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Increasing oxidative damage and loss of mismatch repair enzymes during breast carcinogenesis

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ABSTRACT

This study examined the expression of oxidative damage markers 8-hydroxydeoxyguanosine (8-OHdG), 4-hydroxy-2-nonenal (HNE) and nitrotyrosine using immunohistochemical techniques. In addition, DNA topoisomerase II binding protein 1 (TopBP1) and mismatch repair proteins 2 and 6 (MSH2 and MSH6) were immunostained in a series of 80 stage I invasive breast tumours, 26 *in situ* breast carcinomas and 12 benign breast hyperplasias. 8-OHdG, HNE and nitrotyrosine expression were considerably weaker in hyperplasias than in *in situ* lesions, which, in turn, showed less oxidative damage than T1N0 tumours. Hyperplasias and *in situ* tumours were all, at least moderately, positive for MSH2, and nearly all were positive for MSH6. Nitrotyrosine expression was associated with HNE ($P < 0.0005$) and 8-OHdG ($P = 0.041$) in the T1N0 cohort. To conclude, there is increasing oxidative stress during the early steps of breast carcinogenesis. On the other hand, a significant reduction in expression of mismatch repair proteins occurs during the progression of *in situ* lesions to invasive tumours.

1. Introduction

Breast carcinogenesis is thought to be a multistep process starting with hyperplasia and progressing through atypical hyperplasia to *in situ* and invasive carcinoma. Cancer is now widely regarded as a degenerative disease of DNA, the incidence of which grows exponentially as the population ages and as genomic damage accumulates in the cell. During the last decade, the formation of reactive oxygen and nitrogen species (ROS) has been implicated as a linking factor in certain potential carcinogens, such as hormonal factors, alcohol, microbes and radiation.

ROS is the collective term for certain reactive oxygen and nitrogen metabolites. The term ROS also encompasses free radicals, which are highly reactive molecules with an unpaired electron on their outermost orbital that react with most cellular macromolecules. Physiologically and pathologically the most important ROS are superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($\cdot OH$). $O_2^{\cdot-}$ is formed continuously, predominantly as a by-product of oxidative phosphorylation in mitochondria.[1] $O_2^{\cdot-}$ is further dismutated to less reactive H_2O_2 by superoxide dismutases. H_2O_2 is then converted to molecular oxygen and water by catalase, glutathione peroxidases and recently characterised peroxiredoxins.[2] If the amount of ROS exceeds the capacity of the ROS-suppressing machinery, principally the above-mentioned antioxidant enzymes, oxidative stress is said to occur. This imbalanced redox status is a potent factor in inducing DNA, lipid and protein damage.[3]

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Surplus H_2O_2, which is not eliminated by antioxidant systems, reacts readily with reduced transition metals, mainly via the Fenton reaction when Fe^{2+} comes into contact with H_2O_2. This reaction produces hydroxyl radicals (·OH), which, when generated in the immediate vicinity of DNA, can damage both its deoxyribose backbone and all four DNA bases in various ways, including via the generation of 8-hydroxyguanosine (8-OHG), the hydrolysis product of which is 8-hydroxydeoxyguanosine (8-OHdG). 8-OHdG has been used as a 'fingerprint' of ·OH attack and is the most widely studied DNA oxidation product.[3]

Lipid peroxidation is another quantitatively important target of ·OH-derived damage.[3] It leads to loss or weakening of cell membrane structure and function, and generation of aldehyde products, such as acrolein, crotonaldehyde, malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE).[4] MDA and HNE have been shown to be carcinogenic in animal models.[4,5] Both MDA and HNE are stable aldehydes, which can be assessed by immunological methods.

Nitric oxide ($NO^{\cdot}$) is a free radical generated from L-arginine by three highly conserved $NO^{\cdot}$ synthases (NOS).[6] The primary pathway of $NO^{\cdot}$ metabolism is the reaction of $NO^{\cdot}$ with $O_2^{\cdot-}$, which yields a powerful oxidant, peroxynitrite ($ONOO^-$). $ONOO^-$ is considered an effective carcinogen, and can induce DNA-strand breakage, nitration of tyrosine residues of proteins, and inhibit mitochondrial electron transport.[7] Since $ONOO^-$ readily reacts onwards, it is difficult directly to determine its concentrations *in vivo*. However, $ONOO^-$ reacts with tyrosine groups of proteins and forms nitrotyrosine as a stable end-product that can be visualised using immunohistological methods. Nitrotyrosine is recognised as a somewhat specific marker of nitrosative stress.[8]

