but other authors reported no differences for tobacco smoking in breast and colon mucosa [10,22]. A recent study also indicated that cigarette smoking caused the elevations of urinary F_2-isoprostanes, an indicator of oxidative stress [4].

Conclusions

This study suggests that an increment over the background frequency of M_1dG adduct generated during normal physiological processes can contribute to development and progression of cancer of breast. FNA M_1dG adduct could be used for evaluating cancer risk in future clinical and epidemiological studies. An additional benefit will be their eventual application to chemoprevention of cancer, i.e. to reduce the levels of mutagenic M_1dG adduct to slow the mutation rate of cancer cell.

Acknowledgements

Declaration of interest

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