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Original Paper

Prognostic and Aetiological Relevance of 8-Hydroxyguanosine in Human Breast Carcinogenesis

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In order to estimate the level of oxidative damage and its role in breast cancer, the promutagenic oxidative lesion, 8-hydroxy-2′-deoxyguanosine (8-OHdG), was determined in DNA isolated from 75 human breast tissue specimens and from normal and transformed human breast cell lines, utilising a newly developed solid-phase immunoslot blot assay. The amount of 8-OHdG was found to be 0.25 ± 0.03 pmol/μg in normal breast tissue from reduction mammoplasty, 0.98 ± 0.174 pmol/μg in benign tumours and 2.44 ± 0.49 pmol/μg DNA in malignant breast tissue with invasive ductal carcinoma. The malignant tissue had a statistically significant 9.76-fold higher level of 8-OHdG than normal tissue ($P < 0.001$, Mann–Whitney). A statistically significant 12.9-fold ($P = 0.004$) higher endogenous formation of 8-OHdG was also observed in cultured breast cancer cells compared with normal breast epithelial cells. In addition, a significantly elevated level (3.35-fold higher, $P < 0.05$) of 8-OHdG observed in oestrogen receptor-positive compared with oestrogen-negative malignant tissues, and in breast cancer cell lines (9.3-fold higher, $P = 0.007$) suggests a positive relationship between 8-OHdG formation and oestrogen responsiveness. The extent of 8-OHdG adducts did not show a discernible correlation with either the age or the smoking status of the patients. These results indicate that the accumulation of 8-OHdG in DNA has a predictive significance for breast cancer risk assessment and is conceivably a major contributor in the development of breast neoplasia.

Key words: breast cancer, 8-hydroxydeoxyguanosine, oxidative DNA damage, oestrogen receptor, immunodetection

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INTRODUCTION

Of the total incidence of cancer in women, breast cancer represents about 25–29% and accounts for 15–18% of the mortality [1]. The exact cause of breast cancer is essentially speculative, but the evidence suggests that it could be mediated through xenobiotic-induced structural modifications in DNA nucleotide bases that affect template-directed DNA synthesis [2]. Among the plethora of bulky adducts and subtle oxidative modifications in DNA, the oxidative base lesion 8-hydroxy-2′-deoxyguanosine (8-OHdG) has received considerable attention due to its demonstrated mutagenic potential. This stable premutagenic oxidative modification has also been recognised as a potential marker connoting target organ damage from reactive oxygen species (ROS) [3, 4], and its level is well correlated with the incidence of cancer [5, 6]. The concept that the elevated levels of 8-OHdG adducts in DNA play a fundamental role in carcinogenesis [7] and tumour promotion [8], particularly of human breast cancer [9], is becoming an increasingly acceptable dogma. However, a recent report, unable to show a significant difference between 8-OHdG levels in the DNA of breast cancer and corresponding non-cancerous breast tissues, suggests inconsistency either with the DNA isolation procedures and/or choice of the analytical methods, for example, HPLC-EC and GC/MS-SIM [10]. Thus, it is important to determine the extent of 8-OHdG in normal and cancerous human breast tissue, by independent approaches, to establish firmly its role in the pathogenesis of breast neoplasia.

Despite around 60 classical and modern prognostic factors reported for breast cancer [11], the identification of high- and low-risk breast cancer patients remains an intriguing problem. So far, an aetiological association of promutagenic oxidative

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DNA lesions and breast neoplasia is not well founded and needs more supportive evidence. Indeed, an average of 168 residues of 8-OHdG per cell per day [12], reflects the incessant, endogenous, free radical-induced DNA damage, and the accumulation of modified base lesions may be a significant contributor to ultimate malignancies in humans [13, 14]. Therefore, in the present study, we utilised a newly developed solid-phase immunoslot blot assay to determine quantitatively the extent of 8-OHdG in normal, benign and malignant breast tissue. This study addressed: (i) the magnitude of the biochemical impact of recurring oxidative damage on genetic material in terms of the guanine base modification in human breast; (ii) the aetiological relevance of 8-OHdG in breast carcinogenesis; and (iii) the association of oxidative DNA base modification with age, smoking and hormone receptor status. The overall data provide an important estimate of oxidative damage for the early prognosis of disease, as well as insights into the mechanistic role of oxidative DNA damage in initiation and progression of breast cancer.