If DNA damage occurs, genomic integrity may be maintained either with the help of DNA repair proteins or, as a final option, by apoptosis. Mismatch repair (MMR) proteins are highly conserved DNA caretaker proteins, able to eliminate mispaired nucleotides or extra-helical loops of different size in newly synthesised DNA.[9] Mutations in MMR proteins are associated with malignancies, the best characterised of which is hereditary non-polyposis colorectal cancer syndrome (HNPCC).[10]

DNA topoisomerase II (Top2) is a ubiquitous and highly conserved ATP-dependent enzyme, which is able to cleave and re-ligate double-stranded DNA and thereby catalyse changes in DNA topology.[11] Two isoforms of Top2 are present in human cells; Top2α and Top2β. Since DNA replication and further cell proliferation are dependent on these enzymes, and because Top2 is up-regulated in many malignancies, its inhibitors have been researched extensively in primary breast cancer and adjuvant chemotherapy.[12] The 161 kDa Top2β binding protein 1 (TopBP1) is involved in the DNA damage response and binds to the C-terminal region of Top2β. Following exposure to ionising radiation, TopBP1 is phosphorylated and co-localises with Top2 and various other interacting proteins to sites of DNA breaks.[13] TopBP1 protein expression shows a peak in the S-phase, suggesting that TopBP1 is involved in S-phase checkpoints and possibly also in DNA damage recognition.[13]

The aim of this study was to reveal the extent of ROS-derived DNA, protein, and lipid damage in benign breast hyperplasias, *in situ* tumours and invasive stage I breast tumours. In addition, the study tested whether this damage correlates with expression of DNA repair enzymes in these tumours.

2. Material and methods

2.1. Materials

Eighty formalin-fixed, paraffin-embedded breast carcinoma samples, dating from the period 1982–2001, were collected randomly from the files of the Department of Pathology, Oulu University Hospital, Finland. The tumours were staged as T1N0, i.e. tumour size was 2 cm or less in its greatest dimension and no regional lymph node metastasis were observed. Staging was based on the TNM-classification of breast tumours.[14] The degree of differentiation of ductal carcinomas was classified after Elston and Ellis; there were 12 grade I, 32 grade II and 19 grade III tumours.[15] Sixty-three of the T1N0 tumours were diagnosed as ductal, 10 lobular, 3 medullary, 2 mucinous, and 2 tubular, respectively. The mean age of these patients was 58 years (range 32–86 years) and mean follow-up time 78 months (range 12–235 months). Eight of the patients died of breast cancer during follow-up. The clinical data, such as patient survival, were obtained from hospital records. The oestrogen and progesterone receptor status of the tumours was based on biochemical assessment immunohistochemical data. In addition to invasive lesions, 26 *in situ* breast carcinomas and 12 benign breast tissue hyperplasias were selected in the same way from the archives of the Department of Pathology. Thirteen of the selected *in situ* tumours were grade I or II, and 13 grade III.

2.2. Immunohistochemistry

Paraffin-embedded tissues were cut into 4 μm thick slices and placed on SuperFrostPlus glass, fixed at 37 °C overnight, and processed further within a few days. They were soaked in xylene to remove the paraffin and rehydrated in a graded alcohol series. To predigest the sections, they were heated in a microwave oven in 10 mM citric acid monohydrate (pH 6.0) for 10 min then cooled at room temperature. In order to consume endogenous peroxide, sections were immersed in 3% hydrogen peroxide in methanol for 15 min. The antibody dilution used for nitrotyrosine was 1:100 (Upstate, NY, #06-284) and for 8-OHdG, HNE, TopBP1, MSH2 and MSH6 1:125, 1:250, 1:100, 1:200 and 1:250, respectively (Table 1). Slides were incubated overnight at +4 °C with primary antibody. Immunostaining was performed using the Histostain-Plus Bulk Kit (Zymed Laboratories Inc., South San Francisco, CA, United States of America (USA)) for HNE and nitrotyrosine, whereas in 8-OHdG, TopBP1, MSH2 and MSH6 immunostainings a biotinylated secondary antibody (dilution 1:400) followed by the avidin-biotin-peroxidase complex was applied (Dakopatts, Glostrup, Denmark). Aminoethyl carbazole (AEC) (Zymed Laboratories Inc.) was used as a chromogen for all antibodies. The sections were counterstained in Meyer's haematoxylin, immersed in 2% ammonia water and finally mounted with Immu-Mount (Shandon, Pittsburgh, PA, USA). Negative control stainings were performed with the same procedure and

Table 1 – Antigens and antibodies used in the immunohistochemistry studies

Antigen	Antibody	Dilution	Source of primary antibody
Nitrotyrosine	Rabbit polyclonal nitrotyrosine antibody	1:100	Upstate, NY, United States of America (USA)
HNE	Rabbit polyclonal HNE antibody	1:250	Calbiochem, CA, USA
8-OHdG	Mouse monoclonal 8-OHdG antibody	1:125	Gentaur, Brussels, Belgium
TopB1	Mouse monoclonal TopB1 antibody	1:100	Becton, Dickinson and Company Biosciences, NJ, USA
MSH2	Mouse monoclonal MSH2 antibody (ab-2)	1:200	OncogeneScience, MA, USA
MSH6	Mouse monoclonal MSH6 antibody	1:250	BD Biosciences, CA, USA

HNE, 4-hydroxy-2-nonenal; MSH, mismatch repair proteins; 8-OHdG, 8-hydroxydeoxyguanosine.

same lesions, except that primary antibodies were replaced with phosphate-buffered saline (PBS) and rabbit or mouse isotype controls (Zymed Laboratories Inc.).

Immunostaining results were graded semi-quantitatively into four groups: – = no immunostaining present; + = weak immunostaining; ++ = moderate immunostaining; and +++ = strong immunostaining.

Monoclonal mouse anti-human Ki-67 antibody (Zymed Laboratories Inc.) (dilution 1:50) was used to study cell proliferation in invasive carcinomas. The immunostaining was performed as described for nitrotyrosine, except that the avidin-biotin-peroxidase complex method was used and the chromogen was 3,3′-diaminobenzidine. The results were evaluated as the percentage of positive cells in the whole cell population. The presence or abundance of oestrogen and progesterone receptors was also studied immunohistochemically as above, but excluding the citric acid treatment.

When comparing immunohistochemical stainings with each other or with tumour parameters, cell differentiation, receptor status or survival, immunostaining results were divided into two categories: 0 = no staining or weak staining, and 1 = moderate or strong staining. In the case of MSH2 and MSH6, quantification was either 0 = absence or 1 = presence of each MMR protein. The grading of invasive tumours was into two subgroups: 0 = well or moderately differentiated, and 1 = poorly differentiated.

2.3. *Statistical analysis*

SPSS 12.0 for Windows (Chicago, IL, USA) was used for statistical analysis. The significance of the associations was determined using Fisher's exact probability test, two-tailed *t*-test and Mann-Whitney test. In survival analysis the Kaplan–Meier curve was used and the significance was measured by the log-rank, Breslow and Tarone-Ware tests. Statistical significance was set at $P < 0.05$.

3. Results

HNE expression was seen in 86.8% and nitrotyrosine in 91.2% of the T1N0 lesions (Table 2). Both HNE and nitrotyrosine immunostainings were almost entirely cytoplasmic, also extracellular reactivity was observed (Fig. 1). The third oxidative damage marker, 8-OHdG, showed somewhat weaker expression in invasive tumours, 58.9% of the lesions being positive. 8-OHdG expression was found mainly in the nuclei of tumour cells. TopBP1 immunostaining was observed in 65% of the samples, as a DNA damage response protein, also TopBP1 had a nuclear staining pattern. MSH2 and MSH6 proteins were expressed in 55.9% and 56.5% of the lesions in this cohort. None of the MSH6-positive samples showed strong positivity (Table 3). MMR protein expression was nuclear.