MATERIALS AND METHODS

Chemicals

Calf thymus DNA, RNase A and T1, proteinase K, nitroblue tetrazolium (NBT), bis-chloro-indolyl-phosphate (BCIP), were purchased from Sigma Chemical Company (St. Louis, Missouri, U.S.A.). Alkaline phosphatase conjugate of goat anti-rabbit IgG (GARI-AP) was obtained from Boehringer Mannheim Biochemicals (Indianapolis, Indiana, U.S.A.). Nitro-cellulose filters, Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum were obtained from Gibco BRL. Polyclonal anti-8-OHdG antibodies were a kind gift of Dr B.N. Ames, University of California (Berkeley, California, U.S.A.). All other reagents were of analytical grade.

Human breast tissues and isolation of DNA

Human female breast tissues were obtained from Arthur G. James Cancer Hospital and Research Institute and the Cooperative Human Tissue Network, Midwestern Division, Ohio State University (Columbus, Ohio, U.S.A.). The total number of samples, comprising normal ($n = 15$), benign ($n = 26$) and malignant ($n = 19$) tissues, were collected at the time of surgery and stored immediately at −70°C. Additional archived malignant tissue specimens ($n = 15$) from liquid nitrogen storage were provided by Dr Hussien Abou-Issa, Department of Surgery, Ohio State University hospitals. The archived samples did not exhibit any significant storage-time-dependent variability in the 8-OHdG level compared to the freshly frozen samples. Information on histopathological characteristics, age, smoking and receptor status of the samples was obtained through the pathology reports. For isolation of DNA, the frozen tissue (approximately 0.4 g) was minced with the cross strokes of two scalpel blades on prechilled ground glass blocks. The minched tissue was homogenised in a Dounce glass homogeniser in TE-sucrose, pH 8.0, at 4°C. The homogenate was filtered and supernatant centrifuged at 2000 rpm for 5 min. The nuclear pellet was incubated with a mixture of RNase A (200 μg/ml) and T1 (2 U/ml), 0.25% sarkosyl for 60 min at 37°C. Subsequently, proteinase K (600 μg/ml) was added and incubated at 65°C for 15 min followed by overnight incubation at room temperature. Samples were then extracted four times with an equal volume of chloroform:isoamyl alcohol (24:1). DNA in the aqueous layer was precipitated with ethanol and the pellet dissolved in TE (10 mM Tris–HCl, pH 8.0, 1 mM EDTA). DNA was evaluated qualitatively by measuring absorbance at 260 nm, A_{260}/A_{280} ratios and gel electrophoresis, and quantitated by a micro-fluorimetric assay with Hoechst 33258 [15] or by colorimetric microdiphenylamine assay as previously described [16].

Cell culture and isolation of nuclear DNA

Normal human mammary epithelial cells (HMEC) obtained from Clonetics (San Diego, California, U.S.A.), and derived from reduction mammoplasty tissue, were grown in serum-free MCDB-170 (Clonetics) in 5% CO_2. The human breast adenocarcinoma cell line, MCF-7 (hormone-dependent, receptor-positive), was grown in Dulbecco's modified Eagle's medium (DMEM), pH 7.2, supplemented with heat inactivated fetal calf serum (10%), 2 mM glutamine, 30 μg/ml penicillin and 50 μg/ml streptomycin sulphate in a humidified atmosphere of 5% CO_2. The human breast adenocarcinoma cell line, MDA-MB 231 (hormone-independent, receptor-negative) was grown in Leibovitz's (L-15) medium plus 10% fetal calf serum in air at 37°C. Cells from confluent cultures of HMEC, MCF-7 and MDA-MB 231 were washed with PBS (0.8% NaCl, 0.02% KCl, 0.12% Na_2HPO_2, $2H_2O$, 0.02% KH_2PO_4) and harvested by trypsinisation with 0.05% trypsin-versene. The pellet (approximately 2×10^7 cells) was collected, lysed and incubated in 1 ml of TNE buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 140 mM NaCl) containing 0.25% v/v sarkosyl, and a mixture of RNase A (100 μg/ml) and T1 (2 U/ml) for 60 min at 37°C. Proteinase K (400 μg/ml) was then added and incubated at 65°C for 15 min and left at 37°C for 4 h. The lysate was processed for DNA isolation as described above for the tissue samples.