All used markers of oxidative damage, 8-OHdG, nitrotyrosine and HNE showed noticeably increasing expression from benign hyperplasias through carcinomas *in situ* to T1N0 tumours (Fig. 2(a–c)). However, due to the relatively small amount of material in our non-invasive groups, none of these differences reached statistical significance. T1N0 lesions expressed significantly more nitrotyrosine than did hyperplasias ($P = 0.003$). *In situ* tumours also showed more intense staining for nitrotyrosine than did hyperplasias ($P = 0.011$). In benign breast hyperplasias and *in situ* tumours all MSH2 lesions showed at least moderate positivity. In addition, MSH6 was present in 82.8% of hyperplasias and 84.6% of all *in situ* tumours. MSH6 showed significantly stronger expression in *in situ* lesions than T1N0 tumours ($P = 0.014$). Differences in

Table 2 – Distribution of immunohistochemical staining of oxidative damage markers in all studied cohorts

	8-OHdG				HNE				Nitrotyrosine			
	–	+	++	+++	–	+	++	+++	–	+	++	+++
Hyperplasia	91.7	8.3	0.0	0.0	50.0	33.3	16.7	0.0	33.3	58.3	8.3	0.0
In situ, low-grade	75.0	16.7	8.3	0.0	23.1	30.8	46.2	0.0	15.4	30.8	53.8	0.0
In situ, high-grade	50.0	33.3	16.7	0.0	38.5	38.5	23.1	0.0	25.0	16.7	58.3	0.0
T1N0	41.1	35.6	21.9	1.4	13.2	48.5	26.5	11.8	8.8	33.8	51.5	5.9

Figures represent the percentage of each staining group.
HNE, 4-hydroxy-2-nonenal; 8-OHdG, 8-hydroxydeoxyguanosine.

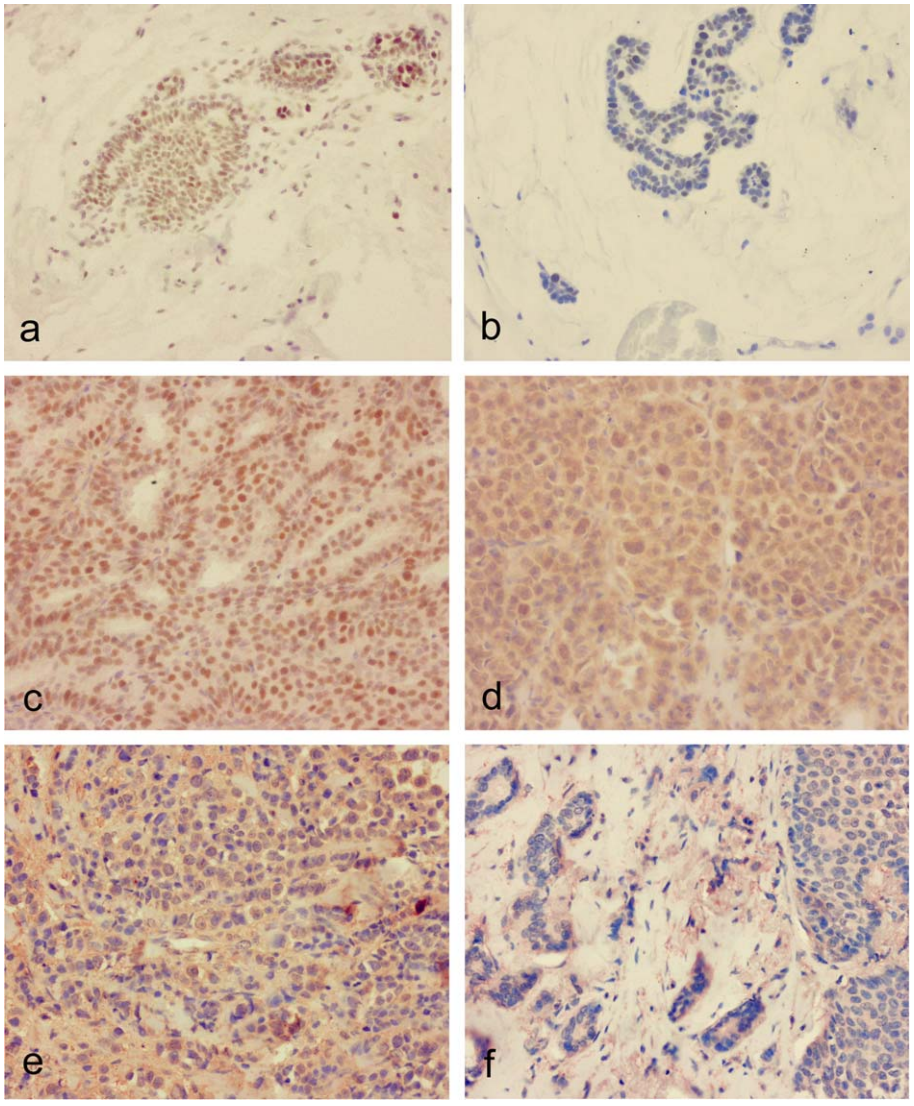

Fig. 1 – (a) Moderate mismatch repair protein (MSH)2 expression can be seen in the nuclei of benign breast hyperplasia cells. (b) Moderate MSH2 expression was found in nuclei of a T1N0 lesion. (c) Strong MSH6 levels were observed in a high-grade *in situ* tumour. (d) Strong TopBP1 expression is seen in nuclei of a T1N0 tumour. Almost all of the studied invasive tumours were positive for nitrotyrosine; (e) moderate cytoplasmic immunostaining; (f) weak to moderate HNE expression.

Table 3 – Distribution of immunohistochemical staining of TopBP1 and mismatch repair (MSH) proteins in all cohorts

	TopBP1				MSH2				MSH6			
	–	+	++	+++	–	+	++	+++	–	+	++	+++
Hyperplasia	50.0	40.0	10.0	0.0	0.0	0.0	91.7	8.3	18.2	45.5	36.4	0.0
In situ, low-grade	61.5	30.8	7.7	0.0	0.0	0.0	41.7	58.3	23.1	15.4	53.8	7.7
In situ, high-grade	18.2	36.4	45.5	0.0	0.0	0.0	50.0	50.0	7.7	53.8	15.4	23.1
T1N0	35.0	30.0	28.3	6.7	44.0	20.3	23.7	11.9	43.5	32.3	24.2	0.0

Figures represent the percentage of each staining group.

MSH6 staining intensity of hyperplasias and T1N0 tumours did not reach statistical significance ($P = 0.103$). Divergence between MSH2 expression in invasive and non-invasive tumours was striking, as MSH2 was constantly positive in hyperplasias and *in situ* tumours. The *P*-value of two-sided Fisher's test was 0.003 between T1N0 tumours and hyperplasias, and <0.0005 between T1N0 and *in situ* cohorts. TopBP1 was expressed in 50% of hyperplasias and 58.3% of all *in situ* tumours. Strong immunostaining was observed only in invasive tumours.