Immunoslot blot assay

The antibody binding to DNA samples immobilised on NC filters was determined by immunoslot blot (ISB) assay as described previously [17]. Briefly, increasing concentrations of DNA were vacuum immobilised on nitrocellulose filters using a 24-well Hybri-slot™ manifold. The filters were rinsed in 1 M ammonium acetate, baked at 80°C for 1 h under vacuum. The filters were blocked overnight with blotto (120 mM glycine, 20 mM Tris–HCl, pH 8.0, 0.5% gelatin, 5% dry non-fat milk, 0.01% thimersal, 0.01% antifoam A emulsion and 0.4% Tween-20) at 37°C to prevent non-specific protein binding and incubated with 1:1000 dilution of anti-8-OHdG polyclonal antibodies. The unbound antibody was removed by extensively washing in PBS–0.1% Triton X-100. The incubation procedure was repeated with a secondary reagent, GARI-AP (1:1000 dilution in blotto) for 2 h. The filters were washed extensively with AP 9.5 buffer (100 mM Tris–HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$). The filter bound enzymatic activity was visualised by colour development with 0.33 mg nitro-blue tetrazolium and 0.17 mg 5-bromo-4 chloro-3 indolyl phosphate/ml in AP 9.5 buffer. The lanes of the filters were scanned for the relative blot intensities with a LKB laser densitometric scanner. The data were transmitted to the interfaced computer and analysed with the LKB XL gelscan software as described [17, 18]. The background of the filter was subtracted and specific peak intensities were computed for each slot and plotted for corresponding DNA concentrations. Quantitation of the 8-OHdG in all the DNA samples was performed upon intensity comparisons with a

reference standard run in parallel. The level of 8-OHdG in reference calf thymus DNA (12.4 8-OHdG/10^5dG) was pre-established by ISB and HPLC-EC analysis of enzymatically hydrolysed DNA as described earlier [17].

Statistical analysis

The average 8-OHdG and standard error of mean (SEM) for each tissue sample were calculated from the repeated determinations. The mean 8-OHdG level and standard error for each group of tissue were calculated from the average value of each sample in the group. The comparison among the groups was made by one-way analysis of variance (ANOVA) and Mann–Whitney unpaired non-parametric test. Correlation of 8-OHdG concentration with age was examined with the linear regression analysis by the least-squares method using Instat software (Graphpad, San Diego, California, U.S.A.).

RESULTS

Quantitative estimation of 8-OHdG in human female breast tissue

The extent of endogenously oxidised DNA base lesion (8-OHdG) was determined in DNA isolated from 75 individual breast tissue specimens obtained from patients undergoing surgery for normal reduction mammoplasty ($n = 15$), benign tumours ($n = 26$) and malignant breast with invasive ductal carcinoma ($n = 34$). The amount of 8-OHdG was assessed quantitatively by immunoslot blot assay using adduct specific polyclonal antibodies. The assay is based upon the specific binding of the antibody to 8-OHdG in DNA absorbed on to a NC filter. The amount of 8-OHdG in the test DNA samples was estimated on the basis of the reference standard calf thymus DNA. A standard response curve exhibiting the relationship between the relative peak area and the absolute amount of 8-OHdG is shown in Figure 1. The concentration of 8-OHdG in this reference DNA sample was pre-established by independently quantitating the modification level (8-OHdG/dG = 12.4×10^{-5}) with a significant correlation ($r = 0.99$) by ISB [17] and HPLC-EC [19, 20]. As shown in the representative filter in the inset (Figure 1), the increasing amounts of single stranded calf thymus DNA from 25 to 400 ng were immobilised to nitrocellulose filter for detection and quantitation of 8-OHdG by specific antisera. The specific binding of antibody as visualised by colour development of cognate bands shows a gradient of slot colour intensities. Quantitative measurement of 8-OHdG was carried out by monitoring the colour intensity of individual bands by direct laser densitometry of NC filters as described earlier [17]. As previously shown [17], the binding of the antibody to 8-OHdG was found to be directly proportional to the amount of 8-OHdG in DNA. The average amounts of 8-OHdG were determined to be 28, 113 and 282 8-OHdG/10^5 dG equivalent to 0.25 ± 0.03, 0.98 ± 0.17 and 2.44 ± 0.49 pmol/μg DNA in normal, benign and cancerous breast tissues, respectively (Table 1). An interindividual variation in the level of 8-OHdG was found to vary in the range of 0.05–0.53 pmol/μg in normal, 0.07–2.96 pmol/μg in benign and 0.21–5.73 pmol/μg DNA in malignant tissues. The analysis of variance (ANOVA) of the data indicate significant differences in the standard deviation of the 8-OHdG level ($P = 0.0001$) between malignant and normal breast tissues. The comparison of 8-OHdG based on non-parametric Mann–Whitney rank sum test

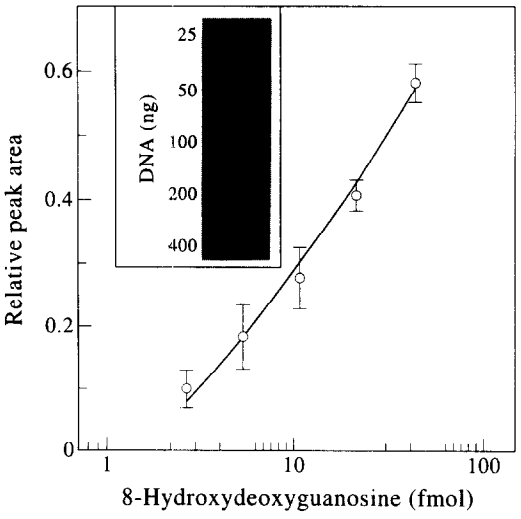

Figure 1. Relationship of antibody binding with absolute amount of 8-OHdG in DNA. Inherently oxidised calf thymus DNA was used as reference standard in non-competitive ISB assay. The modification level of 0.107 pmol/μg DNA was independently predetermined by ISB and HPLC-EC detection. The inset shows NC filter containing varying amounts of heat denatured DNA (25–400 ng) with increasing amounts of 8-OHdG detected upon immunoanalysis with polyclonal antibodies as described in Materials and Methods. The filters were scanned for slot colour intensities and relative peak area plotted as a function of 8-OHdG content in DNA. The data plotted are mean ± SEM of three independent experiments.

Table 1. Comparisons of 8-OHdG content in DNA of histopathologically different human female breast tissues

Tissue groups	Age (years)	n	8-OHdG* (pmol/μg DNA)	8-OHdG/kb	Statistical significance†
Normal	16–53	15	0.25 ± 0.03	0.28	
Benign	17–73	26	0.98 ± 0.17	1.13	$P < 0.02$
Malignant	25–90	34	2.44 ± 0.49	2.82	$P < 0.001$

*Mean ± SEM calculated from the average value of each sample in the group. †Based on Mann–Whitney unpaired non-parametric rank-sum test.
n, total number of patients.

revealed that the cancerous tissue groups have statistically significant 9.76-fold higher level of 8-OHdG ($P < 0.001$) compared with the normal. This is indicative of the fact that the DNA within breast carcinoma cells has received substantial hydroxyl radical-induced oxidative hits. The presence of the relatively high concentrations of 8-OHdG in DNA of the carcinoma tissues appears compatible with the view that 8-OHdG with the potential to cause misreplication and critical target mutations is a culprit lesion in the pathogenesis of breast cancer.