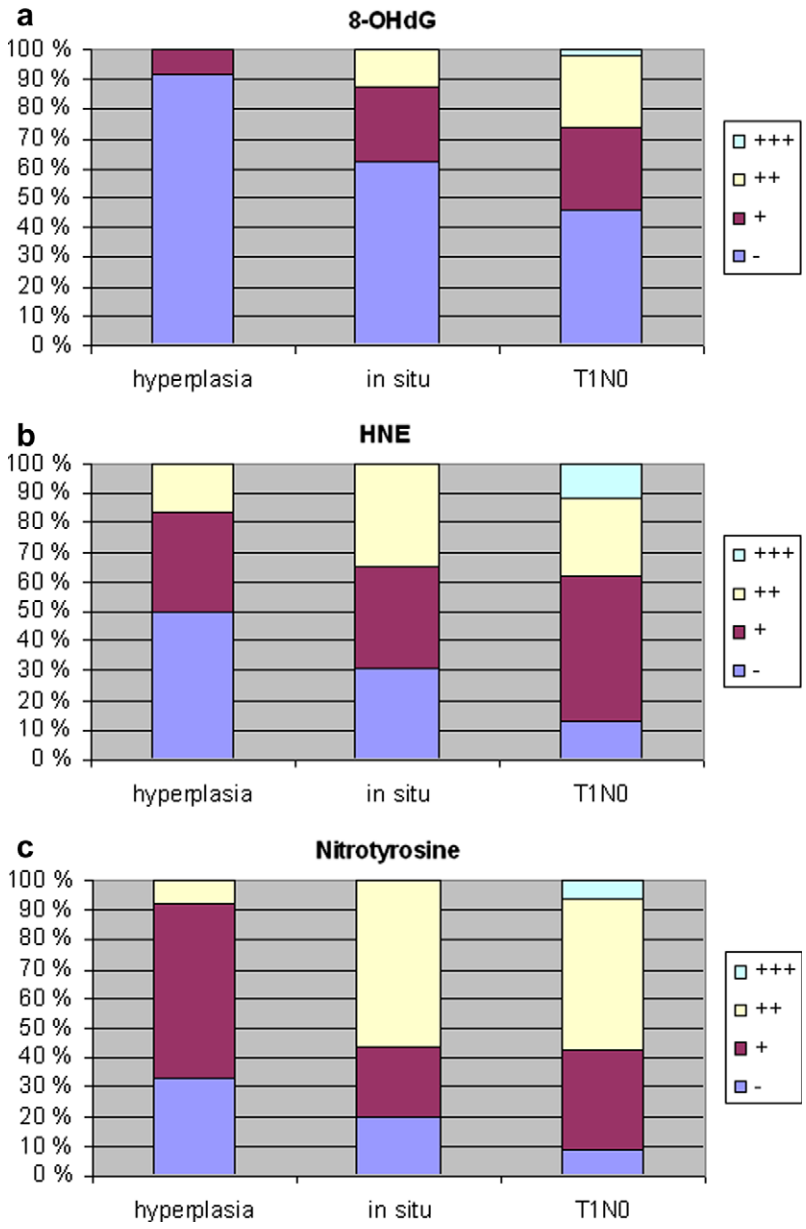

Fig. 2 – (a–c) Piled bar diagrams visualising increasing DNA damage, lipid peroxidation and nitrotyrosine formation during the early steps of breast carcinogenesis.

The immunostaining results were compared within the T1N0 carcinoma group. TopBP1 expression associated with 8-OHdG ($P = 0.008$), but not with nitrotyrosine or HNE. 8-OHdG also associated with nitrotyrosine positivity ($P = 0.041$). The association between HNE and nitrotyrosine was convincing; 22 of the 25 nitrotyrosine negative lesions did not express HNE either, whereas 22 of the 36 nitrotyrosine expressive lesions were also HNE positive ($P < 0.0005$). MSH2 and TopBP1 co-expressed significantly ($P = 0.039$). Also the immunostaining results for MSH2 and MSH6 showed a strong positive association ($P < 0.0005$). 8-OHdG had a near-significant association with decreased proliferation rate ($P = 0.054$). No significant associations with hormone receptors, grade, histological diagnosis, survival or disease-free interval were found.

4. Discussion

High rates of radical generation and persistent oxidative stress are characteristic features of carcinoma cells, both *in vivo* and *in vitro*, caused principally by enhanced glycolytic metabolism, macrophage infiltration to tumours and ROS generation during the reperfusion phase following hypoxia in the defective tumour vascular system.[3] Although certain ROS-derived metabolites, such as H_2O_2, HNE and $NO^{\cdot}$, play a role as essential intracellular messengers, they are also thought to be significant mediators of every step of carcinogenesis.

In breast carcinomas 8-OHdG levels have been found to be elevated 8–17-fold compared with non-malignant tissue.[16] There are no previous studies in which 8-OHdG has been

immunohistochemically assessed in breast lesions. Hence, this is the first study to show that carcinoma cells themselves rather than, for example, inflammatory cells, express high levels of 8-OHdG, suggesting extensive ·OH-derived damage even in stage I tumours. In hyperplasias only 1 of the 12 samples studied showed weak expression of 8-OHdG, the others being negative.

Immunohistochemical methods have been suggested to be the most reliable and specific means of HNE detection.[17] Previously, another important lipid peroxidation product, MDA, has been studied quite extensively in invasive breast carcinomas, and plasma MDA levels have been found either to increase with malignancy stage, or to be elevated in breast cancer patients compared with healthy controls.[18,19] In the T1N0 tumour cells in the present study there was excessive lipid peroxidation; 93.5% of the samples showing HNE positivity. This implies enhanced ROS production and macromolecular damage during breast carcinogenesis. This is further supported by noticeably weaker expression of HNE in the hyperplasia and carcinoma *in situ* cohorts.