Relationship of 8-OHdG with hormone receptor status in cancerous breast

The association between the amount of oxidative damage and hormone responsiveness was evaluated both in hormone receptor-negative and -positive breast cancer cells and tissue specimens. A plot of absolute amount of 8-OHdG adducts as a function of DNA concentration exhibits a linear relationship

of antibody binding with the level of 8-OHdG in the DNA (Figure 2). The level of 8-OHdG in these DNA samples obtained upon comparison of the relative peak intensity with the reference standard was determined to be 0.13 ± 0.04 pmol/μg in HMEC, 0.18 ± 0.03 pmol/μg in MDA-MB 231 and 1.68 ± 0.20 pmol/μg DNA in MCF-7 cells. A statistically significant difference of 12.9-fold ($P = 0.004$) in the level of 8-OHdG was seen between the normal HMEC and MCF-7 cancer cells whereas the difference (1.38-fold, $P = 0.428$) was not found to be significant between HMEC and MDA-MB231 cells. Furthermore, the level of the 8-OHdG was found to be significantly elevated (9.3-fold, $P = 0.007$) in oestrogen receptor-positive (ER^+) MCF-7 cells compared with the receptor-negative (ER^-) MDA-MB 231 cells. A similar relationship was also noticed between the 8-OHdG level and the hormone receptor status in the breast tissue samples. The adduct dependent binding of the antibody to the DNA isolated from the selected normal, and ER^- and ER^+ cancerous tissue samples is shown in Figure 3a. The corresponding plot of absolute amount of 8-OHdG as a function of DNA concentration (Figure 3b) exhibits the differential binding of antibody due to varying levels of 8-OHdG in histopathologically different tissue types. The concentration of 8-OHdG in these representative DNA samples from a normal and cancerous ER^- and ER^+ tissues was 0.12 ± 0.08, 0.31 ± 0.06 and 1.04 ± 0.14 pmol/μg, respectively, exhibiting a statistically significant 3.35-fold ($P < 0.05$) greater amount of 8-OHdG in ER^+ than ER^- tissues. Nevertheless, the average 8-OHdG level in DNA from total ER^- tissues ($n = 17$) and ER^+ tissues ($n = 11$) was estimated to be 1.96 ± 0.25 pmol/μg and 3.08 ± 0.61 pmol/μg DNA, respectively, indicating a 1.57-fold elevated level of 8-OHdG in ER^+ compared with ER^- tissues (Figure 4).

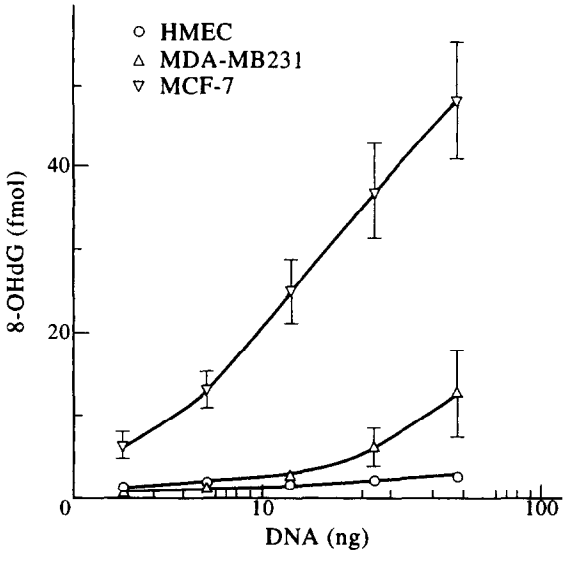

Figure 2. Comparative level of 8-OHdG in cultured human breast cell lines. Varying amounts of DNA isolated from normal HMEC and oestrogen-negative MDA-MB 231 and oestrogen-positive MCF-7 cancer cells were applied to NC filters and processed for adduct quantitation as described in Materials and Methods. The relative peak intensity values obtained upon laser scanning of the filters were transformed to 8-OHdG adduct concentration upon intensity comparison with the reference standard curve using Jandel's Table curve software. The absolute amount of 8-OHdG adducts in DNA of different cell types was plotted as a function of the amount of DNA by non-linear curve fitting (Sigmaplot).

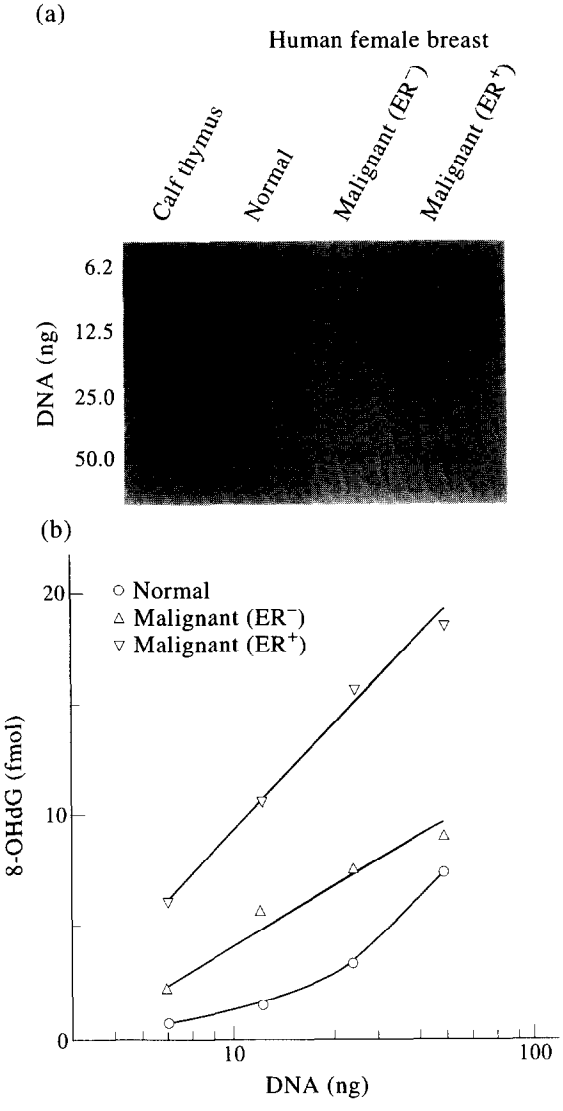

Figure 3. Differential level of 8-OHdG in oestrogen-positive and -negative malignant human breast tissues. DNA isolated from representative normal and malignant (ER^+) and (ER^-) tissues were applied to NC filters in increasing amounts (6.2–50 ng) and processed as described in Materials and Methods. Calf thymus DNA was always included in parallel as a reference standard. The representative filter (a) shows the bands visualised upon colour development with NBT/BCIP substrate and photographed with a Kodak plus-X Pan black and white film. The intensity of the individual band was determined by laser densitometric filter scanning and the data computed after valley to valley subtraction of the peak with LKB XL gel scan software. (b) The amount of 8-OHdG determined upon comparison of the relative peak intensity values with the reference standard DNA (Figure 1) was plotted as a function of the amount of DNA.