In line with this finding, oxidative protein modification was significantly higher in the invasive lesions than in pre-invasive lesions; more than 90% of samples being positive for nitrotyrosine in T1N0 tumours. By contrast, in benign hyperplasias 91.6% of lesions were either negative or, at the most, weakly positive. Low- and high-grade *in situ* lesions expressed nitrotyrosine more than hyperplasias, but the expression was still much weaker than in invasive tumours. Corresponding results have been reported previously from head and neck squamous cell carcinomas, where increased nitrotyrosine levels were found immunohistochemically in reactive, dysplastic and carcinoma samples compared with samples of normal mucosa.[20] Pathologically, one of the most important consequences of lipid peroxidation, protein modification, probably partly explains the strong co-expression of HNE and nitrotyrosine ($P < 0.0005$) in our material. Increased ROS production or insufficient repair mechanisms in these tumours may also explain this finding. Tyrosine nitration was also significantly increased in 8-OHdG positive lesions. To summarise, breast carcinoma cells seem to have noteworthy concurrent $NO^{\cdot}$, ·OH and $O_2^{\cdot-}$ production, which increase during the early steps of breast carcinogenesis. This oxidative stress overwhelms the capacity of ROS counteracting mechanisms and thus accumulates to critical cellular macromolecules, including DNA.

ROS-derived or other defects in MMR genes lead to microsatellite instability and predisposition to cancer.[10] In the light of knock-out studies and clinical observations, two of the most important MMR proteins are MSH2 and MSH6.[21,22] These two proteins form a complex in the nucleus and recognise base-base mispairing of DNA subsequent to its synthesis and correct these mispairings.[10] Simultaneous MSH2 and MSH6 expression has been assessed previously in breast carcinomas only in a small single study.[23] Considering their interrelated roles in DNA repair, it was not surprising that we found very close co-expression between MSH2 and MSH6 in T1N0 lesions. MSH2 positivity has previously been found to be 53–100% in breast carcinomas, depending on the material used and the immunoscoring method.[24–27] MSH2 was expressed, at least moderately, in all of the benign hyperplasias and in *in situ* lesions in the present study; however, MSH6 was absent in approximately 15.2% of the hyperplasias and 18.4% of the *in situ* lesions. The discrepancy with invasive stage I tumours was still striking; there was a loss of both MMR proteins in approximately 44% of the lesions.

There are contradictory reports about the role of MMR proteins in breast tumour development. Bock and colleagues found decreased MSH2 immunostaining in invasive carcinomas compared with *in situ* carcinomas. Furthermore, they reported associations between MSH2-positive tumours and positive lymph node status, higher grade and enhanced proliferation rate.[28] The authors suggested that, during breast cancer development from *in situ* to invasive carcinoma, MSH2 would be down-regulated, but in the invasive type its expression would associate with an aggressive phenotype. Several other recent studies support this down-regulation, leading to diminished MSH2 levels when breast tumour became more malignant.[27,29] By contrast, Friedrich and colleagues found up-regulation of MSH2 in invasive carcinomas compared with benign breast tissue in their relatively small immunohistochemical study.[24] Based on our results, it appears that, during pre-invasive stages, breast carcinoma cells do not show loss of MMR proteins. However, a significant proportion of both MSH2- and MSH6-negative tumours, even in stage I, indirectly suggests their importance in breast cancer suppression.

DNA topoisomerase II is up-regulated in many malignancies, and both its isoforms, α and β, are important targets of several anti-cancer drugs.[12,30] Top2β expression is unknown in breast tumours. Sandri and colleagues found Top2β expression in more than 90% of breast tumours and also that it correlates with shortened patient survival.[30] In our material TopBP1 that specifically binds Top2β was expressed in 77.2% of the T1N0 tumours. It has been hypothesised previously that TopBP1 plays a central role in recognition of damaged DNA.[13] Our results further support to this hypothesis, since TopBP1 was distinctly associated with increased ROS-derived DNA damage, measured by 8-OHdG formation ($P = 0.008$). Furthermore, in line with this idea, we noted increasing TopBP1 expression during cancer progression.

To summarise, there is simultaneous loss of MSH2 and MSH6 proteins in stage I breast carcinomas compared with benign hyperplasias and carcinomas *in situ*. There is also increased oxidative stress during breast carcinoma development, demonstrated here as enhanced levels of oxidatively modified macromolecules from pre-invasive to early stage invasive lesions. These results further support the hypothesis of persistent oxidative stress in breast carcinomas *in vivo*.

Conflict of interest statement

None declared.

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