Correlation of 8-OHdG level with age and smoking status

The relationship of 8-OHdG level in breast tissue with patients' age is shown in Figure 5. The distribution pattern of 8-OHdG in histologically different tissue types shows that the malignant tissue group has the highest amount of oxidative modification, particularly with an average age for patients of 56 ± 6.4 years. However, the linear regression analysis as

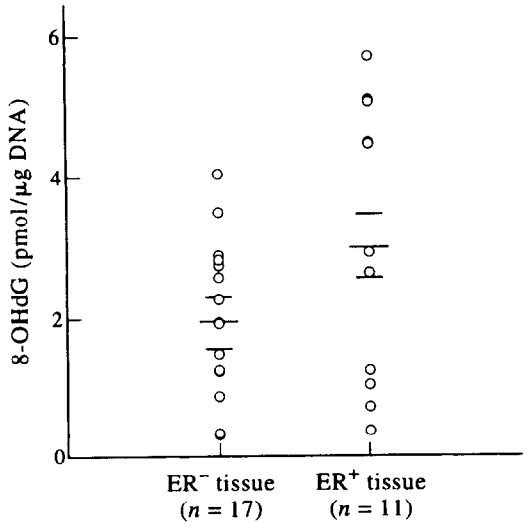

Figure 4. Relationship of 8-OHdG levels with oestrogen receptor status in malignant human breast. The amount of 8-OHdG in DNA isolated from total ER⁻ ($n = 17$) and ER⁺ ($n = 11$) tissues was determined by ISB assay as described in Materials and Methods. Each data point (○) represents an average of replicate determinations and the long dash for each group indicates the mean value and the two short dashes the SEM.

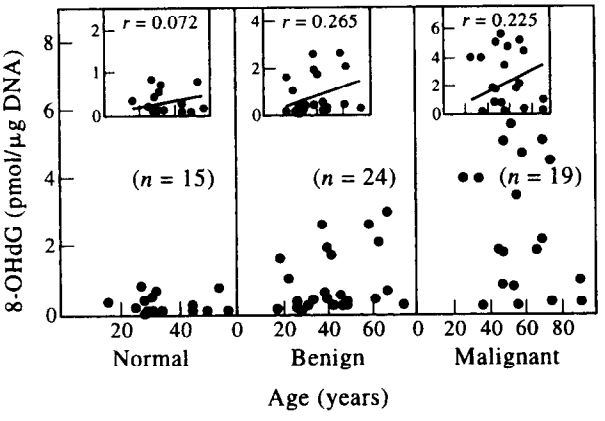

Figure 5. Correlation of 8-OHdG level with the patients age. The amount of 8-OHdG in DNA isolated from total normal, benign and malignant tissue samples (with ages available) was graphed on a scatter plot. Each data point (●) represents the DNA from one single individual donor and the mean value of triplicate determinations of 8-OHdG in respective DNA. Details of the methods are described in the text. The correlation coefficient (r) as shown in the inset was determined by the linear regression analysis of 8-OHdG as a function of age. $r^2 = 0.005$, $P = 0.79$ (two-tailed) for normal; $r^2 = 0.07$, $P = 0.20$ (two-tailed) for benign; $r^2 = 0.05$, $P = 0.35$ (two-tailed) for malignant tissues. The slope is not significantly different than zero.

shown in Figure 5 (inset) did not reveal any significant correlation between 8-OHdG level and the patients' age. Similarly, no correlation was observed between the 8-OHdG level in human breast DNA and smoking status of the donor patients (data not shown).

DISCUSSION

In the present study, we assessed the extent of oxidative DNA damage by immuno-quantitating the surrogate marker 8-OHdG both *in vivo* in the genome of histologically different human breast tissues and *in vitro* in cultured human breast cell lines to investigate the prognostic relevance and role of this promutagenic oxidative base lesion in the aetiology of breast cancer. This comprehensive study further provides important supportive evidence that significant and readily discernible differences exist in the oxidised base lesion profile between normal, benign and malignant human breast tissues. The data indicate the presence of a substantially higher level of 8-OHdG, both in DNA from malignant breast tissues as well as cultured breast adenocarcinoma cells. In comparison to the malignant breast tissue, exhibitng 2.44 ± 0.49 pmol 8-OHdG/μg DNA corresponding to about 2.82 8-OHdG/kb DNA, the normal and benign tissues were estimated to contain 0.28 and 1.13 8-OHdG/kb DNA, respectively. A statistically significant 9.76-fold higher induction of 8-OHdG in malignant breast than in normal breast is in agreement with previously reported results [9]. However, in that study, the reported values were based on a limited number of tissue samples and the control used was a commercially available calf thymus DNA rather than the DNA from an actual normal breast tissue. Nevertheless, the overall magnitude of 8-OHdG for cancerous and normal breast tissues, identified in the two studies, clearly supports the occurrence of elevated oxidative stress in breast cancer.

It is well known that about 40% of all cancers in women are hormonally mediated [21], and both oestrogens and progestagens play critical roles in the development of breast cancer [22]. In relation to this, our data, indicating a relatively higher level of 8-OHdG modification in DNA from ER⁺ compared to the ER⁻ cancerous tissues, suggest a positive correlation between the oxidative damage and oestrogen receptor status. The data also showed a significantly higher level of 8-OHdG in the receptor-positive human breast adenocarcinoma cell line, MCF-7, than the receptor-negative, MDA-MB 231 cells. It is presumed that, in this case, the major factor contributing to the elevated levels of 8-OHdG in MCF-7 cells could be the steroidal constituents including phenol red present in the culture medium. Phenol red is demonstrated to have significant oestrogenic activity at the concentration of 15–45 μM at which it is found in tissue media [23]. In light of the pivotal role of oestrogen in breast carcinogenesis cited in literature, it is hypothesised that exposure to certain xenoestrogens elevates endogenous hormone levels, especially 16-α-hydroxyestrone (16α-OHE1), which in turn stimulates breast cell proliferation and thereby induces or promotes breast cancer. Recent studies have also demonstrated that the reaction of 2-hydroxy-catechol of estradiol (2-OH-E2) by copper (Cu^{2+}) causes the reduction of Cu^{2+} to Cu^{+} with concomitant generation of hydrogen peroxide [19]. Since copper is reported to be present at higher concentrations in many tissues [24], its preferential binding to guanine may result in the site-specific formation of 8-OHdG in the DNA [19]. Also, the endogenously generated hydrogen peroxide can be readily transported across the nuclear membrane and produce the hydroxyl radical via the iron (Fe^{2+}) catalysed Fenton reaction [7], causing oxidative modifications in DNA.

A considerable association has been suggested between the oxidative DNA damage and aging [25]. Individual's age is recognised as a major risk factor in the development of breast cancer [26]. Our data pertaining to the age-related distribution of oxidised base adducts in DNA suggest that the cancer patients with an average age of 56 ± 6.4 years have an immense oxidative burden as compared to the individuals

with benign tumours or normal breast. The 8-OHdG has been shown to accumulate with age in rat liver, kidney and intestine, but no correlation between age and the concentration of 8-OHdG has been observed in human brain and breast [20, 25, 27]. Neither did linear regression analysis of our data reveal any significant correlation either with the patients' age or smoking status. These deductions are consistent with the earlier observation showing an absence of any discernible relationship between smoking and breast cancer risk [28].

In conclusion, based upon the results of this study, it is postulated that the attack of diversely originating reactive oxygen species on the DNA in human breast causes excessive formation of 8-OHdG and other oxidative lesions. In susceptible individuals, this excess is beyond the threshold normally maintained by the specific cell surveillance systems. Thus, the accumulation of promutagenic oxidative DNA adducts in cells could ultimately result in genetic instability leading to the activation and dysregulation of well-recognised targets, such as oncogenes (*C-HA-RAS, C-FOS, C-JUN*) [29] and tumour suppressor genes (*TP53, P16, C-RET*) [30–32]. Consequently, a normal cell is triggered on to the pathway of malignancy, further mediated and modulated by a host of endogenous and exogenous factors, involving multiple events in temporal phases during the course of 25–30 years of the gradual process of developing malignancy in human breast. In depth and systematic studies are needed to link various facets of this complex process centrally driven by the oxidative damage to cellular DNA.

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